eISSN: 2564-6524
ISSN: 1015-3918 (1971-2010)

ANKARA ÜNİVERSİTESİ

ECZACILIK FAKÜLTESİ DERGİSİ

JOURNAL OF FACULTY OF PHARMACY OF ANKARA UNIVERSITY

Cilt / Vol : 49
Sayı / No : 3
Yıl / Year : 2025

eISSN: 2564-6524
ISSN: 1015-3918 (1971-2010)

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Cilt: 49, Sayı: 3, Yıl: 2025

(Ankara Ecz. Fak. Derg.) *eISSN: 2564-6524*
ISSN: 1015-3918 (1971-2010)

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Cilt: 49, Sayı: 3, Yıl: 2025

(Ankara Ecz. Fak. Derg.) *eISSN: 2564-6524*
ISSN: 1015-3918 (1971-2010)

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(Ankara Ecz. Fak. Derg.)

eISSN: 2564-6524
ISSN: 1015-3918 (1971-2010)

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JOURNAL OF FACULTY OF PHARMACY OF ANKARA UNIVERSITY
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(J. Fac. Pharm. Ankara) *eISSN: 2564-6524*
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JOURNAL OF FACULTY OF PHARMACY OF ANKARA UNIVERSITY
Volume: 49, Issue: 3, Year: 2025

(J. Fac. Pharm. Ankara) *eISSN: 2564-6524*
ISSN: 1015-3918 (1971-2010)

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(J. Fac. Pharm. Ankara) *eISSN: 2564-6524*
ISSN: 1015-3918 (1971-2010)

Journal of Faculty of Pharmacy of Ankara University (*J. Fac. Pharm. Ankara*) is official scientific journal of Ankara University Faculty of Pharmacy. It was published between 1971 and 2010 as a print.

Journal of Faculty of Pharmacy of Ankara University is published three times (January-May-September) a year. It is an open access, peer-reviewed journal for the publication of original research reports, reviews and short communications in English or Turkish on relevant developments in pharmaceutical sciences. Proceeding of scientific meetings, conference paper, and meeting abstract may be published as special issues of supplements to the journal. All the articles appeared in this journal are published on the responsibility of the author(s). The manuscript submitted to the journal should not be published previously as a whole or in part and not be submitted elsewhere. The manuscripts should be prepared in accordance with the requirements specified.

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J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 49(3): 615-630, 2025
Doi: 10.33483/jfpau.1526137

ORIGINAL ARTICLE / ÖZGÜN MAKALE

DIPEPTIDYL-PEPTIDASE-4 INHIBITORS FROM TINOSPORA CRISPA AS REVEALED BY METABOLOMICS STUDY, MOLECULAR DOCKING AND MOLECULAR DYNAMICS SIMULATION APPROACHES

METABOLOMİ ÇALIŞMALARI, MOLEKÜLER DOKUNMA VE MOLEKÜLER DİNAMİK SİMÜLASYON YAKLAŞIMLARIYLA ORTAYA ÇIKAN TİNOSPORA CRİSPA'DAN DİPEPTİDİL-PEPTİDAZ-4 İNHİBİTÖRLERİ

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ABSTRACT

Objective: *This study aims to identify potential compounds as inhibitors of DPP-4 from Tinospora crispa.*
Material and Method: *Tinospora crispa stem powder was extracted by ultrasonication. The DPP-4 inhibition test used the MAK 203 screening kit protocol. LC-MS/MS was used to determine the chemical profile. MetaboAnalyst5 and SIMCA were used to analyze the dataset. Molecular docking was performed using Molegro virtual docker, and molecular dynamics simulations were performed using YASARA dynamics employing the AMBER14 force field.*
Result and Discussion: *The percentage inhibition of DPP-4 results showed that the most active was 96% ethanol extract. Orthogonal projection to latent structure (OPLS) analysis provides that 6'-O-LactoylBorapetoside B correlates most with DPP-4 inhibitory activity based on VIP value and Y coefficient. In the docking molecular analysis, 6'-O-LactoylBorapetoside B was predicted to be active as DPP-4 inhibitors with a lower rerank score (−106.51 Kcal/Mol) than alogliptin as a reference (−96.02 Kcal/mol). In molecular dynamics simulation for 100 ns, 6'-O-LactoylBorapetoside B complex of binding with DPP-4 protein was stable with the movement of the RMSD value below 3Å. 6'-O-Lactoyl Borapetoside B has a potential of being a DPP-4-inhibitor. But these results must be tested in vitro and in vivo in order to confirm its activity as a DPP-4 inhibitor.*
Keywords: *6'-O-LactoylBorapetoside B, computer-assisted drug discovery, DPP-4, Tinospora crispa*

ÖZ

Amaç: *Bu çalışmanın amacı, Tinospora Crispa'dan DPP 4'ün inhibitörleri olarak potansiyel bileşikleri tanımlamaktır.*
Gereç ve Yöntem: *Tinospora crispa sapının tozu ultrasonikasyonla ekstrakte edildi. DPP-4 inhibisyon testinde MAK 203 tarama kiti protokolü kullanıldı. Kimyasal profilin belirlenmesinde*

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Submitted / Gönderilme : 08.08.2024
Accepted / Kabul : 09.04.2025
Published / Yayınlanma : 19.09.2025

LC-MS/MS kullanıldı. Veri setinin analizinde MetaboAnalyst5 ve SIMCA kullanıldı. AMBER14 kuvvet alanını kullanan YASARA dinamikleri kullanılarak moleküler dinamik simülasyonları yapıldı.

Sonuç ve Tartışma: *DPP-4'ün yüzde inhibisyonu sonuçları en aktif olanın %96 etanol ekstraktı olduğunu gösterdi. Latent yapıya dik projeksiyon (OPLS) analizi, 6′-O-LactoylBorapetoside B'nin, VIP değeri ve Y katsayısına dayalı olarak DPP-4 inhibitör aktivitesi ile en fazla korele olduğunu sağlar. Yerleştirme moleküler analizinde, 6′-O-LactoylBorapetoside B'nin, referans olarak alogliptin'den (−96.02 Kcal/mol) daha düşük bir yeniden sıralama skoru (−106.51 Kcal/Mol) ile DPP-4 inhibitörleri olarak aktif olduğu tahmin edildi. 100 ns'lik moleküler dinamik simülasyonunda, 6′-O-LactoylBorapetoside B kompleksinin DPP-4 proteini ile bağlanma kompleksi, RMSD değerinin 3Å'un altındaki hareketi ile stabildi. 6′-O-Lactoyl Borapetoside B, bir DPP-4 inhibitörü olma potansiyeline sahiptir. Ancak bu sonuçların, bir DPP-4 inhibitörü olarak aktivitesini doğrulamak için in vitro ve in vivo olarak test edilmesi gerekir.*

Anahtar Kelimeler: *6′-O-LactoilBorapetosit B, bilgisayar destekli ilaç keşfi, DPP-4, Tinospora crispa*

INTRODUCTION

Diabetes mellitus is a metabolic disease. Defects cause diabetes in insulin secretion, or the insulin does not work optimally, or because of both [1]. Patients with this disease are at high risk for long-term macro- and microvascular complications due to its chronic metabolic imbalance. If they do not receive high-quality care, this puts the patients at risk for complications such as frequent hospitalization and an increased risk of cardiovascular diseases [2]. More than one in ten persons worldwide today have diabetes. A growing number of nations have adult populations where diabetes affects one in five or even more people. The projected prevalence of diabetes in adults aged from 20 to 79 has more than tripled since the first edition in 2000, which rises from an estimated 151 million (4.6%) of the world' population at the time to 537 million (10.5%) today. By 2030, 643 million individuals, or 11.3% of the world's population, will have diabetes, based on our forecast. If the current trends hold, the figure will increase to an astounding 783 million (12.2%) by 2045 [1].

Dipeptidyl Peptidase-4 (DPP-4) is a serine protease enzyme that cleaves peptides on amino acids 2 and 3 of the N-terminal, especially when the amino acid is alanine or proline [3]. The incretin hormone glucagon-like peptide 1 (GLP-1) is a substrate for DPP-4. DPP-4 will cleave amino acid alanine at number 2 GLP-1, so GLPI-1 is not active. GLP-1 lowers blood glucose by increasing insulin secretion and inhibiting glucagon secretion [4]. DPP-4 inhibitors have a mechanism of action in reducing blood glucose in type 2 diabetes patients by inhibiting DPP-4 [5]. The inhibition of DPP-4 prevents the inactivation of the incretin hormone GLP-1.

Hypoglycemic conditions should be avoided in older adult diabetic patients [6]. The combination of metformin and dipeptidyl-peptidase-4 (DPP-4) inhibitors can control blood glucose levels without increasing the risk of hypoglycemia [7,8]. In all the patients with diabetes mellitus, DPP-4 inhibitors are efficient and well-tolerated. These do not raise the risk of CV disease, weight gain, or excessive hypoglycemia [9]. DPP-4 inhibitors also reduce the risk of hypoglycemia, So it may be the drug of choice in adults and older patients [6,10,11]. However, the cost of treating diabetes with DPP-4 inhibitors is much higher than that of the cost of other antidiabetic drugs [12]. Therefore, alternative natural medicines are needed

Tinospora crispa is a medicinal plant of the genus Tinospora from the family Menispermaceae, which is widely distributed in Southeast Asia, especially in Thailand, Malaysia, the Philippines, Indonesia, and the northeastern region of India [13]. *Tinospora crispa* has long been used as a traditional antidiabetic drug. *Tinospora crispa* is known as Brotowali and has been used traditionally in treating several diseases, like diabetes, in Indonesia [14]. The ethanol extract *Tinospora crispa* was shown to be a DPP-4 inhibitor in vitro at a concentration of 2.5 µg/ml with a percentage of inhibition of 65.86 [15]. However, the active compound responsible for the inhibition of DPP-4 is not yet known. *Tinospora crispa* contains a lot of secondary metabolites. The main biologically active compounds of *Tinospora crispa* are terpenoids and terpenoid glycosides. Most terpenoid glycosides are Borapetoside A-F [16,17]. Furthermore, *Tinospora crispa* contains diterpenoids as main components and is predicted to be an

active compound [18]. The use of computational techniques has reduced the time and expense needed for the drug design and discovery process. Molecular docking is one of these techniques used to clarify the binding mechanism for therapeutic candidate molecules The stability level of the resultant target-compound complexes derived from the docking is then assessed using molecular dynamics (MD) simulation [6].

The novelty of this study is identifying bioactive compounds from the stem of *Tinospora crispa* as inhibitors of DPP-4 by using a metabolomic study. The laborious repeated chromatographic separation required in identifying active compounds was eliminated by metabolomics research. In this research, we identified compounds acting as DPP-4 inhibitors and evaluated their molecular activities using a metabolomics study, molecular docking, and dynamic simulation. The goal of molecular dynamics simulation is to see the stability of the protein-ligand complex from the best molecular docking results. The compounds contained in *Tinospora crispa* were obtained by using the metabolomics approach.

MATERIAL AND METHOD

Materials

Tinospora crispa was obtained from the Tawangmangu plantation, Central Java, Indonesia. Plant determination was performed at the Center for Research and Development of Traditional Medicinal, and Medicinal Plants (B2P2TOOT), the Ministry of Health of the Republic of Indonesia. The specimen vouchers were stored at the Faculty of Pharmacy, University Pancasila. Acetonitrile, analytical grade methanol, DPP-4 screening kit, and sitaglipin (MAK 2023) were purchased from Sigma-Aldrich (Sigma-Aldrich Gmbh, Sternheim, German).

Extraction

Ten grams of *Tinospora crispa* stem powder was extracted by ultrasonication for 60 minutes at 40 °C with various solvents such as 96% ethanol, 50% ethanol, and pure water. The ratio of *Tinospora crispa* powder and the extracting solvent volume was 1:10. Extraction was done three times. The solvent evaporated until thick using a rotary evaporator temperature of 40°C.

DPP-4 Inhibition Activity Assay

The DPP-4 inhibition test used the MAK 203 screening kit protocol. The sitaglipin was used as a positive control. Forty-nine microliters of buffer solution, 1 µl of DPP IV enzyme, and 25 µl of sample solution (100 ppm) were put into the well, shaken, and then incubated for 10 minutes at 37°C. Then, 23 µl of buffer solution and 2 µl of the substrate were added and shaken. In the control wells, the inhibitor was replaced with a buffer. Then, it was measured at an excitation wavelength of 360 nm and an emission wavelength of 460 nm using a microplate reader [19].

% Relative Inhibition

$$\text{Slope} = \frac{(\text{FLU2}-\text{FLU1})}{(\text{T2}-\text{T1})} = \Delta\text{FLU/minute} \quad (1)$$

$$\%\ \text{Relative Inhibition} = \frac{(\text{Slope}_{\text{EC}} - \text{Slope}_{\text{SM}})}{\text{Slope}_{\text{EC}}} \times 100\% \quad (2)$$

where: slope_{SM} = the slope of the Sample Inhibitor, slope_{EC} = the slope of the enzyme control

Data were analyzed using Tukey's Honest Significant Difference (HSD) test to determine the statistical significance of differences between treatment groups.

Metabolomic Study with LC-MS/MS

Ultra-high–performance liquid chromatography (UHPLC) 1290 Infinity II HPLC System (Agilent, UK) with a binary pump and an autosampler was used for HPLC analysis. The samples were separated using a Zorbax XDB C18 column (150 mm 4.6 mm i.d., 3.5 µm, Agilent Technologies,

Germany) at room temperature. The samples were separated using a Zorbax XDB C18 column (3.5 µm, 150 × 0.5 mm, Agilent Technologies, Germany). Acetonitrile (B) and 0.1% (v/v) formic acid in water (A) made up the mobile phase, and the elution gradient was configured as follows: 0–1.82 min, 8–12% B; 1.82–10.22 min, 12–16% B; 10.22–18.62 min, 16–25% B; 18.62–31.22 min, 25–46% B; 31.22–33.32 min, 46–58% B; 33.32–39.62 min, 58–65% B; 39.62–43.82 min, 65–68% B. A flow rate of 1.4 ml/min was used.[1] Tandem 6545 QTOF (Agilent, UK) mass spectrometer was linked to the HPLC system by using a dual AJS-ESI interface. Negative ion mode complete scan data was gathered from m/z 250 to 1000. The data collection and analysis were performed on a mass Hunter qualitative analysis.

ProteoWizard was used in converting first raw LC-MS spectra from vendor format to mzML and centroid them [20]. An automated pipeline with a built-in parameter optimization processes, as described in MetaboAnalys5, was used in processing all centroided spectra [21]. According to the database, manual corrections were made to the remaining unclear compounds and peaks. All the compounds were found to have exact mass errors within 5 ppm [22]. The mass error was calculated by.

$$\text{ppm error} = \frac{\text{Theoretical } \frac{m}{z} \text{ value} - \text{Observed } \frac{m}{z} \text{ value}}{\text{Theoretical } \frac{m}{z} \text{ value}} \times 10^6 \quad (3)$$

By using the appropriate functions in MetaboAnalyst5, a chemometrics analysis (PCA) was performed based on the normalized peak tables. Large datasets are reduced in dimension by a vector space transform in PCA. A data matrix with N observations (rows) and X variables is the foundation for PCA (columns). PCA visualizes the data by converting a multivariate data table into a low-dimensional plane. A score plot was used to show the reduction of spectral variation into a set of principal components (PC), each representing connected spectral differences, new PC variables, and orthogonal to one another. The significant correlation's confidence interval was established at 0.95. Simca-P was used in creating the OPLS model, and the primary active ingredients for DPP-4 inhibition were identified by examining the VIP and regression coefficients. The resulting datasets were transferred to the SIMCA-P software version 17.0 (Umetrics, Umea, Sweden) in order to perform the OPLS. Both the models' data were mean-centered and Pareto-scaled before undertaking these multivariate data analyses. OPLS was applied to flatten the dataset, improve class separation, and identify possible biomarkers. R2Ycum (goodness of fit) and cumulated Q2 were used in order to validate the models' quality (Q2cum, goodness of prediction). In classifying models as having strong (Q2cum 0.5) or poor (Q2cum 0.5) prediction skills, a threshold of 0.5 is commonly used. We used the permutation test to validate the OPLS model and decrease the possibility of overfitting and false-positive results. The plots show the correlation coefficients of the original Y and the permuted Y against the cumulative two R2Ys. The model was deemed accurate if both the regression line's intercepts on the y-axis were negative and all Q2 values from the permuted dataset to the left were lower than the Q2 value on the actual dataset to the right [23]. Subsequently, supervised studies were used to identify variable importance in projection (VIP) values. VIP is a readout of the model's contribution from each x-axis variable. It is totaled over all components and weighted to the Y represented by each element. Thus, VIP ranking indicates the role played by metabolites in the model. In broadening our selection in this investigation, we chose primary metabolites with VIP >0.75. The coefficient plot shows a clear, understandable illustration of the relationships between the X- and Y-variables. The figure shows how the factors in the input affect the outcome.

Molecular Docking

The DPP-4 inhibitory enzyme with code 3G0B was obtained from the protein data bank at RCSB website [24-26]. The crystal structure csomprises the four chains (A, B, C, and D) from which chain A is selected. The water molecules were removed using Molegro virtual docker. We downloaded the structure of the compound which resulted from the metabolomic analysis from PubChem, and then its energy was minimized using the ChemDraw 3D 16 program.

Eight compounds that were identified from the metabolomics LC-MS/MS and sitagliptin as the standard were docked with the DPP-4 with PDB Code 3G0B using Molegro virtual docker. The docking protocol uses a protocol which has been validated in the previous studies [27]. which was the binding site X: 42.21Å, Y: 34.47Å, and Z: 14.97Å, radius 10Å, MolDock Optimizer algorithm (differential

evolution) and Moldock Score scoring function. We compared our result analysis according to the lowest rerank score with alogliptin as the reference.

Molecular Dynamic Simulation

Scene mode in YASARA structure was constructed using the default mode after the best pose of the hit in the virtual screening, which utilizes the Molegro virtual docker, was chosen. Molecular dynamics simulations were performed using YASARA dynamics employing the AMBER14 force field [28]. The scene mode was then put through MD simulations using the YASARA structure macro for the MD run's default settings [29] except for the length of the MD and the intervals for capturing pictures. This study's MD run lasted 100 ns, and the photographs were taken every 10 ps. Trajectory analysis was performed to measure the root mean square deviations (RMSDs). Complex binding of compounds with DPP-4 inhibitors was considered stable if the overall RMSD did not exceed 3.2Å [30].

RESULT AND DISCUSSION

Percentage of DPP-4 Inhibition of *Tinospora crispa* Result

DPP-4 inhibition tests on ethanol extract (96% and 50%) and *Tinospora crispa* stem water with a concentration of 100 ppm have been performed, and the results show that all extracts have activity as inhibitors of DPP-4. The results showed that 96% of the ethanol extract of *Tinospora crispa* stem had the highest percentage of DPP-4 inhibition compared to 50% of the ethanol extract and aqueous extract (Figure 1). DPP-4 inhibition activity of the three extracts was compared using one-way ANOVA statistical analysis. Results indicated a statistically significant difference in inhibition percentages ($p < 0.05$). Tukey's HSD test (post hoc analysis) showed that the DPP-4 inhibition by 96% ethanol extract was significantly higher (67.9%±2.5%) than that by both 50% ethanol extract ($P < 0.01$) and aqueous-extract ($P < 0.01$: mean inhibition = 44.8%±3.1% and 54.1%±2.8% respectively. The main components of *Tinospora crispa* were diterpenoids which were known to have an antidiabetic activity [31–33]. Borapetoside A, B, C, D, E, and F comprised most of the terpenoid glycosides. Both insulin-dependent and insulin-independent mechanisms mediated borapetoside A's hypoglycemic effects.

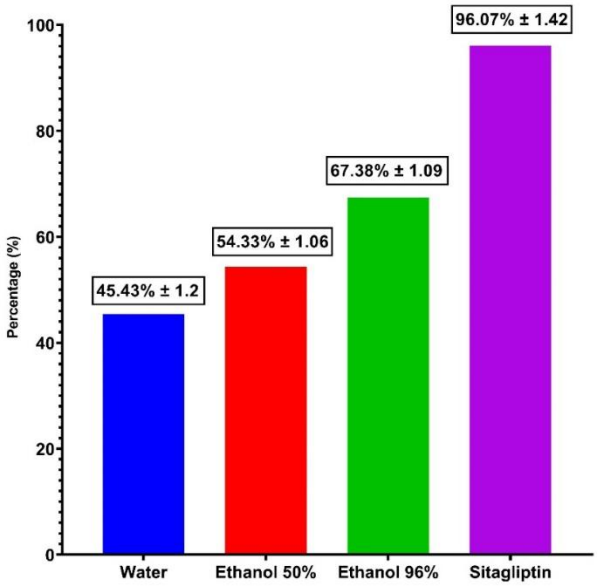


Figure 1. Inhibitory activity of DPP-4 ethanol extract (96% and 50%) and stem water of *Tinospora crispa*

In addition, it was shown that borapetoside A increased glucose consumption in peripheral tissues, decreased hepatic gluconeogenesis, and activated insulin signaling system, which helped in lowering

the plasma glucose levels [34]. Borapetoside C can improve insulin sensitivity, postpone the onset of insulin resistance, and promote glucose utilization. The hypoglycemic effects of Borapetoside C in diabetic mice may be attributed to the stimulation of IR-Akt-GLUT2 expression and the improvement of insulin sensitivity [32]. Diterpenoids are soluble in ethanol but difficult to dissolve in water, so the 96% ethanol extract has the highest percentage of a DPP-4 inhibitor since the diterpenoid content in ethanol extract is 96% which is more than the 50% ethanol extract and water. This observation was consistent with Lam et al [17]. In support of previous studies showing that high-concentration ethanol solvents optimize the extraction ratio of bioactive diterpenoids and elevate the bioactivity in vitro. Additionally, Lam et al. explained that the biological activity of diterpenoids is weak in water-based extractions owing to their low solubility, including DPP-4 inhibition. For this reason, these comparative results further highlight the potential application of tailored solvents to enhance diterpenoid yield and bioactivity.

Metabolomic Study Result

LC-MS/MS was used to conduct metabolomic analysis using the method's recommended configuration parameters. LC-MS/MS analysis presents information on precursor and fragment ions in negative ion mode $[M-H]^-$. The chromatograms obtained from LC-MS/MS were processed by using the MetaboAnalyst5 software. We got the LC-MS/MS chromatogram in a table mass array containing information on normalized peak intensity, retention time, and accurate mass of detected peaks. The complete details on statistically significant secondary metabolites contributing to the inhibition of the DPP-4 enzyme were obtained using multivariate statistical analysis methods. By grouping or splitting data on the score plot, analysis techniques like PCA and OPLS can display the correlation between each class [23]. Then, the data were analyzed by using PCA. PCA analysis (Figure 2) results present that on the score plot, PC1(54.5%) and PC2 (30.8%) explain 85.3% of the total variance in the dataset. The results of the multivariate analysis using the PCA method (Figure 2) indicate the clear separation of sample groups3 based on differences in treatment (differences in solvent). Therefore, the metabolite profiles of the three solvents were different from each other. The group separation of the ethanol extract (96%) was clear as compared to the ethanol extract (50%) and water, which indicated that the metabolite profile of the ethanol extract (96%) was different from that of the other two extracts.

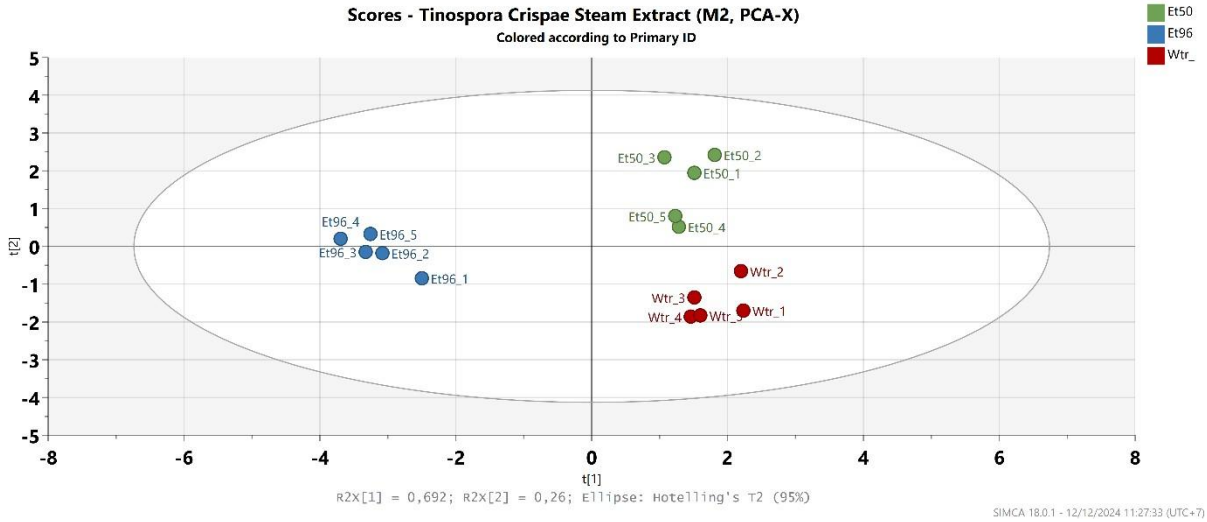


Figure 2. PCA score plots were generated using the Simca from LC-MS data on sample *Tinospora crispa* stem extract metabolites. Different colors indicate different solvents; purple (water), pink (ethanol 50%), and green (ethanol 96%)

According to the fragment pattern and m/z retrieved from METLIN [35], PubChem [36], and other databases, the potential metabolites were searched for and chosen. The compounds were identified from the peaks $[M-H]^-$ at m/z 533.2036, 535.2191, 537.1985, 551.2142, 567.2092, 581.2249, 623.2354,

and 697.2717, respectively. The compounds were Borapetoside F [$C_{27}H_{34}O_{11}$], Borapetoside C [$C_{27}H_{36}O_{11}$], borapetoside A [$C_{26}H_{34}O_{12}$], Borapetoside B[$C_{27}H_{36}O_{12}$], Rumphioside A [$C_{27}H_{36}O_{13}$], Rumphioside B [$C_{28}H_{38}O_{13}$], 6'-O-Lactoyl Borapetoside B[$C_{30}H_{40}O_{14}$], and Borapetoside D [$C_{33}H_{46}O_{16}$]. For detailed data, see Table 1, which lists down the determined molecular formulas, theoretical masses, observed m/z values, mass errors (in parts per million), and hypothesized structures for the eight metabolites found from the *Tinospora crispa* stem extract.

Table 1. Exact mass data from QTOF LC-MS/MS of *Tinospora crispa* stem

No	Mass			Assigned adduct	Formula	Identification
	M/Z Theoretical	M/Z Observed	Error (ppm)			
1	533.2017	533.2036	−3.54	$[M-H]^-$	$C_{27}H_{34}O_{11}$	Borapetoside F
2	535.2174	535.2191	−3.30	$[M-H]^-$	$C_{27}H_{36}O_{11}$	Borapetoside C
3	537.1967	537.1985	−3.50	$[M-H]^-$	$C_{26}H_{34}O_{12}$	Borapetoside A
4	551.2123	551.2142	−3.45	$[M-H]^-$	$C_{27}H_{36}O_{12}$	Borapetoside B
5	567.2072	567.2092	−3.49	$[M-H]^-$	$C_{27}H_{36}O_{13}$	Rumphioside A
6	581.2229	581.2249	−3.51	$[M-H]^-$	$C_{28}H_{38}O_{13}$	Rumphioside B
7	623.2334	623.2354	−3.17	$[M-H]^-$	$C_{30}H_{40}O_{14}$	6'-O-LactoylBorapetoside B
8	697.2702	697.2717	−2.13	$[M-H]^-$	$C_{33}H_{46}O_{16}$	Borapetoside D

The results of the OPLS score plot from the LC-MS data indicate that there has been a grouping of samples based on their activities (Figure 3). The model which correlated well with the inhibitory activity of the DPP-4 enzyme was the 96% ethanol extract of *Tinospora crispa*, located on the upper right of the X-ordinate axis. In comparison, the ethanol extract of 50% of *Tinospora crispa* was located in the middle of the X-ordinate axis, and the aqueous extract of *Tinospora crispa* had the least activity. It was located on the top left X-ordinate axis. As can be gleaned from this, it can be concluded that the OPLS model can completely separate samples based on their activities.

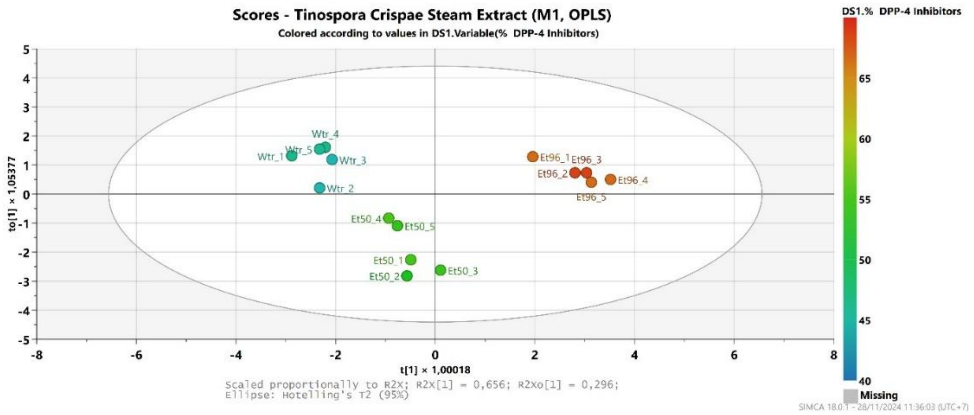


Figure 3. *Tinospora crispa* extracts in a scatter plot of LC-MS OPLS scores. The values which followed stood for extraction solvents (Et96 = ethanol 96%, Et50 = ethanol 50%, and Wtr = water, respectively) and replication. Lower to higher values are represented by the colors blue and red

We have validated the OPLS model by evaluating its quality with cross-validation ANOVA (CV-ANOVA) and permutation methods (as can be shown in Figure 3). CV-ANOVA has been performed by calculating the *p*-value to estimate the validity of the OPLS model. The requirement for the *p*-value of CV-ANOVA is below 0.05 [37]. The goal of validating the OPLS model with a permutation test is to avoid false findings and reduce the risk of overfitting. The permutation test was performed by developing a new model in which the data on the inhibition of the DPP-4 enzyme was reordered randomly. R2Y and Q2Y values of the new model were compared to the original model [23]. The permutation test results obtained two values, R2 (goodness of fit) and Q2 (excellence of prediction). If all the R2Y and Q2Y scores of the new model must be lower than that of the original model or minimize the overlap between the two groups, then the model is considered to be valid. If the $Q2 \geq 0.5$, the OPLS model has a good predictive ability [38]. The results of the OPLS LC-MS validation were considered valid since the *p*-value of CS-ANOVA is 1.06364×10^{-5}, and the Q2 value of the permutation test (Figure 4) was 0.583, R2Y and Q2Y of the new model which was consistently lower than that of the original.

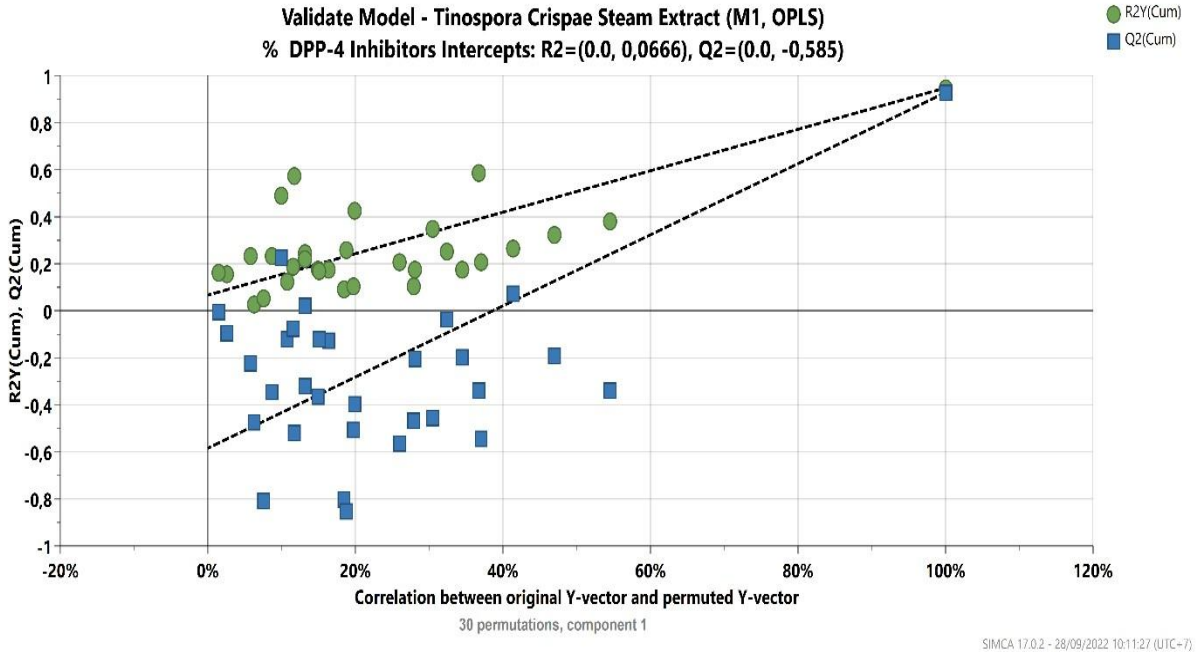


Figure 4. Permutation test model graphs for OPLS scores. 30 randomly selected permutations were used for the permutation tests

The loading plot of the variable importance projection (VIP) model shows the contribution of each X variable to the projection. VIP scores indicate that the X variable plays a vital role in explaining the variation in Y. The VIP score in this study was used to assess the contribution of compounds from *Tinospora crispa* to inhibitors of the DPP-4 enzyme. We use the VIP score ≥ 0.75 as the criterion for the variable choice [39]. The VIP score results (Figure 5) presented five compounds that have a VIP score of ≥0.75 such as Borapetoside C, 6'-O-LactoylBorapetoside B, Rumphioside A, Borapetoside D, and Rumphioside B, Borapetoside B, Borapetoside F, and borapetoside A. These compounds significantly affected the effect on the percentage of DPP-4 enzyme inhibition, but the VIP score could not determine whether the impact was positive or negative on the DPP-4 enzyme inhibition. Thus, we have analyzed it further by using the coefficient score analysis.

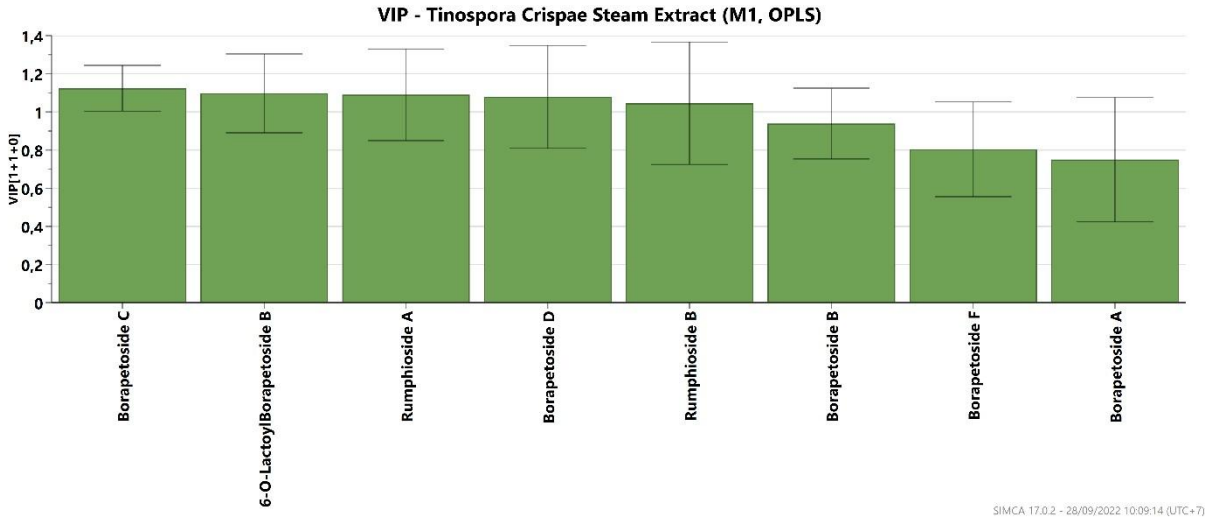


Figure 5. Results of the OPLS analysis which used the VIP plot indicating the importance of each variable in relation to the predictive component

A positive coefficient value indicates that the compound has an activity as an inhibitor of the DPP-4 enzyme. In contrast, a negative coefficient value indicates that the compound has no activity as an inhibitor of the DPP-4 enzyme. The results of the score coefficient analysis (Figure 6) provide that 6'-O-LactoylBorapetoside B, Borapetoside C, Rumphioside B, and Borapetoside D have an activity as an inhibitor of the DPP-4 enzyme since they had a positive coefficient score. Base on the score coefficient, 6'-O-LactoylBorapetoside has the best activity as a DPP-4 inhibitor. Borapetoside A, Borapetoside F, and Borapetoside B did not have activity as inhibitors of the DPP-4 enzyme since they all had negative coefficient scores.

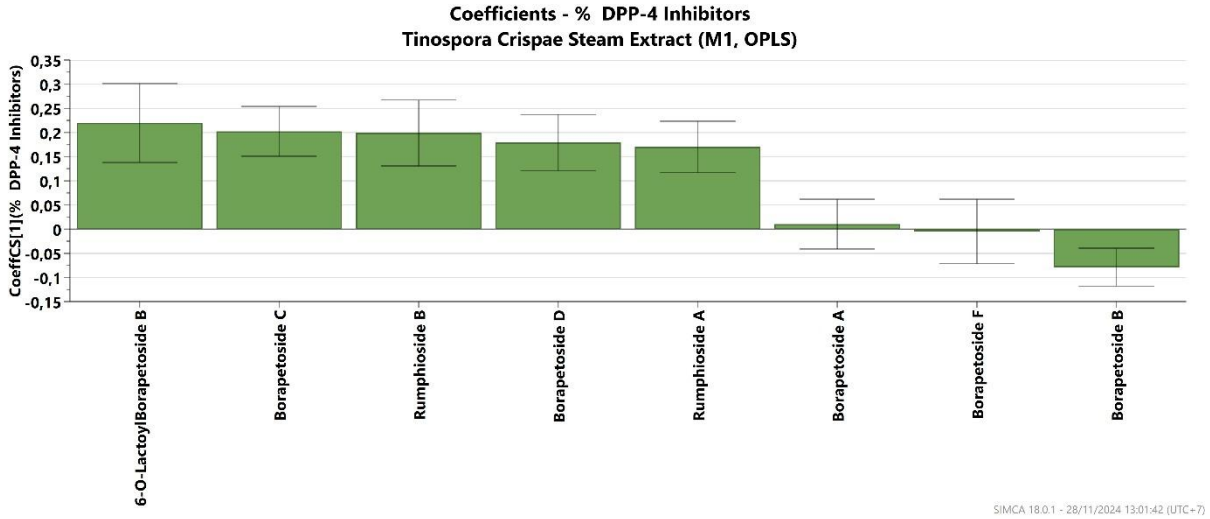


Figure 6. Profile coefficient for Y plot in identifying the substances which cause the samples to separate in the score scatter plot

Molecular Docking Simulation Result

The compounds from the stem extract of *Tinospora crispa*, which were predicted to have positing correlations as inhibitors of DPP-4 from the results of the metabolomic study, were confirmed by

molecular docking simulation. Docking validation ensures that the native ligand can re-occur with the binding site—docking validation success parameters based on the results of the root mean square deviation (RMSD) value. We used the criteria RMSD value of less than 1Å as a parameter for validation success [40]. The results of the redocking validation obtained an RMSD value of 0.5844Å, which indicates that the validation has met the excellent criteria since the RMSD value is below 1Å. Figure 7A presents the native ligand pose after docking (yellow) occupying the binding site accurately with the native ligand pose before docking (red). The bound ligand interacts with the structure very well. The ligand interacts with the structure with six hydrogen bonds Glu205, Glu206, Tyr547, Tyr631, Tyr662, Asn710 with the enzyme (Figure 7B). Similar hydrogen bonding locations were found in the prior experimental investigation. The quantity of hydrogen bonds in this investigation and those reported by the experimental study were comparable [24]. Therefore, it was discovered that the results of this study's docking of the bound ligand to the crystal structure were comparable to those of earlier experiments.

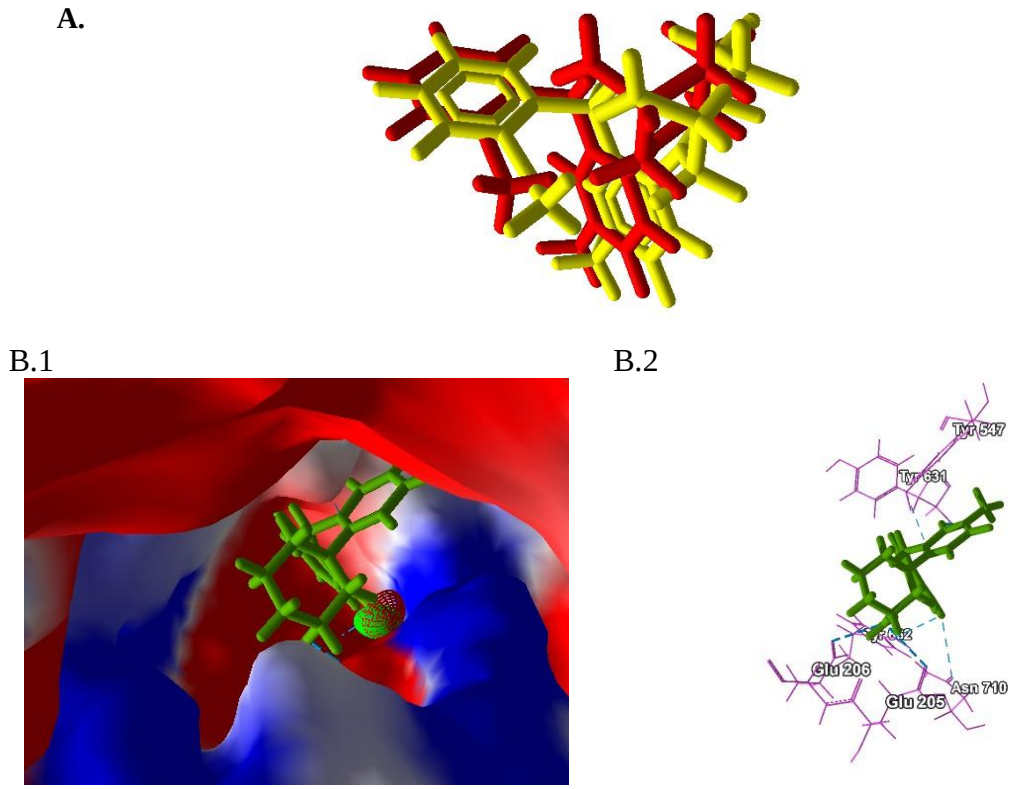


Figure 7. A) Redocking validation, native ligand (yellow) after docking and native ligand (red) before docking, B) Binding mode of DPP-4 inhibitor, B.1 its binding pose in the binding site, B.2 its 3D binding

The results of the molecular docking simulation of the five compounds (see Table 2) which are positively correlated as DPP-4 inhibitors show a rerank score of 6'-O-Lactoyl Borapetoside B, Borapetoside C, Rumphioside B, and Borapetoside D (−97.43 to −111.43 Kcal/mol) lower than alogliptin as reference (−96.02 Kcal/mol). Additionally, the hydrogen bond interaction analysis of amino acid residues showed that Rumphioside B and 6'-O-LactoylBorapetoside had hydrogen bonds Glu205 and Glu206, where the amino acid residues Glu205 and Glu206 had a vital role in inhibiting the DPP-4 enzyme [41]. However, 6'-O-Lactoyl Borapetoside B was predicted to have a better DPP-4 inhibitory activity than Rumphioside B since 6'-O-LactoylBorapetoside B, besides having amino acid residues Glu205 and Glu206 also has Ser630. However, 6'-O-Lactoyl Borapetoside B is predicted to have a better

DPP-4 inhibitory activity than Rumphioside B since 6'-O-Lactoyl Borapetoside B, besides having amino acid residues Glu205 and Glu206, also has Ser630. The amino acid residue Ser630 is one of the Triad catalytic enzymes which can increase its ability as a DPP-4 inhibitor. These molecular docking simulation results also confirm the metabolomics study's findings. Molecular dynamics simulations further confirmed the molecular docking simulation results, ensuring that the 6'-O-LactoylBorapetoside B complex bond with the DPP-4 enzyme was stable.

Table 2. Rank score and hydrogen bonds of amino acid residues (kcal/mol) of the five compounds obtained from OPLS

Number	Compounds	Rerank score (Kcal/mol)	Hydrogen bonding interactions of amino acid residues
1	Alogliptin (Reference)	−96.02	Glu205, Glu206, Tyr547, Tyr631, Tyr662, Asn710
2	Rumphioside B	−111.44	Glu205, Arg669, Glu206, Tyr662, Tyr547, Tyr666, Ser209
3	Borapetoside D	−107.09	Glu205, Tyr631, Gly632, Tyr662
4	6'-O-LactoylBorapetoside B	−106.51	Glu205, Glu206, Ser630, Arg125
5	Rumphioside A	−100.63	Glu206, Tyr547, Tyr666, His126, Ser209
6	Borapetoside C	−97.43	Glu206, Tyr547, Tyr666, His126, Ser209

Molecular Dynamic Simulation Result

The stability of the protein structure upon ligand binding and the system's equilibrium process is monitored by the radius of gyration and RMSD of the protein structure as a function of time [42]. The radius of gyration analysis (Rg) for the MD simulation indicates the compactness of the protein structure. Rg was computed here to analyze the structural stability of DPP-4 protein bound with test ligand during 100 ns simulation. The Rg plot (Figure 8) displayed an average value ranging between 26.5–27.6 Å, verifying the protein structure stabilized itself without drastic changes in compactness during the simulation. Small changes in Rg values (<0.5 Å) indicate the stable interaction of the protein with the ligand, and suggest no unfolding of the protein by the ligand. The RMSD of Cα atoms To examine the structural stability of the DPP-4 protein during the molecular dynamics (MD) simulation in YASARA, the root mean square deviation (RMSD) of the C-alpha atoms was analyzed. C alpha RMSD simulation results for 100 ns (Figure 9), we found that the protein-ligand complex experienced equilibrium after the first 10 ns, while the RMSD value was between 1.6–2.6 Å. Such a narrow range indicates that there is little perturbation of the initial conformation and thus validates the stability of the system during the short simulation time. Cα RMSD results as well, are consistent with other stability indicators, including radius of gyration (Rg), which characterizes the compactness and maintenance quality of the protein-ligand complex structure. The RMSD ligan movement (in Figure 10) is used in MD simulation to evaluate structural departure from the starting protein structure [42]. The stability of the enzyme-inhibitor interaction was investigated next using molecular dynamics simulations by utilizing the optimal pose of 6'-O-Lactoyl Borapetoside B in 3G0B obtained from the prior molecular docking studies as the beginning point. As can be seen from Figure 8, a rise in the RMSD values of the 3G0B's 6'-O-Lactoyl Borapetoside B was seen throughout 1–100 ns. In the production run of the MD simulations, the 6'-O-Lactoyl Borapetoside B RMSD values ranged from 2.068 to 2.539Å. The RMSD's average deviation was 0.223, and its standard deviation was 0.070. The poses of 6'-O-Lactoyl Borapetoside B remained steady during the MD's production run, as indicated by the RMSD values below 3.2Å during equilibrium processes. The maximum value of RMSD is 3.2Å [43], which indicates a better docking position, and the enzyme structure is not disturbed. The results of molecular dynamic simulations have confirmed the findings from metabolomic studies and molecular docking simulations that 6'-O-Lactoyl Borapetoside B has a great potential activity as a DPP-4 inhibitor.

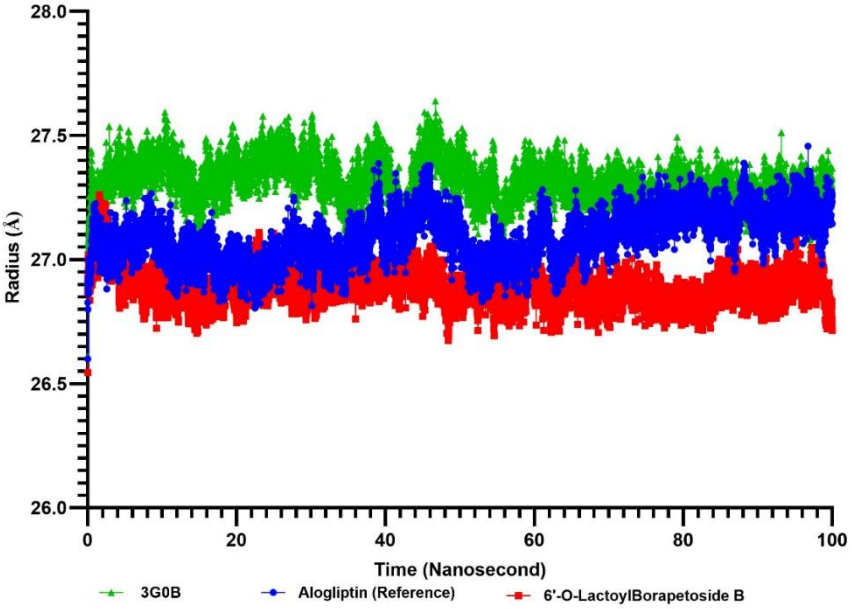


Figure 8. The radius of gyration for the ligand atoms in relation to each complex's initial structure and simulation time

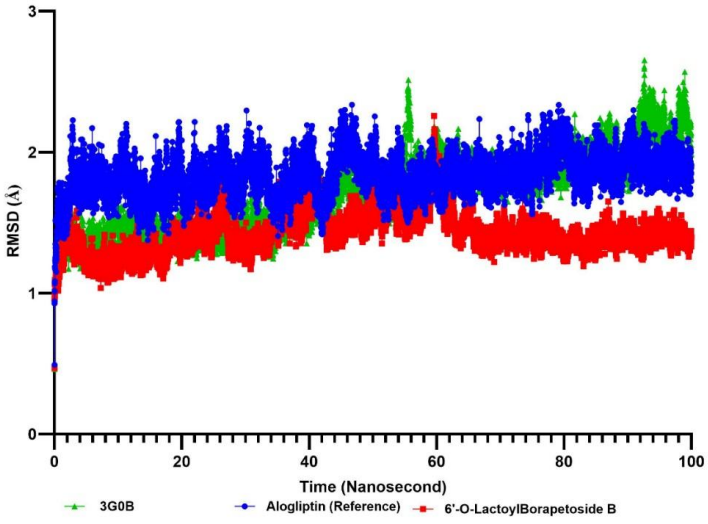


Figure 9. The RMSD Cα for the ligand atoms in relation to each complex's initial structure and simulation time

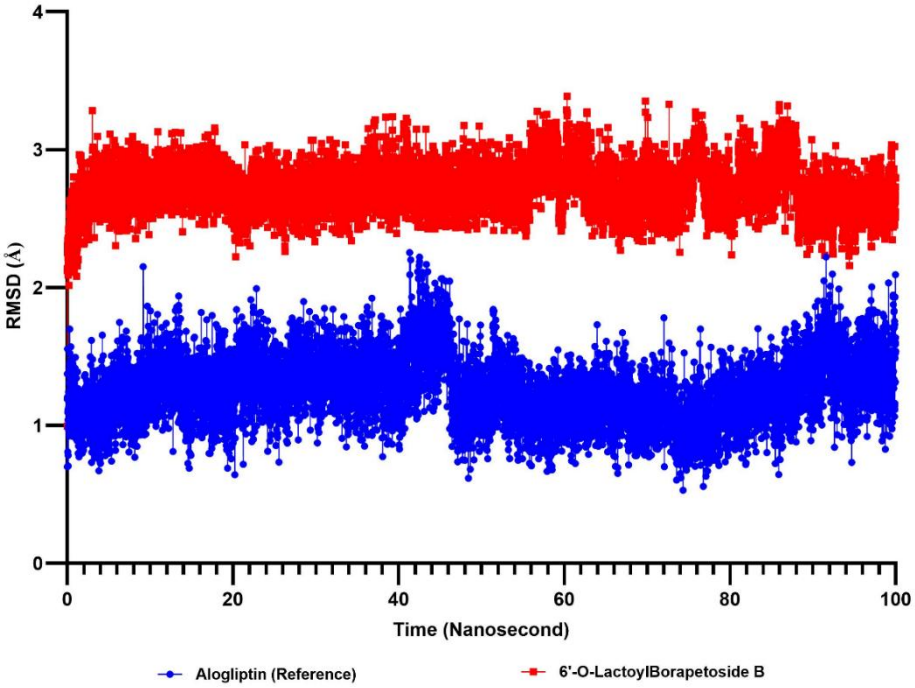


Figure 10. The RMSD for the ligand atoms in relation to each complex's initial structure and simulation time

The compound 6'-O-LactoylBorapetoside B has been identified which has excellent potential as an inhibitor of DPP-4 from *Tinospora crispa* stems from a metabolomic study approach based on LC-MS/ms, molecular docking, and molecular dynamic simulation. Information on these compounds can be used as a quality control marker.

ACKNOWLEDGEMENTS

The author would like to thank Prof. Dr. Siswandono, Apt, Department of Pharmaceuticals Chemistry, Airlangga University, for permission and support in software facilities Molegro virtual docker.

AUTHOR CONTRIBUTIONS

Concept: A.P., R.R.T.; Design: A.P., S.K.; Sources: A.P., S.K., E.M., R.R.T., N.D.Y.; Materials: S.K., E.M.; Data Collection and/or Processing: A.P., N.D.Y.; Analysis and/or Interpretation: S.K., E.M., R.R.T.; Manuscript Writing: A.P., S.K.; Critical Review: E.M., R.R.T., N.D.Y.; Other: -

CONFLICT OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

The authors declare that the ethics committee approval is not required for this study.

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J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 49(3): 631-650, 2025
Doi: 10.33483/jfpau.1582539

ORIGINAL ARTICLE / ÖZGÜN MAKALE

ORIGANUM ONITES L. ETHANOLIC EXTRACT ALLEVIATES ACETIC ACID-INDUCED ULCERATIVE COLITIS IN RATS BY REGULATING OXIDANT, INFLAMMATORY, AND APOPTOTIC MARKERS

ORİGANUM ONİTES L. ETANOLİK EKSTRAKTI OKSİDAN, İNFLAMATUAR VE APOPTOTİK BELİRTEÇLERİ DÜZENLEYEREK SIÇANLARDA ASETİK ASİT İLE İNDÜKLENEN ÜLSERATİF KOLİTİ HAFİFLETİR

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ABSTRACT

Objective: *This study aims to evaluate the antioxidant and anti-inflammatory effects of Origanum onites ethanolic extract (O. onites EE) in vitro and its effects on inflammation, apoptosis, oxidative stress, and tissue damage-related parameters in the acetic acid (AA)-induced ulcerative colitis (UC) model in rats.*
Material and Method: *Dried parts of O. onites were extracted with ethanol using the maceration method. Then, the extract's anti-DPPH and anti-ABTS free radical scavenging, anti-5-lipoxygenase (LOX) activities, and total secondary metabolic quantities were evaluated in vitro. The rat groups were determined as control + saline (C), AA + saline (A), AA + O. onites EE (O), AA + Sulfasalazine (S). The treatments were applied once a day for 3 consecutive days.*
Result and Discussion: *O. onites EE showed high amount of total phenolic, total flavonoid, and total triterpene contents, high antioxidant activity against ABTS, DPPH radicals, and anti-inflammatory effects against 5-LOX enzyme. Due to AA-induction, increased luminol-lucigenin, TNF-α, IL-1β, IL-17, TLR-9, caspase-3, caspase-9, MMP-3, macroscopic and microscopic scores attenuated by O. onites EE and sulfasalazine treatments. In conclusion, O. onites EE has a significant therapeutic effect on rats' colitis due to its total flavonoid, phenolic, and triterpene content, and its Anti-5-LOX and free radical scavenging activity.*
Keywords: *Anti-5-LOX, apoptosis, IL-17, IL-10, Origanum onites, TLR-9, ulcerative colitis*

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Submitted / Gönderilme : 08.08.2024
Accepted / Kabul : 09.04.2025
Published / Yayınlanma : 19.09.2025

ÖZ

Amaç: *Bu çalışmada Origanum onites etanolik özütünün (O. onites EE) antioksidan ve anti-inflamatuar etkileri in vitro olarak ve inflamasyon, apoptoz, oksidatif stres ve doku hasarıyla ilişkili parametreler üzerindeki etkileri asetik asit (AA) ile indüklenen sıçan ülseratif kolit (UC) modelinde değerlendirilmiştir.*

Gereç ve Yöntem: *O. onites'in kurutulmuş kısımları maserasyon yöntemi kullanılarak etanol ile ekstre edildi. Daha sonra, ekstrenin anti-DPPH ve anti-ABTS serbest radikal temizleme, anti-5-lipoksijenaz aktiviteleri ve toplam sekonder metabolik miktarları ölçüldü. Sıçan grupları kontrol + salin (C), UC + salin (A), UC + O. onites EE (O), UC + Sülfasalazin (S) olarak belirlendi. Tüm tedaviler 3 ardışık gün boyunca günde bir kez uygulandı.*

Sonuç ve Tartışma: *O. onites EE, toplam fenolik, flavonoid ve triterpen içeriklerine iyi miktarda, sahipti ve ABTS, DPPH radikallerine karşı yüksek bir antioksidan aktivite ve 5-LOX enzimine karşı yüksek bir anti-inflamatuar aktivite gösterdi. AA ile uygulanması ile artan luminol-lusigenin, TNF-α, IL-1, IL-17, TLR-9, kaspaz-3, kaspaz-9, MMP-3, makroskobik ve mikroskobik skorlar, O. onites EE ve sülfasalazin tedavileriyle azaldı. Sonuç olarak, O. onites EE'nin sıçanlarda kolit üzerinde iyi bir terapötik etkisi vardır; bu da esas olarak total flavonoid, fenolik ve triterpen içeriği ve Anti-5-LOX ve serbest radikal temizleyici aktivitesinden kaynaklanmaktadır.*

Anahtar Kelimeler: *Anti-5-LOX, apoptoz, IL-17, IL-10, Origanum onites, TRL-9, ülseratif kolit*

INTRODUCTION

Ulcerative colitis (UC) is classified as a treatment-resistant disease by World Health Organization [1]. Since UC is characterized by inflammation and ulcers in the mucosal membrane of the colon, it causes symptoms such as weight loss, diarrhea, and even bloody stools in patients. UC patients (including those whose symptoms are under control) are under significant psychosocial pressure due to the disease-related impact on their quality of life [2]. The incidence of UC, which can develop in individuals from all walks of life, regardless of gender and age (dominant age range of 10-30), due to genetic, environmental, and immunological factors, is increasing worldwide [3,4].

Oxidative stress is a condition that arises when there is an imbalance between the production of free radicals and the biological system's ability to detoxify these reactive substances or repair the resulting damage [5]. Free radicals such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) are highly reactive molecules that possess one or more unpaired electrons, making them unstable and capable of initiating chain reactions. Free radicals are naturally produced during cellular metabolic processes. In addition, external factors, such as toxins and certain chemicals, can also amplify free radical production leading to damage to cellular molecules and playing an essential role in developing inflammatory diseases [6]. Oxidative stress stimulates various transcription factors, resulting in the altered expression of genes that upregulate inflammatory pathways. The inflammation initiated by oxidative stress plays a pivotal role in the development of numerous chronic diseases including ulcerative colitis [7,8]. Inflammation is a physiological response that serves as the organism's defensive system against injury. Inflammation happens after cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) are released by activated inflammatory cells. The etiology of UC is multifaceted, with mucosal inflammation resulting from a complex interaction of several variables. Colonocytes, mucosal, and epithelial barriers are somehow disrupted, which is important in UC pathogenesis [9]. With all of this, the severity of UC can be directly linked to oxidative stress-induced tissue damage. Reactive oxygen species (ROS) are essential signaling molecules that significantly influence inflammation, highlighting their critical role in our body's response to injury and disease [10,11]. Controlling inflammation is crucial to therapy success in many chronic diseases. IL-10 deficiency or aberrant expression may boost the inflammatory response but also contribute to the development of certain autoimmune disorders [12]. IL-17 triggers a potent inflammatory response, activating granulopoiesis factors, neutrophil-specific chemokines, IL-6, pro-inflammatory cytokines (TNF-α and IL-1β), and matrix metalloproteinases (MMP) [13]. Toll-like receptor (TLR)-9 has been known to play a part in combating pathogens and inflammatory diseases [14]. IL-17 enhances recruitment of neutrophils and also epithelial barrier disruption and causes worsening mucosal inflammation in UC [15]. IL-1β also promotes neutrophil recruitment, enhances the production of other

inflammatory mediators, and disrupts epithelial integrity. Its overexpression in UC is linked to the persistence of mucosal inflammation and tissue damage [16]. MMPs are enzymes involved in extracellular matrix remodeling. In UC, overexpression of MMPs, contributes to tissue destruction by degrading the extracellular matrix and basement membrane [17]. Dysregulated TLR-9 signaling in UC contributes to excessive inflammation by activating downstream pathways caused by pro-inflammatory cytokines. Overactivation of TLR-9 in UC disrupts epithelial barrier integrity, increasing intestinal permeability. This causes luminal antigens and microbial substances to cross the mucosa, spreading chronic inflammation [18].

The fact that the medications employed in the treatment of UC do not give adequate success (treatment success rate is 33%) increases disease resistance and complicates treatment [19]. Natural medicine has existed almost since humankind's inception, and its impact on the advancement of clinical medicine is indisputable. The valuable ingredients found in medicinal plants have guided countless studies in quest of the best and most successful therapies, laying the groundwork for today's pharmaceutical industry [20]. Due to the limitations of current treatment, the search for a therapeutic regimen that can benefit from UC regulatory and preventive medications is ongoing. In this context, plants with medicinal uses known or tested to have anti-inflammatory, antioxidant, antiapoptotic, and neuroprotective properties have become the pivot. Many medicinal plant extracts are helpful in UC models [21,22]. When therapeutic plants with antioxidant, anticancer, antimicrobial, antiapoptotic, and anti-inflammatory activities are evaluated in terms of content, it is noted that the extracts of these plants contain high contents of flavonoids, phenolics, and polyphenols [23].

The genus Origanum (Lamiaceae) has around 43 species in the eastern Mediterranean region. The leaves of the *Origanum onites* (O. *onites*) plant is widely used in traditional medicine as they have a wide range of pharmacological properties [24-26]. *O. onites* contains fatty acids, hydroquinones, flavonoids, phenolic acids, sterols, pigments, terpenoids, and tocopherols. In compliance with the pharmacological studies, *O. onites* has shown the following activities: antimicrobial, antioxidant, anticancer, antidiabetic, antiviral, anti-inflammatory, hepatoprotective, and analgesic, effects, as well as effects on colitis and gastrointestinal tract [24]. *O. onites* L. essential oil, applied in various doses in experimental colitis, has been shown to have a considerable anti-inflammatory impact, especially when compared to traditional treatment approaches, particularly 5-aminosalicylic acid (5-ASA); it was shown to be more prominent than either. As a result of that study, the protective effect of oil applied rectally or intraperitoneally against colitis was shown [27]. Another study found that *O. onites* L. essential oil has antioxidant, anti-inflammatory, and antiapoptotic features [28].

The diagnosis rate in UC is low, and one of the main reasons is the scarcity of biomarkers for UC detection. In the long-term process of UC, before the ulcer or tissue deterioration becomes evident, different biochemical pathways, such as an elevation in epithelial permeability, activation of mucosal invasion of inflammatory cells, and mucosal cell apoptosis can be observed [29]. The underlying mechanisms of mucosal epithelial cell damage include TNF-α, which induces intestinal cell apoptosis [30], reactive oxidants such as superoxide and nitric oxide [31], and increased matrix metalloproteinases [32]. Studies evaluating the potential of biomarkers to increase mucosal healing and remission rates in patients and thus facilitate early intervention are of great importance. Depending on the data, biomarkers that indicate effective immune cell infiltration, and anti-inflammatory, and anti-apoptotic actions in UC should be further explored [33].

In our study, firstly, we targeted to investigate the in vitro anti-2,2-Diphenyl-1-picrylhydrazyl radical scavenging (DPPH) and anti-2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) free radical scavenging activities and anti-lipoxygenase type V (LOX-5) activity of ethanolic extract (EE) of O. onites in addition to the amounts of total phenolic (TPC), total flavonoid (TFC) and total triterpene content (TTC). Secondly, we aimed to determine the effects of *O. onites* EE in terms of inflammation markers (TNF-α, IL-1β, IL-10, IL-17, TLR-9), apoptotic caspases (casp) (casp-3 and casp-9), oxidative stress markers (Superoxide dismutase (SOD), luminol, and lucigenin chemiluminescence (CL)) and tissue damage parameters (MMP-3, and histological evaluation with H&E) in AA-induced UC model in rats.

MATERIAL AND METHOD

Chemicals and Drugs

Ketamine and sulfasalazine were purchased from Pfizer, Türkiye (Ketalar and Salazopyrin, respectively). AA (100%), folin-ciocalteu reagent, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), Folin–Ciocalteu's phenol reagent, luminol, lucigenin and ethanol were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol (≥ 99.8%) and neutral buffered formalin were purchased from Merck (Darmstadt, Germany). All ELISA kits [(TNF-α, Cat. No. E0764Ra), (IL-1β, Cat. No. E0119Ra), (IL-10, Cat. No. E0108Ra), (IL-17, Cat. No. E0115Ra), (TLR-9, Cat. No. E0082Ra), (SOD, Cat. No. E1376Ra), (casp-3, Cat. No. E1648Ra), (casp-9, Cat. No. E1898Ra), and (MMP-3, Cat. No. E031Ra)] used in the experiments were obtained from Bioassay Technology Laboratory (BT LAB, China).

Plant Collection and Extraction

The aerial parts of *O. onites* were collected from Akpınar village of Bayramiç district of Çanakkale province (Türkiye) in July 2023 by Dr. Leyla Bitiş and identified by Dr. Ahmet Doğan. The plant sample was numbered (23479), recorded, and stored in the herbarium (Marmara University (MU), Faculty of Pharmacy, MARE). Dried and ground aerial parts of *O. onites* L. (21.83 g) were extracted with 90% ethanol (3 x 100 ml) using the maceration method. The solvent of the extract was evaporated to dryness at a temperature not exceeding 45 °C using a rotary evaporator to obtain EE with 11.3% yield (g/g) [34].

Assessment of the Antioxidant Activity of EE of *O. onites*

DPPH and ABTS, which demonstrate the radical scavenging activity of *O. onites* EE, were determined briefly as follows, as implemented by Zou et al. (2011): 190 µl of ABTS or DPPH radical solution was added to each well containing 10 µl of the extract (Stock solutions in the range 5000-9.77 µg/ml) in DMSO and mixed [35]. After incubation in the dark for half an hour, the absorbance by a spectrophotometer (Agilent, BioTek, Epoch) of all samples (triplicated for each sample), including Trolox or Ascorbic acid as a reference standard, was measured at 517 or 734 nm, respectively.

Assessment of the Anti-Inflammatory Activity of EE of *O. onites*

O. onites EE (10 µl), purified water (20 µl), ethanol (20 µl), 0.1 M sodium borate buffer (25 µl), and finally 25 µl type V soybean lipoxygenase enzyme solution (20,000 U/ml) dissolved in sodium borate buffer were added to each well. After incubating the cocktail for 5 min., linoleic acid solution (100 µl, 0.6 mM) was added and mixed. Using indomethacin as a reference standard, the absorbance values of the samples in the wells were measured at the wavelength of 234 nm. The IC_{50} (µg/ml) was calculated as the amount of *O. onites* EE and indomethacin needed to inhibit LOX activity by 50% [36,37].

Assessment of the Total Secondary Metabolic Amount of EE of *O. onites*

TPC of the *O. onites* EE was analyzed using the Folin-Ciocalteu colorimetric assay as described previously [38,39]. After mixing 20 µl of Folin-Ciocalteu reagent with 10 µl of *O. onites* extract (Stock solution at 5000 µg/ml concentration), 200 µl of H_2O, and 100 µl of 15% Na_2CO_3, the absorbance value was measured at 765 nm after leaving at room temperature for 2 h. The TPC value (mg GAE/g extract) was expressed using gallic acid (GA) (Stock solutions in the range 500 - 15.63 µg/ml) as the reference standard.

TFC analysis using the method of Zhang et al. (2013) and Yildirim et al. (2019) was performed briefly as follows: 125 µl of water (distilled) was added to the well containing 25 µl of *O. onites* EE, then 7.5 µl of sodium nitrite (5% w/v) was added and waited for 6 min, and finally 15 µl of $AlCl_3$ (10% w/v) was added [37,38]. After waiting for 5 min NaOH (50 µl) and distilled water (27.5 µl) were added to the mixture. The absorbance value was read with a spectrophotometer (510 nm). TFC was calculated as mg quercetin equivalent (mg QUE/g extract).

The TTC method of Chang et al. (2012) was adapted to the microplate [40]. After preparation of 10 µl of each of the stock solutions at a concentration of 5000 µg/ml these solutions were mixed with 15 µl of vanillin-glacial acetic acid solution (5% w/v) and 50 µl of perchloric acid solution. The plate was heated at 60°C for 45 min and then cooled to room temperature in an ice-water bath. After adding

225 µl of gallic acetic acid, the absorbance of the solutions was measured at 548 nm by a spectrophotometer. Absorbances corresponding to each concentration were measured using oleanolic acid (1200-37.5 µg/ml) for the standard curve plot. The TTC of the extract was calculated from this graph, and the results were expressed as mg oleanolic acid equivalent per g extract (mg OLE /g plant extract). The extract measurements were repeated three times, and the standard curve measurements were repeated five times.

Experimental Animals

All experimental protocols were carried out after obtaining ethical approval from Marmara University Animal Experiments Local Ethics Committee (50.2023mar. Date: 2023). Sprague-Dawley strain rats (3-4 months, male and female, 200-250 g, each group consisting of 6 rats) obtained from MU (Experimental Animal Application and Research Center) were housed under the following environmental conditions one week before the experiments: in a ventilated room with 12:12 h of day/night lighting, a temperature of 22 ± 2 °C, and a constant relative humidity of 60-65%. All rats were fed *ad libitum*.

Experimental Design

In the current study, in vitro, anti-LOX activity, ABTS, and DPPH free radical scavenging activity, and the total secondary metabolic amount of the EE of *O. onites* measurements were first executed. *In vivo* experiments were conducted, and after euthanasia, *in vitro* experiments were carried out (Figure 1).

In this study, 24 rats were divided into four experimental groups by randomly allocating six rats to each group (no casualties occurred). The four experimental groups and the treatments that the rats received based on the experimental group are as follows:

i) Control group (C), and ii) AA-induced colitis model group (A): The rats under mild ether anesthesia were through 8 cm proximal to the anus, 1 ml of saline (0.9% NaCl) or 1 ml of AA (5%; v/v in distilled water) (AA) intrarectally (i.r.) using a soft polyethylene catheter for 30 s. One ml/day saline was given orally to the C and A group rats once daily for 3 days [41,42].

iii-iv) Treatment groups: The rats with AA-induced colitis were given *O. onites EE* (O) (250 mg/kg/orogastric/day) [43] or sulfasalazine (S) (100 mg/kg/orogastric/day) [44] once daily for three days following UC. All rats in the control and model groups were fasted for 12 hours before creating an UC model.

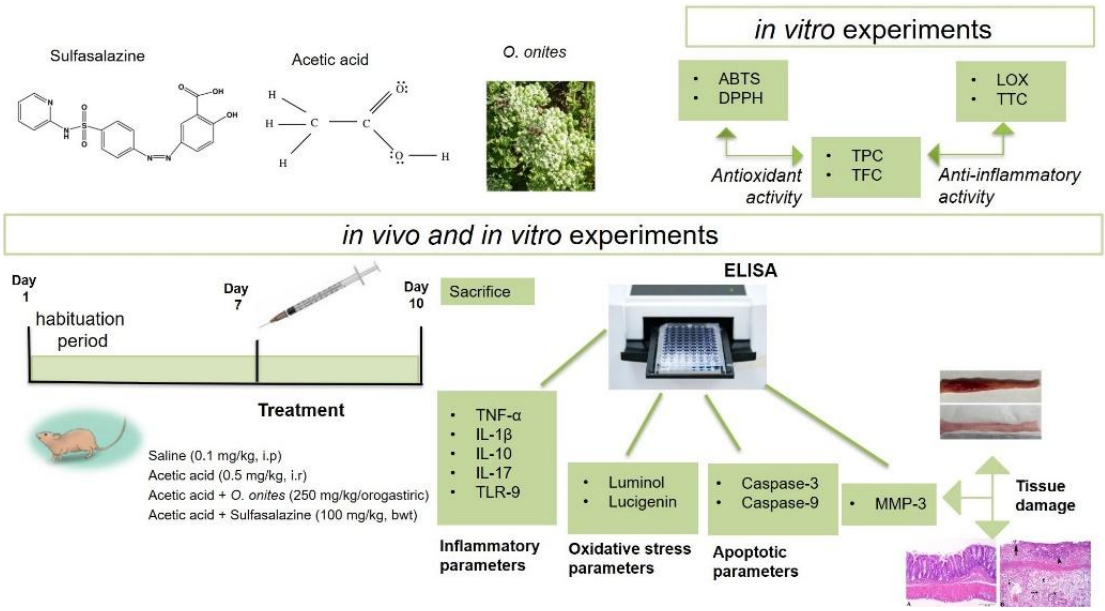


Figure 1. Experimental design

Appraisal of Colitis Degree: Macroscopic Measurement

At the end of 72 h, all rats were euthanized under ketamine and xylazine anesthesia (100 mg/kg and 10 mg/kg, respectively) and 8 cm of distal colon tissue was resected. After the luminal contents of the resected tissues were removed, they were washed with saline and allowed to dry with a blotting paper for a few sec. Macroscopic evaluation of colon lesions was carried out as instructed by Iseri et al. (2009) [45] (Table 1).

Table 1. Criteria for the macroscopic grading of lesions in the colon

Grade	Appearance
0	No injury
1	Local hyperemia
2	Hyperemia or gut wall thickening
3	Ulceration or inflammation in one area
4	Two or more areas of ulceration or inflammation
5	Major area of injury extending more than 1 cm along the length of the colon
6-10	If the damage extends more than 2 cm, increase by one for each + 1 cm

Evaluation of Colitis Severity

The Release of Cytokine and Reactive Oxygen Species of the Colon Tissues

Changes in inflammatory cytokine levels (TNF-α, IL-1β, IL-10, IL-17, and TLR-9), mitochondria-mediated apoptosis markers (casp-3 and casp-9), and tissue damage indicators (MMP-3) in colon tissue were assessed using ELISA tests carried out using a Bio Tek Epoch Microplate spectrophotometer (450 nm) (Agilent Technologies) following the manufacturer's guidelines.

The release of reactive oxygen species in the colon was determined using luminol and lucigenin probes CL levels were taken at 1-minute intervals for 5 min. The data is reported as relative light units per mg of tissue (rlu/mg) [46].

Histological Evaluation of the Colon Tissues

The rat colon tissues were fixed in formalin (10% neutral buffer), dehydrated in a graded alcohol series, and embedded in paraffin. To evaluate the overall histologic structure regarding damage/necrosis, submucosal edema, inflammatory cell infiltration, vasculitis, and perforation, 4 μm sections were cut from the tissues and stained with hematoxylin and eosin. The sections were examined under a BX51 photomicroscope and graded on a scale of 0 to 3 (n= 6) [47] (Table 2).

Table 2. Histopathologic assessment of the colonic injury was performed using the previously described criteria [39]

	None	Localised	Moderate	Severe
Damage/necrosis	0	1	2	3
Submucosal edema	0	1	2	3
Inflammatory cell infiltration	0	1	2	3
Vasculitis	0	1	2	3
	Absent		**Present**	
Perforation	0		1	
With a maximum score of 13				

Statistical Analysis

Statistical analysis of biological activity and total secondary metabolite content of *O. onites* EE was carried out using with Student's t-test. Each value was represented as mean ± SD. A p-value of less than 0.05 indicates a significant difference (GraphPad Prism 6.0, San Diego, CA, USA).

After verifying the normal distribution of other data of analyses by the Kolmogorov-Smirnov test, for group comparisons, a one-way analysis of variance (ANOVA) was performed, followed by Tukey's testing for multiple comparisons. The data were provided as mean ± SEM. (GraphPad Prism 6.0, San Diego, CA, USA). A p-value of less than 0.05 indicates a significant difference.

RESULT AND DISCUSSION

Results of the Antioxidant and Anti-inflammatory Activity of *O. onites* EE

The IC_{50} value determined by DPPH radical was 82.51 ± 0.08 for *O. onites* EE and 40.23 ± 2.08 µg/ml for ascorbic acid, which was used as a reference. According to the ABTS test result, the IC_{50} value for Trolox was used as a reference standard, and *O. onites* EE was determined as 4.54 ± 0.08 and 88.38 ± 0.06, respectively. The extract showed low antioxidant activity compared to standards ($p<0.05$, Table 3). However, in an article by Indarti et al., antioxidant activity was classified as very strong when IC_{50} was <50 µg/ml, strong when IC_{50} values were 50-100 µg/ml, moderate when IC_{50} was 101-250 µg/ml, weak when IC_{50} was 250-500 µg/ml, and inactive when IC_{50} was >500 µg/ml [48]. According to this classification, the extract exhibited strong antioxidant activity against both DPPH and ABTS radicals.

TPC was determined using the equation [y = 0.093x + 0.062 (R2: 0.9987)] obtained from the calibration curve as GAE (mg GAE/g extract). The TPC of *O. onites* EE was determined at 125.20 ± 1.50 mg GAE per g of dried extract. The TFC of *O. onites* was determined using the equation [y= 0.003x + 0.015 (R2: 0.9644)] obtained from the calibration curve as QUE (mg/g extract). The TFC of *O. onites* EE was determined at 362.10 ± 0.92 mg QUE per g of dried extract.

The TTC of *O. onites* EE was determined using the equation [y = 0.027x + 0.016 (R^2: 0.9981)] obtained from the calibration curve as OLE (mg/g extract). The total triterpene of *O. onites* EE was determined at 194.50 ± 1.35 mg OLE per g of dried extract.

O. onites EE showed substantial anti-inflammatory action against a LOX enzyme, with an IC_{50} value of 19.08 ± 0.11 µg/ml (21.42 ± 0.48 µg/ml for standard indomethacin) ($p<0.05$, Table 3).

Table 3. Biological activity and total secondary metabolite content of *O. onites* EE

Tests		*O. onites* EE	Ascorbic acid	Trolox	Indomethacin
Antioxidant activity	DPPH radical scavenging activity ($IC_{50,}$ µg ml^{-1})	82.51±0.08[b]	40.23±2.08[a]		
	ABTS radical scavenging activity ($IC_{50,}$ µg ml^{-1})	88.38±0.06[b]		4.54±0.08[a]	
Anti-inflammatory activity	Anti-lipoxygenase activity ($IC_{50,}$ µg ml^{-1})	19.08±0.11[a]			21.42±0.48[b]
Total secondary metabolite contents	Total Phenolic (mg GAE/g extract)	125.20±1.50			
	Total Flavonoid (mg QE/g extract)	362.10±0.92			
	Total Triterpene (mg OAE/g extract)	194.50±1.35			

* OOE: Aqueous ethanol extract of *Origanum onites* aerial parts; GAE: Gallic acid equivalent; QE: Quercetin equivalent; OAE: Oleanolic acid equivalent.

** Each value in the table is represented as mean ± SD (*n*=3). The values with different letter superscripts in the same row indicate statistically significant differences between the means ($p<0.05$).

Changes in Inflammatory, Oxidative Stress, Apoptotic, and Damage Parameters

The current study determined that the AA-induced colitis group (A) dramatically elevated levels of pro-inflammatory cytokines such as TNF- α ($p < 0.001$) and IL-1β ($p < 0.001$) compared to the C group, which explains the increase in inflammatory response (Figures 2A, B). It was determined that TNF-α and IL-1β levels of the O and S groups after AA induction were significantly decreased compared to the A group ($p < 0.01$-0.001). IL-10 level was significantly reduced in the A group compared to the C group (Figure 3C). IL-10 level was significantly increased in the O group ($p < 0.05$) and S groups ($p < 0.05$) compared to the A group. IL-17 ($p < 0.001$) and TLR-9 ($p < 0.01$) levels were significantly increased in the A group compared to the C group (Figures 2D and E). IL-17 and TLR-9 levels were reduced considerably in the O group ($p < 0.01$-0.001) and S groups ($p < 0.001$) compared to the A group.

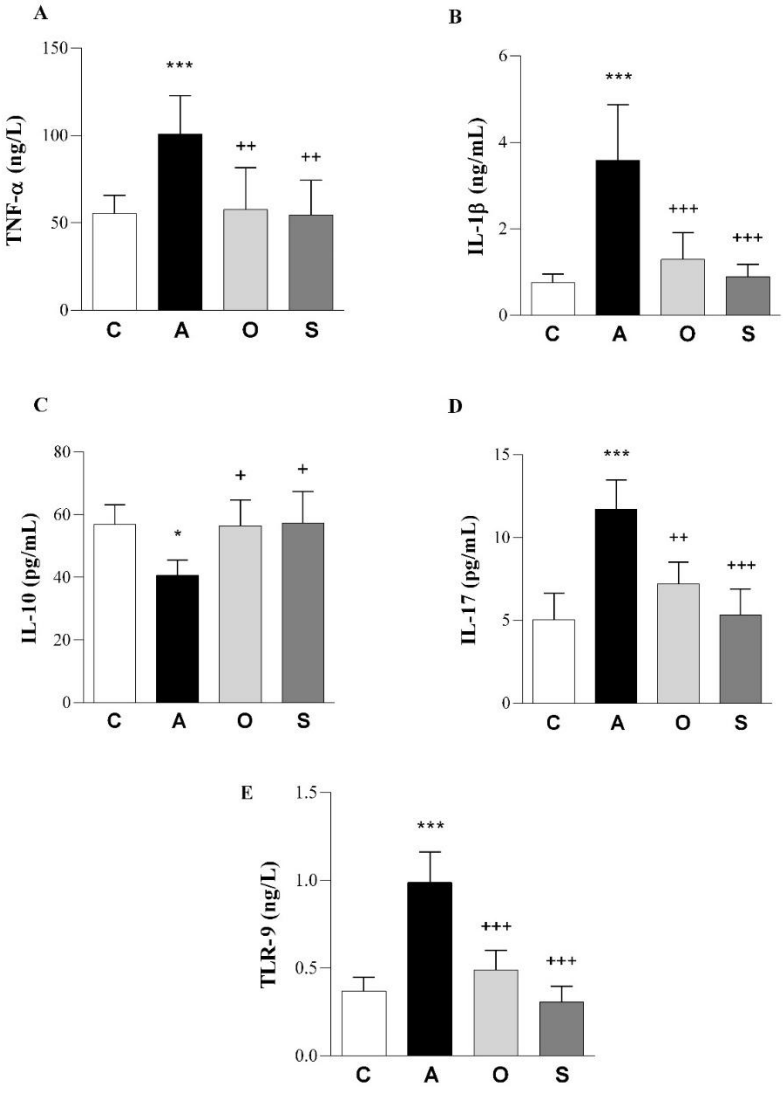


Figure 2. Evaluation of the levels of TNF-α (A), IL-1β (B), IL-10 (C), IL-17 (D), and TLR-9 (E) in colon tissue after *O. onites* EE or sulfasalazine treatment in the acetic acid-induced ulcerative colitis rat model, using ELISA method. Saline-treated control group (C), saline-treated AA-induction group (A), AA + *O. onites* treated group (O), AA + sulfasalazine treated (S) group (n=6). The results were presented as mean ± SEM. *$p < 0.05$, ***$p < 0.001$ control vs. AA; $^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$ AA vs. treatment group

The activity of SOD, an antioxidative enzyme, was not found to be considerably different between the groups (Figure 3A). In the current study, it was discovered that the rats in the A group had higher levels of lucigenin and luminol than the values in the C group ($p < 0.001$, for both markers). The A group's increased lucigenin level dropped dramatically in both treatment groups and eventually recovered to control levels ($p < 0.001$, for both treatment groups) (Figure 3B). When the amount of luminol-enhanced CL in each treatment group was compared to that in the A group, it was found that the amount had decreased in both treatment groups ($p < 0.01$ for the O group and $p < 0.05$ for the S group) (Figure 3C).

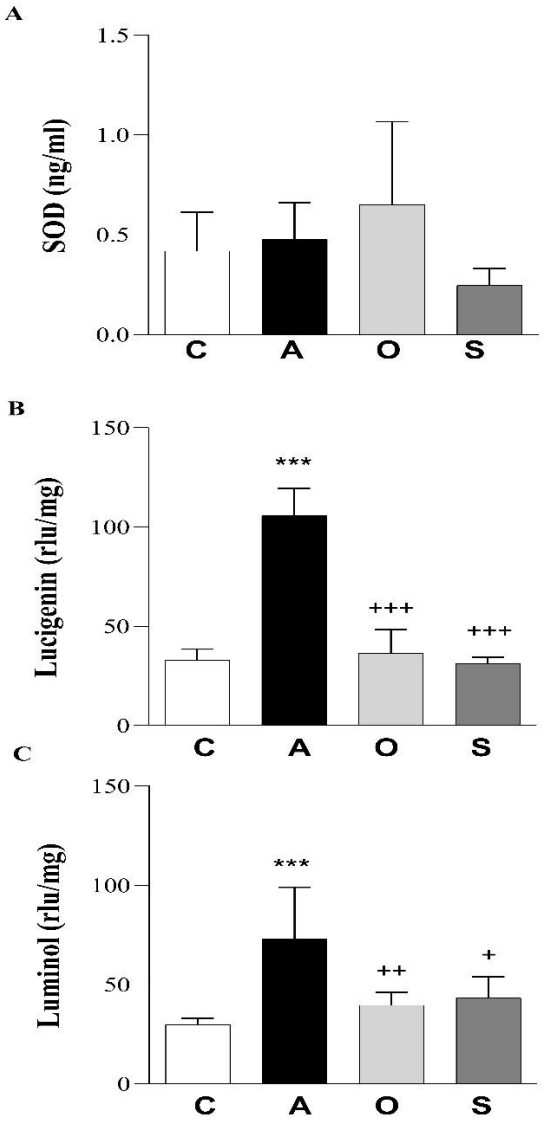


Figure 3. Evaluation of the levels of SOD (A), lucigenin (B), and luminol (C) in colon tissue after *O. onites* EE or sulfasalazine treatment in the AA-induced ulcerative colitis rat model, using ELISA method. Saline-treated control group (C), saline-treated AA-induction group (A), AA + *O. onites* treated group (O), AA + sulfasalazine treated (S) group (n=6). The results were presented as mean ± SEM. ***$p < 0.001$ control vs. AA; $^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$ AA vs. treatment group

The activity of apoptotic markers, casp-3 and casp-9, were increased significantly in the A-group compared with the C group ($p < 0.001$, for both markers). *O. onites* EE and sulfasalazine treatment ameliorated the activity values of casp-3 ($p < 0.05$ for O group, $p < 0.01$ for S group) and casp-9 ($p < 0.01$ and $p < 0.001$, respectively) almost to the C group level (Figure 4A, B).

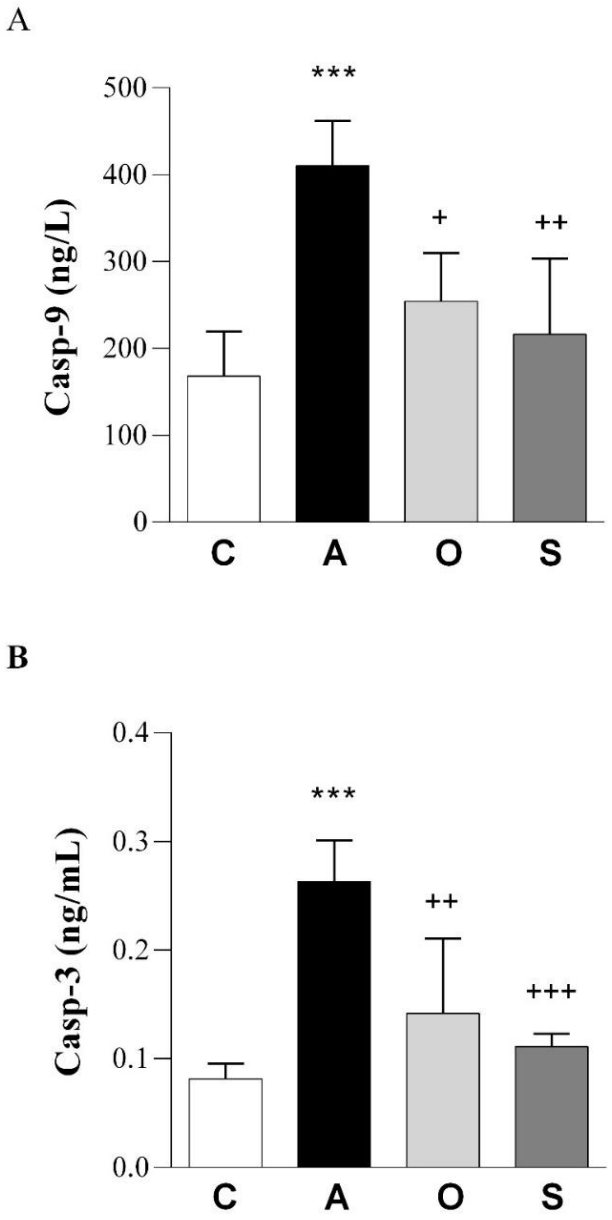


Figure 4. Evaluation of the levels of caspase-9 (A), and caspase-3 (B) in colon tissue after *O. onites* EE or sulfasalazine treatment in acetic acid-induced ulcerative colitis rat model, using ELISA method. Saline-treated control group (C), saline-treated AA-induction group (A), AA + *O. onites* treated group (O), AA + sulfasalazine treated (S) group (n=6). The results were presented as mean ± SEM. ***$p < 0.001$ control vs. AA; $^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$ AA vs. treatment group

Sample colon sections belonging to the experimental groups were photographed (Figure 5).

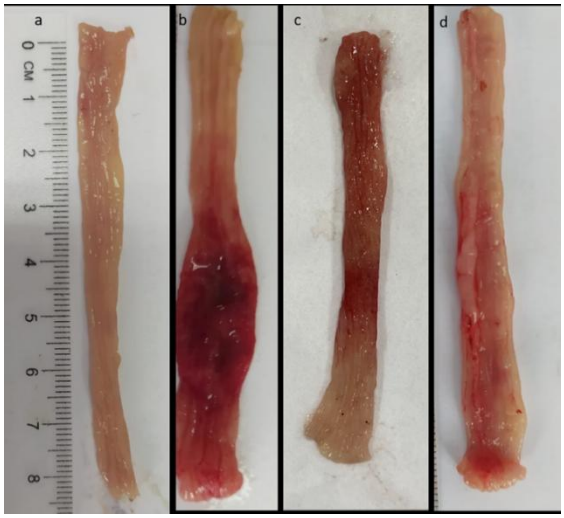


Figure 5. The effect of *O. onites* EE on macroscopic images of rat colons with AA-induced ulcerative colitis. (a) Saline-treated control group (C), (b) saline-treated AA-induction group (A), (c) AA + *O. onites* treated group (O), (d) AA + sulfasalazine treated (S) group

The A group had higher MMP-3 activity (an indicator of tissue damage) than the C group ($p < 0.01$). Both *O. onites* EE and sulfasalazine treatment groups showed decreased levels compared to the A group ($p < 0.05$, Figure 6A).

The total macroscopic damage score data obtained by evaluating the pathologic changes in the colon tissues of the experimental groups are given in Figure 6B. It was determined that macroscopic damage in the A group treated with AA was significantly higher than in the control group ($p < 0.001$). Compared to the A group, a decrease in the macroscopic damage was observed in the groups treated with *O. onites* EE or sulfasalazine ($p < 0.001$, in both treatments). In addition, while there was a significant decrease in macroscopic damage in the O and S groups compared to the A group, it was observed that this decrease in damage was almost at the level of the C group.

The total microscopic damage data obtained by evaluating the histopathologic changes in the colon tissues of the experimental groups are given in Figure 6C. It was determined that microscopic damage in the A group treated with AA was significantly higher than in the control group ($p < 0.01$). Compared to the A group, a decrease in the microscopic damage was observed in the groups treated with *O. onites* or sulfasalazine (O and S group, $p < 0.05$ and $p < 0.01$, respectively).

Figure **7**A-D illustrates how the EE of *O. onites* affected histopathological changes in the colon tissue of the experimental groups in terms of the following parameters: submucosal edema, inflammatory infiltration, and vasculitis. As a result of the microscopic examination of the colon tissue of the control (C) group, the images obtained were found to have a regular, that is, normal colonic mucosa, submucosa, and muscularis muscularis histologic structure (Figure 7A). Microscopic analysis of the AA-induced colitis group revealed markedly reduced and distorted colonic crypts, extensive inflammatory cell infiltration, vasculitis, and submucosal edema (Figure 7B). In the O group, mucosal, glandular degeneration, submucosal edema, and mild inflammatory cell infiltration were lower than in the A group (Figure 7C). In the S group, epithelial surfaces appeared more regular, and glandular structures were preserved (Figure 7D).

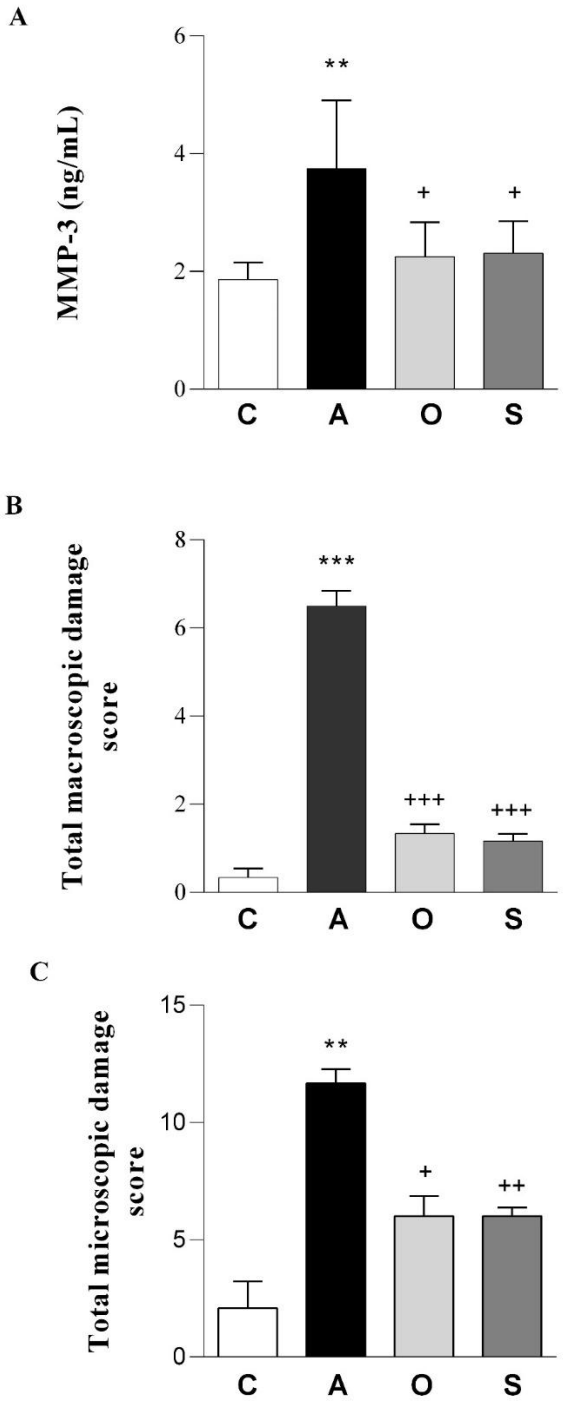


Figure 6. Evaluation of (A) MMP-3 level by ELISA method and evaluation of colon tissue macroscopically (B) and microscopically (C) after *O. onites* EE or sulfasalazine treatment in AA-induced ulcerative colitis rat model. Saline-treated control group (C), saline-treated AA-induction group (A), AA + *O. onites* treated group (O), AA + sulfasalazine treated (S) group (n=6). Results were presented as mean ± SEM. ** $p < 0.01$, ***$p < 0.001$ control vs. acetic acid; $^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$ AA vs. treatment group

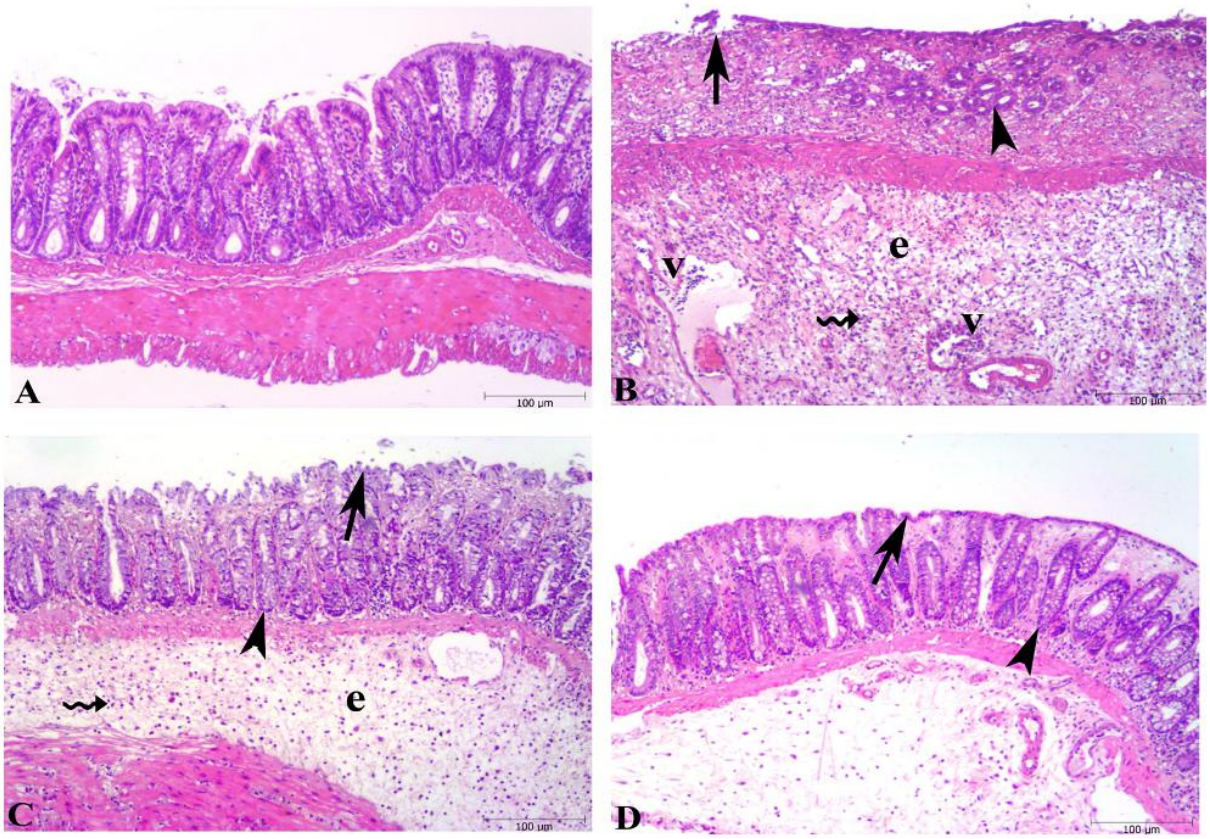


Figure 7. Histopathologically evaluated the effect of *O. onites* EE on the colon tissue of rats with acetic acid-induced UC. Representative photomicrographs shown were colons of (A) control (C group) rats [exhibited regular intestinal mucosa, submucosa, and muscularis], (B) AA (A group) rats [showed significant loss of colonic crypts (arrow) and glands (arrowhead), along with submucosal edema (e), vasculitis (v), and substantial inflammatory cell infiltration (wavy arrow)], (C) *O. onites*-treated (O group) rats [had less mucosal and glandular degradation (arrowhead), edema (e), and inflammatory cell infiltration (wavy arrow)], and (D) sulfasalazine treated (S group) rats [exhibited more homogeneous epithelial surfaces (arrow) and intact glandular structures (arrowhead)]-scale bar: 100 µm

In this study, total phenol, flavonoid, and triterpene compounds were found in very high amounts in *O. onites* EE. The extract also showed a high anti-LOX activity. In addition, it was demonstrated that *O. onites* EE while decreased colonic TNF-α, IL-1β, IL-17, casp-3, casp-9, MMP-3, TLR-9, CL (luminol and lucigenin) levels, macroscopic and microscopic damage score, and elevated IL-10 levels but had no effect on SOD levels.

Rodent models are widely used in studies evaluating the physiopathology of UC and the anti-inflammatory, antiapoptotic, antioxidative, and antiulcerative activities of new compounds. The intrarectal AA induction model is one of the most effective at mimicking the histological and biochemical characteristics of the human UC. AA induction results in acute and non-transmural inflammation of the colon tissue with overproduction of inflammatory mediators, edema, vascular dilatation, and necrosis [49]. In the present study, the model successfully mimicked human UC, which aligns with previous reports.

Multifaceted pro-inflammatory cytokines are involved in the development of UC, the worsening of the inflammatory response, and the maintenance of the inflammatory response. Downregulation of inflammatory responses is a critical component of homeostasis and is required to recover and maintain tissue and organ function in the presence of inflammation. In mice with induced colitis, elevated IL-1β, a critical biomarker associated with pyroptosis in the colon, was associated with worsening of colitis

symptoms in UC and targeting IL-1β significantly attenuated dextran sulfate sodium (DSS)-induced colitis [50,51]. TNF-α is one of the central cytokines involved in the pathogenesis of UC and promotes the production of interleukins such as IL-1β and IL-6. It also increases in AA-induced UC models [42, 50,52]. Pro-inflammatory cytokine IL-17 has an active role in mediating immune responses and inflammation [53]. Studies have revealed the importance of the IL-23/IL-17 immune axis in the pathogenesis of UC and the importance of IL-17 in the progression of UC [54]. In a study report assessment of the influence of a plant extract on the UC model, it was reported that the extract alleviated colitis by reducing inflammatory cell infiltration, TNF-α and IL-1β concentrations, and IL-17 expressions [55]. Other research results also mention the curative effect of inhibiting IL-17 on the disease in the rat UC model [56,57]. IL-17 expression levels in the intestinal mucosa and serum increased in humans with inflammatory bowel disease (IBD) [58]. In addition, a previous study in active IBD patients reported that the disease activity index, histological, and endoscopic score correlated with increased IL-17A expression [59]. Our findings are similar to those of previous studies; the IL-17 level increased in colitis, and we suggest that O. onites treatment might have anti-inflammatory properties that can regulate immunological responses by reducing IL-17 in UC. Our in vitro results supported that *O. onites* EE was rich in triterpenoid compounds known for their anti-inflammatory activities. Previously reported that oleanolic acid, a terpenoid, ameliorates UC in mice by repairing the equilibrium of Th17/Treg cells, downregulating IL-17, TNF-α, IL-1β, and increasing IL-10 levels [60]. Our data support the previous data, suggesting that the IL-17, IL-1β, and TNF-α lowering effect of *O. onites* EE may be due to its triterpene content.

Elevated cytokines and the destroying the intestinal barrier through increased apoptosis of intestinal epithelial cells contribute to UC [61]. Previously, it has been shown that Casp-3 and Casp-9 increased in UC, and inhibition of apoptosis alleviated UC by protecting the physical and immune barrier [62,63]. A limited number of colitis and toxicity studies have reported the anti-apoptotic activity of *Origanum* species L. extract in the literature [64-66]. The observed healing effect of *O. onites* EE could be the direct interruption of mitochondrial apoptotic signaling pathways.

Oxidative stress elevates lipid peroxidation, resulting in structural cellular damage, apoptosis, and necrosis [67]. The resultant necrotic/apoptotic tissues/cells exacerbate the inflammatory response, triggering the vicious cycle of UC pathogenesis [68,69]. In addition, oxidative stress, which results from an unregulated imbalance between the creation and elimination of ROS, is thought to contribute to the advancement of colon inflammation in UC [70]. The increase in luminol and lucigenin levels, which are oxidative stress factors increased by AA induction, was effectively suppressed by *O. onites* treatment. The present study revealed that *O. onites* EE has good DPPH/ABTS radical scavenging activity, which may be a reason for the decrease in the amount of luminol and lucigenin-enhanced CL.

Previously, it has been reported that pattern-recognition receptor TLR-9 expression increased in the colon with UC rats [71]. Furthermore, a positive correlation was found between the brutality of inflammation and TLR-9 expression in UC patients [72]. Our results show that *O. onites* EE and sulfasalazine treatment inhibit the AA-induced TLR-9 expression. This can lead to alleviating colonic inflammation.

SOD, an essential antioxidant enzyme, protects cells against ulcerative damage by facilitating the dismutation of superoxide anion and reducing lipid peroxidation [73]. SOD also inhibits leukocyte adherence in colon tissues. In UC, SOD enzyme levels are decreased due to oxidative damage mediated by free radicals in colon tissues [74]. However, in this study, neither the reducing effect of UC on SOD nor the extract on SOD activity was detected. It is observed that neutrophil-mediated reactive oxygen species increase more in tissues with inflammatory damage, such as colitis. On the other hand, although it was not analyzed in this study, it can be said that the nitric oxide molecule increases due to the activation of inducible nitric oxide synthase through neutrophils. Increased nitric oxide interacts with the superoxide radical to form the peroxynitrite radical, which causes greater oxidative damage. While the SOD enzyme should catalyze the conversion of superoxide radical to hydrogen peroxide, inflammation-mediated free radicals cannot meet the substrate requirement for SOD activity. Although SOD activity was found to be decreased in the colitis group in our study, the most important reason why it was not found to be significant is oxidative stress mediated by inflammation damage [75].

In the current study, the content of *O. onites* EE was analyzed qualitatively and quantitatively using TPC, TFC, and TTC assays. The blue, red, and violet colors in the tests indicated the presence of phenolic, flavonoid, and triterpene compound groups, respectively (Data not shown) [76-78]. It was also found that these compounds were present in very high amounts in the extract. Previous studies have revealed that TPC (such as phenolic acids) and flavonoids have antioxidant [79,80] and anti-inflammatory [81] activity, while triterpenes have anti-inflammatory activity [82,83]. It has also been reported that phenolic compounds [84-87] and triterpenes [83] are effective against UC. Therefore, phenolic (e.g. phenolic acids), flavonoid and triterpene compounds may have been responsible for the healing effect of *O. onites* EE against UC since it contains a good amount of these compounds.

5-LOX is an enzyme for the production of leukotrienes, which play a role in many inflammatory conditions. Hence, 5-LOX inhibitors are often used to investigate UC, and a 5-LOX inhibitor exerted curative effects in UC [88]. Injury of colonic tissue was lowered in 5-LOX-deficient mice with colitis when compared with wild-type ones [89]. In a previous study, *O. onites* essential oil was shown to have anti-LOX activity [90]. Our study showed *that O onites EE* has also anti-LOX activity.

Our macroscopic and microscopic scores data demonstrated that AA treated rats had severe colon tissue destruction, whereas *O. onites* or Sulfasalazine treatments significantly ameliorated this condition. Furthermore, our findings were very similar to the biochemical results stated above. Previously, it was reported that MMP-3 increased in UC [91,92]. Our results were consistent with previous studies that reported that MMP-3 was a potential marker of UC in terms of morphology and histopathology [91, 92]. Moreover, *O. onites* EE or Sulfasalazine treatments diminished MMP-3. Previously, a terpenic phenolic compound, carvacrol, suppressed the protein expression levels of MMP-3 in human chondrocytes [93] so we think our finding is due to the terpenoid content of the extract.

In our study, the potential therapeutic effects of *O. onites* EE in treating UC were evaluated based on antioxidant, anti-inflammatory, oxidative stress, and apoptotic markers. Our findings suggest that *O. onites* EE treatment can significantly alleviate colonic damage and decrease inflammation. In conclusion, *O. Onites* EE may be effective for treating UC.

AUTHOR CONTRIBUTIONS

Concept: D.O., A.S.; Design: D.O., A.S.; Control: A.S., L.B., A.D.; Sources: A.S., L.B, N.O.Y., A.N.H.Y., D.M.T., M.Y.; Materials: A.S., L.B., N.O.Y., D.M.T., M.Y.; Data Collection and/or Processing: D.O., A.S., N.O.Y., A.N.H.Y., D.M.T., A.D.; Analysis and/or Interpretation: D.O., A.S., A.N.H.Y., N.O.Y., M.Y. A.D.; Literature Review: D.O., A.S., A.A.; Manuscript Writing: D.O., A.S., N.O.Y., A.A.; Critical Review: D.O., A.S., M.Y., A.A.; Other: -

CONFLICTS OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

All procedures for experimental protocols of the present study involving animals were performed following the ethical standards of the institution or practice at which the studies were conducted. Approval was granted by the Ethics Committee of University Marmara (Date: 2023, No:50.2023mar). All experimental practices concerning rats were executed following "The Guide for the Care and Use of Laboratory Animals" (www.nap.edu/ catalog/5140.html) and the principles of ARRIVE guidelines.

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J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 49(3): 651-658, 2025
Doi: 10.33483/jfpau.1552812

ORIGINAL ARTICLE / ÖZGÜN MAKALE

EVALUATION OF THE ANTIBACTERIAL ACTIVITIES OF NATURAL COUMARINS FROM APIACEAE PLANTS

APIACEAE BİTKİLERİNDEN ELDE EDİLEN DOĞAL KUMARİNLERİN ANTİBAKTERİYEL AKTİVİTELERİNİN DEĞERLENDİRİLMESİ

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ABSTRACT

Objective: *This study aimed to determine the antibacterial activity of fourteen natural coumarin derivatives against various bacterial strains including Enterococcus faecalis (ATCC 29212), Staphylococcus aureus (ATCC 6538), Staphylococcus epidermidis (ATCC 12228), Escherichia coli (ATCC 8739), and Salmonella enterica serovar Typhimurium (ATCC 14028).*
Material and Method: *The antibacterial activity of the following fourteen natural coumarins: 4'-senecioyloxyostol (1), deltoin (2), smyrnioridin (3), karatavicinol (4), badrakemin (5), colladonin (6), badrakemone (7), colladonin acetate (8), anatolicin (9), 14'-hydroxycolladonin (10), 14'-hydroxybadrakemin (11), 14'-acetoxycolladonin (12), 14'-acetoxybadrakemone (13), and 14'-acetoxybadrakemin (14) was investigated by the disc diffusion method.*
Result and Discussion: *Among the microorganisms tested, Staphylococcus epidermidis showed the highest sensitivity to coumarin derivatives, while Escherichia coli was the least sensitive. Colladonin and colladonin acetate showed activity against all bacterial strains tested, except E. coli and S. enterica serovar Typhimurium, respectively. In particular, 4'-senecioyloxyostol exhibited the strongest antibacterial activity at a concentration of 64 µg/disc, with zones of inhibition ranging from 6.6 to 7.5 mm.*
Keywords: *Antibacterial, Apiaceae, biological activity, coumarin, disc diffusion method*

ÖZ

Amaç: *Bu çalışmanın amacı, on dört doğal kumarin türevinin Staphylococcus epidermidis (ATCC 12228), Staphylococcus aureus (ATCC 6538), Enterococcus faecalis (ATCC 29212), Escherichia coli (ATCC 8739) ve Salmonella enterica serovar Typhimurium (ATCC 14028) bakteri suşlarına karşı antibakteriyel aktivitesini değerlendirmektir.*
Gereç ve Yöntem: *On dört doğal kumarinin antibakteriyel aktivitesi: 4'-senecioyloxyostol (1), deltoin (2), smyrnioridin (3), karatavicinol (4), badrakemin (5), colladonin (6), badrakemone (7), colladonin acetate (8), anatolicin (9), 14'-hydroxycolladonin (10), 14'-hydroxybadrakemin (11),*

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Submitted / Gönderilme : 22.09.2024
Accepted / Kabul : 15.04.2025
Published / Yayınlanma : 19.09.2025

14'-acetoxycolladonin (12), 14'-acetoxybadrakemone (13) ve 14'-acetoxybadrakemin (14) disk difüzyon yöntemi kullanılarak belirlenmiştir.
Sonuç ve Tartışma: *Test edilen mikroorganizmalar arasında, Staphylococcus epidermidis kumarin türevlerine karşı en yüksek duyarlılığı gösterirken, Escherichia coli en az duyarlılık gösteren suş olarak belirlenmiştir. Colladonin ve colladonin asetat, sırasıyla E. coli ve S. enterica serovar Typhimurium bakterilerine karşı antibakteriyel aktivite göstermezken, test edilen diğer tüm bakteri suşlarına karşı antibakteriyel aktivite göstermiştir. Özellikle, 4'-senecioyloxyostol 64 µg/disk konsantrasyonunda en güçlü antibakteriyel aktiviteyi 6.6 -7.5 mm zon çapları ile göstermiştir.*
Anahtar Kelimeler: *Antibakteriyel, Apiaceae, biyolojik aktivite, disk difüzyon yöntemi, kumarin*

INTRODUCTION

People have used natural remedies to treat diseases since ancient times, and many contemporary medicines have been developed using natural product leads [1].

The screening of plant extracts and natural products is still offering the best choices for finding new structures that could lead to active agents in various human diseases. Researchers are actively working on discovering and developing new drugs from natural product sources to treat numerous illnesses [2-5].

It was reported that approximately 50,000 men, women, and children die of microbial infections around the world every day, and 700 thousand deaths occur yearly from multidrug-resistant (MDR) bacterial strains [6,7,8]. The increasing antibiotic resistance of microorganisms has revived intensive studies on the antimicrobial activities of many plant extracts and phytochemicals as alternative strategies for treating bacterial infections [1,9,10].

Phenolic acids, tannins, quinones, saponins, terpenoids, flavonoids, coumarins, and alkaloids found in natural resources are reported to be responsible for antimicrobial activity [11].

Coumarins are the largest class of 1-benzopyran derivatives and a significant group of natural compounds widely distributed in a large number of plants and have several biological activities that include anti-inflammatory, anticoagulant, antibacterial, antimycobacterial, antioxidant, antifungal, antiviral, anticancer, antihypertensive, anticonvulsant, antiadipogenic, antiallergic, anticholinesterase and antihyperglycemic [12-16]. It was reported that more than 1300 coumarin derivatives have been isolated from natural resources [5]. Several coumarin derivatives have clinical applications, such as warfarin, phenprocoumon, and acenocoumarol are well-known as potent anticoagulant agents; novobiocin and armillarisin A are antibiotics; ensaculin is also well-known for its anti-dementia effects; and hymecromone is a choleretic and antispasmodic agent [6]. Antibacterial activities of natural and synthetic coumarin derivatives against several microorganisms have been reported [7,14,17-33].

As a part of our investigations on the biological activities of coumarins, 14 natural coumarin derivatives, namely 4'-senecioyloxyostol (**1**), deltoin (**2**), smyrnioridin (**3**), karatavicinol (**4**), badrakemin (**5**), colladonin (**6**), badrakemone (**7**), colladonin acetate (**8**), anatolicin (**9**), 14'-hydroxycolladonin (**10**), 14'-hydroxybadrakemin (**11**), 14'-acetoxycolladonin (**12**), 14'-acetoxybadrakemone (**13**) and 14'-acetoxybadrakemin (**14**) (Fig. 1), were examined for their antimicrobial activities against *E. coli* (ATCC 8739), *S. aureus* (ATCC 6538), *E. faecalis* (ATCC 29212), *S. epidermidis* (ATCC 12228), and *S. enterica* serovar Typhimurium (ATCC 14028).

MATERIAL AND METHOD

Coumarin Derivatives

The coumarin derivatives used in this study were isolated from the roots of the following Apiaceae species: *Neocryptodiscus papillaris* (Boiss.) Herrnst. & Heyn; 4'-senecioyloxyostol (**1**); *Petroedmondia syriaca* (Boiss.) Tamamsch; deltoin (**2**), and smyrnioridin (**3**); *Heptaptera anatolica* (Boiss.) Tutin; karatavicinol (**4**), badrakemin (**5**), colladonin (**6**), badrakemone (**7**), anatolicin (**9**), 14'-hydroxycolladonin (**10**), 14'-acetoxybadrakemin (**14**); *Heptaptera cilicica* (Boiss. & Balansa) Tutin; colladonin acetate (**8**), 14'-acetoxycolladonin (**12**), 14'-acetoxybadrakemone (**13**); *Heptaptera triquetra* (Vent.) Tutin; 14'-hydroxybadrakemin (**11**) [34-38].

Determination of Antibacterial Properties

Disc diffusion method was performed to investigate the antibacterial properties of coumarin derivatives [39]. The test microorganisms used in this study *S. epidermidis* (ATCC 12228), *S. aureus* (ATCC 6538), *E. faecalis* (ATCC 29212), *E. coli* (ATCC 8739), and *S. enterica* serovar Typhimurium (ATCC 14028) were obtained from İstanbul Medipol University, School of Pharmacy, Microbiology Research Laboratory.

Test microorganisms were inoculated into Tryptic Soy Broth (Neogen, United Kingdom) and incubated for 24 hours at 37°C. Then, they were inoculated onto 5% Sheep Blood Agar (Becton Dickinson GmbH, Germany) and incubated for 24 hours at 37°C.

The 24h culture was used to obtain a bacterial suspension and adjusted to 0.5 McFarland using a densitometer (Biosan). For the investigation of the antibacterial properties of 14 natural coumarin derivatives, the disc diffusion method was applied (Figure 1). Each of the sterile discs (Bioanalyse, 6 mm in diameter) was impregnated with 20 µl of each natural coumarin derivative. Each of 0.5 McFarland adjusted bacterial suspension was inoculated onto Mueller Hinton Agar (Neogen, United Kingdom) plates with sterile swabs. After that, the impregnated discs were gently pressed onto the inoculated Mueller Hinton Agar plates. The incubation period was 24 hours at 37°C. Finally, the diameters of the inhibition zones (IZs) were measured with a digital caliper. Commercially available antibiotic discs (Amoxicillin 25 µg) were positive control. Discs impregnated with DMSO were used as a negative control. To obtain precise results, assays were performed three times under aseptic conditions. The diameters of the IZs were taken as the average of the three replicates.

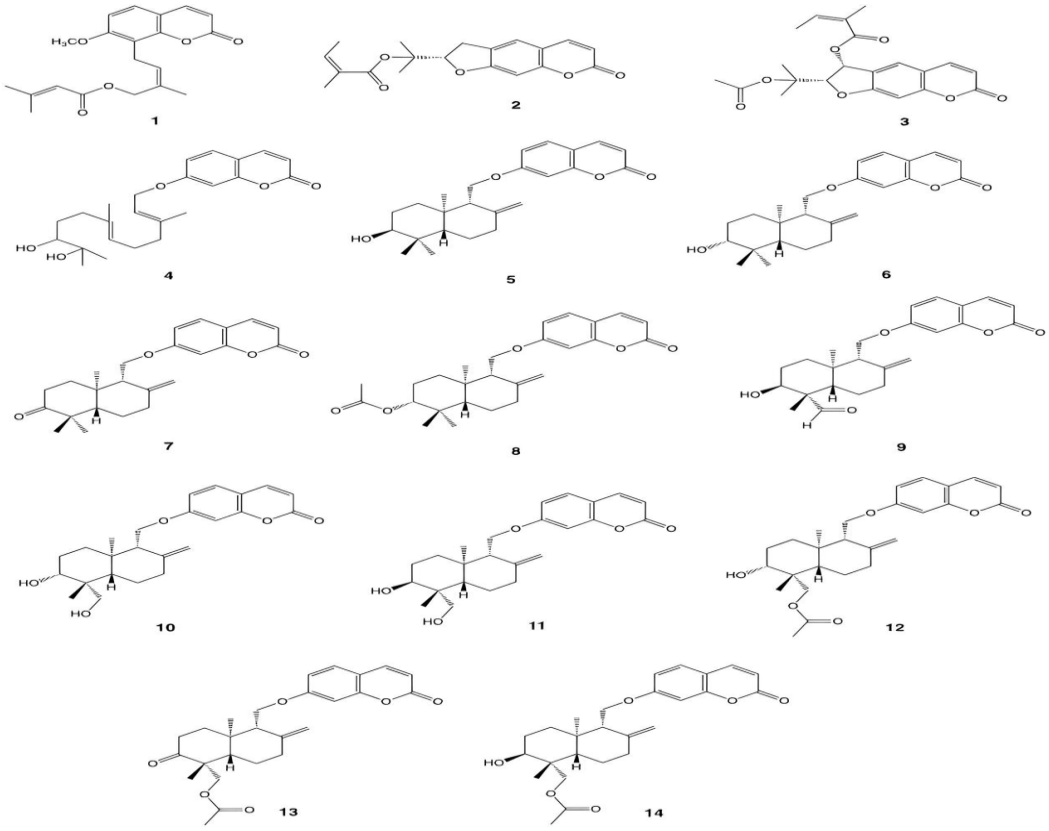


Figure 1. Structures of the natural coumarins from Apiaceae plants used in the antibacterial activity testing [4'-senecioyloxyostol (**1**), deltoin (**2**), smyrnioridin (**3**), karatavicinol (**4**), badrakemin (**5**), colladonin (**6**), badrakemone (**7**), colladonin acetate (**8**), anatolicin (**9**), 14'-hydroxycolladonin (**10**), 14'-hydroxybadrakemin (**11**), 14'-acetoxycolladonin (**12**), 14'-acetoxybadrakemone (**13**) and 14'-acetoxybadrakemin (**14**)]

RESULT AND DISCUSSION

This research study investigated the antibacterial properties of 14 natural coumarin derivatives, namely 4'-senecioyloxyostol (**1**), deltoin (**2**), smyrnioridin (**3**), karatavicinol (**4**), badrakemin (**5**), colladonin (**6**), badrakemone (**7**), colladonin acetate (**8**), anatolicin (**9**), 14'-hydroxycolladonin (**10**), 14'-hydroxybadrakemin (**11**), 14'-acetoxycolladonin (**12**), 14'-acetoxybadrakemone (**13**) and 14'-acetoxybadrakemin (**14**), against *S. epidermidis*, *S. aureus*, *E. faecalis*, *E. coli* and, *S. enterica* serovar Typhimurium. The antibacterial activities of these natural coumarin derivatives against test microorganisms were represented in Table 1.

Table 1. Inhibition zone diameters of 14 natural coumarin derivatives against test microorganisms

Inhibition zone diameters (mm)					
Coumarin derivatives	Test microorganisms				
	S. aureus ATCC 6538	*E. feacalis* ATCC 29212	*S. epidermidis* ATCC 12228	*E. coli* ATCC 8739	*S.* Typhimurium ATCC 14028
Badrakemin (200 µg/disc)	0	0	0	0	0
Colladonin (200 µg/disc)	7.01±1.38	6.36±0.35	6.84±0.33	0	6.67±0.85
14'-acetoxybadrakemin (200 µg/disc)	8.37±0.28	6.44±0.76	7.12±0.53	0	0
Anatolicin (200 µg/disc)	7.72±0.31	0	0	0	0
14'-hydroxycolladonin (200 µg/disc)	6.66±0.57	0	8.16±0.49	0	0
Badrakemone (200 µg/disc)	7.45±0.02	7.50±0.35	9.59±0.56	0	0
Karatavicinol (200 µg/disc)	6.53±0.46	0	9.75±1.88	0	7.59±1.42
4'-senecioyloxyostol (64 µg/disc)	6.64±0.62	0	7.48±0.48	0	7.15±0.34
14'-acetoxycolladonin (200 µg/disc)	0	0	8.17±0.72	7.90±0.02	0
Deltoin (128 µg/disc)	7.62±0.54	0	7.30±0.55	0	6.83±0.72
14'-hydroxybadrakemin (200 µg/disc)	0	8.04±0.34	6.88±0.95	8.97±0.46	0
Colladonin acetate (152 µg/disc)	7.53±0.27	6.32±0.56	7.51±0.40	7.52±0.30	0
14'-acetoxybadrakemone (120 µg/disc)	8.20±1.57	7.10±0.96	7.02±0.12	0	0
Smyrnioridin (320 µg/disc)	0	0	6.51±0.60	0	0
Amoxicillin (25 µg/disc)	19.17±0.79	17.00±0.50	28.53±0.50	20.80±0.2	20.43±0.60
DMSO	0	0	0	0	0

±Standard deviation

In the light of the findings, the most susceptible against the coumarin derivatives used in this study was shown by *S. epidermidis* among the test microorganisms, but *E. coli* has been shown the least susceptibility against the coumarin derivatives. When comparing the antibacterial activity of the coumarin derivatives, colladonin and colladonin acetate showed activity against all the bacteria strains tested except *E. coli* and *S. enterica* serovar Typhimurium respectively. Deltoin, karatavicinol, and 4'-senecioyloxyostol have shown antibacterial activity on the same bacteria, which were *S. aureus, S. epidermidis,* and *S. enterica* serovar Typhimurium. Besides, it was found that 14'-acetoxybadrakemin, badrakemone, and 14'-acetoxybadrakemone have an antibacterial activity against to the same bacteria, which were *S. aureus, E. feacalis,* and *S. epidermidis.* 14'-hydroxycolladonin showed antibacterial activity against to *S. aureus* and *S. epidermidis* whereas 14'-acetoxycolladonin against to *S. epidermidis* and *E. coli.* Anatolicin and smyrnioridin showed activity against only one bacteria strain tested, *S. aureus* and *S. epidermidis*, respectively. Badrakemin showed no inhibition on the all test microorganisms.

In previous studies, the antimicrobial activities of some natural coumarins were determined against to Gram-negative and Gram-positive bacterial strains. They reported that as strongly or moderately active on these microorganisms [27-29,32,33,40-44]. It has been declared that the presence of the prenyl residue leads to increased antibacterial activity of the coumarin derivatives [43,45]. In agreement with these observations, despite its 64 µg/disc concentration, 4'-senecioyloxyostol (a prenylated coumarin derivative) exhibited better antibacterial activity, with inhibition zones ranging from 6.6-7.5 mm (Table 1), which is more potent than the other compounds tested in this study.

Hitherto, deltoin (**2**), and smyrnioridin (**3**), karatavicinol (**4**), badrakemin (**5**), colladonin (**6**), badrakemone (**7**), colladonin acetate (**8**), anatolicin (**9**), 14'-hydroxycolladonin (**10**), 14'-hydroxybadrakemin (**11**), 14'-acetoxycolladonin (**12**), 14'-acetoxybadrakemone (**13**), 14'-acetoxybadrakemin (**14**) have not been tested against the bacteria strains including *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Salmonella* Typhimurium, *Escherichia coli* and *Enterococcus faecalis* and 4'-senecioyloxyostol (**1**) against *Salmonella* Typhimurium. Previously, 4'-senecioyloxyostol has been examined against nine standard strains, including *E. coli* ATCC 10799, *S. epidermidis* ATCC 12228, *S. aureus* ATCC 25923, and *E. faecalis* ATCC 29212, and five clinically isolated strains and found to be active on all test microorganisms [27].

According to our literature research, there is some antibacterial evaluation of natural pyranocoumarins, furanocoumarins, and prenylated coumarins but no study dealing with the antimicrobial activities of the sesquiterpene coumarin derivatives except ferulsinaic acid [27-29,40-44]. Thereby, this is the 1st study on the antimicrobial activities of the sesquiterpene coumarin derivatives tested in this research.

AUTHOR CONTRIBUTION

Concept: F.T., A.H., A.İ., A.Ç.; Design: F.T., A.H., A.İ., A.Ç., M.M.; Control: F.T., A.H., A.İ., A.Ç.; Sources: F.M., D.A.A., F.T., A.H., A.İ., A.Ç.; Materials: F.T., A.H., A.İ., A.Ç., F.M., D.A.A., M.M.; Data Collection and/or Processing: F.T., A.H., A.İ., A.Ç.; Analysis and/or Interpretation: F.T., A.H., A.İ., A.Ç.; Literature Review: F.T., A.H., A.İ., A.Ç., F.M., D.A.A.; Manuscript Writing: F.T., A.H., A.İ., A.Ç., M.M.; Critical Review: F.T., A.H., A.İ., A.Ç., F.M., D.A.A. M.M.; Other: -

CONFLICT OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

ETHICAL APPROVAL

The authors declare that ethics committee approval is not required for this study.

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J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 49(3): 659-671, 2025
Doi: 10.33483/jfpau.1603920

ORIGINAL ARTICLE / ÖZGÜN MAKALE

DEVELOPMENT AND VALIDATION OF AN HPLC METHOD FOR THE QUANTIFICATION OF BENZOCAINE AND FUSIDIC ACID SIMULTANEOUSLY IN MICROEMULSION FORMULATION

MİKROEMULSİYON FORMÜLASYONUNDA BENZOKAİN VE FUSİDİK ASİT MİKTAR TAYİNİ İÇİN HPLC METODU GELİŞTİRİLMESİ VE VALİDASYONU

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ABSTRACT

Objective: *The purpose of this study was to develop and validate an analytical technique for quantification in the microemulsion formulation containing fusidic acid and benzocaine developed for the treatment of mild and moderate wounds.*
Material and Method: *Acetonitrile and acetic acid (0.1%) were used as a mobile phase in the HPLC method. The flow rate and injection volume were determined. The ICH Q2 guideline was followed in the validation of the developed method. Benzocaine (2%) and fusidic acid (2%) were added to the blank microemulsion prepared using ethyl oleate, propylene glycol, ethanol, and Cremophor EL, and the selectivity of the HPLC method for both these substances was investigated.*
Result and Discussion: *The ratio of acetonitrile to acetic acid (0.1%) was determined to be 70:30 (v/v). The injection volume of 20 µl and the flow rate of 1 ml·min^{-1} were set. The developed method, which demonstrated a high correlation coefficient (0.9999) and a low variation coefficient (<2%), was simple, inexpensive, convenient, selective, and suitable for the analysis of both substances at the same wavelength (210 nm).*
Keywords: *Analytical method, benzocaine, fusidic acid, HPLC, microemulsion*

ÖZ

Amaç: *Bu çalışmanın amacı, hafif ve orta dereceli yaraların tedavisi için geliştirilen benzokain ve fusidik asit içeren mikroemülsiyon formülasyonunda miktar tayini için analitik yöntem geliştirilmesi ve valide edilmesidir.*
Gereç ve Yöntem: *HPLC metodunda mobil faz olarak asetonitril ve asetik asit (0.1%) kullanıldı. Akış hızı ve enjeksiyon hacmi belirlendi. Geliştirilen metodun doğrulamasında ICH Q2 kılavuzuna uyulmuştur. Etil oleat, propilen glikol, etanol ve Cremophor EL kullanılarak hazırlanan boş mikroemülsiyona benzokain (%2) ve fusidik asit (%2) ilave edildi ve HPLC metodunun bu iki madde için seçiciliği araştırıldı.*
Sonuç ve Tartışma: *Asetonitrilin asetik aside (%0.1) oranı 70:30 (h/h) olarak belirlendi. Enjeksiyon hacmi 20 µl ve akış hızı 1 ml·dk^{-1} olarak ayarlandı. Yüksek korelasyon katsayısı (0.9999)*

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Submitted / Gönderilme : 21.12.2024
Accepted / Kabul : 15.04.2025
Published / Yayınlanma : 19.09.2025

ve düşük varyasyon katsayısı (<%2) gösteren geliştirilen yöntem basit, ucuz, seçici, kullanışlı ve her iki maddenin aynı dalga boyunda (210 nm) analizi için uygundu.
Anahtar Kelimeler: *Analitik metot, benzokain, fusidik asit, HPLC, mikroemülsiyon*

INTRODUCTION

The skin, which constitutes approximately 15-16% of body weight, is the largest organ in the body and serves as a protective barrier in opposition to external factors. However, it may be damaged due to various internal and external influences, leading to the loss of its integrity and the formation of acute or chronic wounds [1,2]. The effectiveness of active pharmaceutical agents in wound healing can be influenced by various factors such as age, gender, physiological conditions, etc. as well as dosage form and administration route. Novel drug carrier systems have the potential to improve treatment effectiveness, reduce costs, and enhance patient compliance compared to conventional dosage forms.

Dermal delivery, which provides high patient compliance and easy application, is an important alternative compared to oral administration due to the advantages that high amounts of drugs are localized in the application area, decreasing the systemic effect and, consequently, reducing systemic side effects [3,4]. Microemulsions are modern drug delivery systems with nano-sized vesicles consisting of water and oil phases. High drug loading capacity, the ability to present both oil- and water-soluble drug substances simultaneously, easy preparation, and thermodynamically stable are among the advantages of microemulsions that have made them stand out for dermal delivery in recent years. They provide high permeability with their nano size and the penetration-enhancing properties of surfactants and cosurfactants in their structure [5]. Thanks to these attractive advantages, improving pharmacological efficacy by increasing drug permeability is considered a favorable option for the delivery of different active substances in wound treatment [6-9].

Benzocaine, a local anesthetic, has a fairly widespread use for pain relief in the treatment of local and temporal wounds and burns [10]. Benzocaine, when applied dermally, has a short-duration anesthetic effect because it is hydrolyzed by esterases and undergoes enzymatic biotransformation inside the skin layers rather quickly [11]. Therefore, it requires the need for frequent application for the aimed effectiveness. It is also stated that the onset of the effect of benzocaine in the topical application is between 30 min and 2 h and that its permeability is poor [12,13].

Fusidic acid, a narrow-spectrum steroid antibiotic that is predominantly active against gram-positive bacteria, usually has an effect by inhibiting the protein synthesis of bacteria [14]. This molecule, which has bactericidal and bacteriostatic activity, is used in topical dosage forms for treating mild and moderate infections such as erythrasma, burns, and painful wounds [15]. Thanks to the unique advantages of the microemulsion system, it was shown that microemulsion formulations of these active substances have the potential for clinical use by providing an extended and increased therapeutical effect [8,16]. Moreover, microemulsion formulations in which these two active substances coexist were developed by our group [6].

High performance liquid chromatography (HPLC) is a very convenient and reliable technique that is often preferred as a determination and quantification method in the pharmaceutical area [17,18]. For novel combined formulations of known active substances, new methods that determine the substances with a single method are needed to reduce time loss and cost. Although numerous HPLC methods exist for the analysis of fusidic acid and benzocaine, no single analytical technique has been developed to quantify simultaneously both active substances. This work aimed to develop and validate a novel HPLC method that allows the quantification of these active substances by a single analytical method in the microemulsion formulation, which provides high patient compliance and high pharmacological efficacy for treating mild and moderate-severe wounds and burns.

MATERIAL AND METHOD

Materials

Fusidic acid was kindly donated by Berko İlaç (Türkiye). Benzocaine was purchased from Acros Organics (USA). Acetonitrile, acetic acid, ethyl oleate, propylene glycol, Cremophor EL, and ethanol

were obtained from Sigma (USA). All chemicals used in this study are analytical grade.

Instrumentation

An HPLC method was developed to determine and quantify the amount of benzocaine and fusidic acid simultaneously. The HPLC device (1100 Series, Agilent, USA) has a UV detector, gradient pump, and thermostable column. Also, a C18 column (4.6 mm x150 mm, 5 µm, InterSustain, GL Sciences, Japan) was used in the study.

Determination of UV Spectra

Fusidic acid and benzocaine were separately dissolved in acetonitrile at a concentration of 100 µg/ml. Then, these solutions were diluted with acetonitrile, resulting in a final concentration of 10 µg/ml. UV-Vis Spectrophotometer device (UV 1800, Shimadzu, Japan) was utilized with a wavelength of 190-400 nm and 0.5 nm of sensitivity, and the measurements were made against a blind sample in quartz cuvettes. The most suitable single wavelength (λ) for the active substances was determined by evaluating the obtained spectra.

Chromatographic Conditions

Chromographic conditions (HPLC method) were created with reference to the studies of Curbete and Salgado [19] and Hirpara et al. [20]. The most suitable concentration of the mobile phase (acetonitrile and acetic acid (0.1%) was determined as 70:30 (v/v). While preparing the mobile phase, ultra-pure water was degassed in an ultrasonic bath (Super RK 255 H, Sonorex, Bandelin, Germany) for 30 min. The rate of flow was detected to be 1 $ml \cdot min^{-1}$, the injection volume was determined to be 20 µl, and the detector temperature was set to 25°C. The analyses were made at the single wavelengths obtained as a result of measurements made with the UV spectrophotometer analyses. As a result, the studies were performed at 210 nm. In the studies conducted as a gradient, the HPLC system and the column were conditioned for a minimum of one hour with the mobile phase before the analyses.

Preparation of Stock Solutions and Reference Samples

For the preparation of the stock solution of fusidic acid, 100 mg of the active substance was dissolved in 50 ml of acetonitrile. Subsequently, the volume was finalized with acetonitrile in a 100 ml flask, yielding a stock solution (1000 µg/ml). In the same way, the stock solution of benzocaine (1000 µg/ml) was obtained. The reference samples (5, 7.5, 10, 15, 20, 30, 40, 50, 60, 80, and 100 µg/ml) were separately prepared by diluting the stock solutions using acetonitrile for both active substances. All samples were filtered using membrane filters (0.2 µm, Merck Millipore, Germany).

Validation of the HPLC Method

Linearity, precision, accuracy, stability, robustness, specificity/selectivity, limit of detection (LOD), and limit of quantification (LOD) analyses were conducted within the scope of the validation procedure following the guidelines of the International Council for Harmonization [21]. Furthermore, the system suitability was assessed by calculating parameters, including the tailing factor, capacity factor, resolution, height equivalent to a theoretical plate (HETP), etc.

Linearity

Linearity test for an analytical procedure is performed to reveal that different concentrations of the substance are proportional in certain intervals [22]. The linearity is confirmed by the graph plotted using concentration values and areas obtained from the HPLC chromatograms. The correlation coefficient (R^2) and equation of the curve were determined for both active substances.

Accuracy and Recovery

The accuracy of a method is presented as a percentage of recovery [23]. In this test, it is determined whether the substance in the sample is within the confidence intervals. Three different samples were analyzed for both substances (5, 40, and 100 µg/ml for benzocaine; 5, 40, and 100 µg/ml for fusidic acid), and the recovery was calculated by Equation 1.

$$\text{Recovery (\%)} = (C_p / C_t) \times 100 \quad \text{(Eq. 1)}$$

C_p: Practical (measurement) concentration
C_t: Theoretical (nominal) concentration

Precision

Precision is expressed as the proximity of repeated measurements of a sample of the same concentration to each other under certain conditions [24]. Precision evaluation is carried out with repeatability, intermediate precision, and reproducibility tests [21]. The coefficient of variation being below 2% in the conducted studies indicates the high precision of the method [22]. For the precision study, the samples (30 µg/ml) were analyzed ten times, and the mean, standard deviation, and variation coefficient were calculated.

Intermediate precision denotes differences within laboratories, encompassing different days, analysts, equipment, and similar factors. Within the scope of the intermediate precision study, measurements were obtained by preparing six samples from the stock solution at the same concentration (5 µg/ml) by two analysts. The mean, standard deviation, and variation coefficient of the obtained results were calculated. In these analyses, the same concentrations were studied for both active substances. Furthermore, the sample prepared from the stock solution (30 µg/ml) was analyzed five times on two days (interday) under the same conditions.

Stability of the Solution

The solution of both active substances at a concentration of 30 µg/ml was analyzed by HPLC at 15, 30, 60, 120 min, 24, and 48 h. It was assessed whether there was a change in the concentration values corresponding to the obtained area values [21].

Robustness

The method's robustness was tested separately by changing the flow rate, the column temperature, and the ratio of the organic phase [21]. The samples of 100 µg/ml were analyzed for both substances. The flow rate of 0.9 and 1.1 ml, the column temperature of 30°C and 35°C, and the mobile phase ratio of 75:25 (v/v) and 65:35 (v/v) were adjusted. These studies were made by changing each condition separately. The retention time and the area of peak obtained as a result of the analyses were compared with the results of the optimal conditions.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

The lowest concentration value at which a substance can be determined is expressed as the limit of detection (LOD). It is also expressed as the limit of detectability. To determine the ratio of signal to noise, the standard deviation of the area values obtained by analyzing the samples at the lowest known concentrations was determined. LOD was calculated using the standard deviation by Equation 2. The limit of quantification (LOQ) refers to the lowest concentration at which the active substance is calculated. It is also expressed as the limit of computability. The sensitivity of the detector is determined thanks to this analysis. LOQ was calculated using Equation 3 [21,25]. In Equation 2 and Equation 3, "σ" refers to the standard deviation of the received response, and "S" refers to the slope of the linearity curve.

$$\text{LOD} = (3.3 \times \sigma) / S \quad \text{(Eq. 2)}$$

$$\text{LOQ} = (10 \times \sigma) / S \quad \text{(Eq. 3)}$$

LOD and LOQ of the active substances were calculated using the above formulas by analyzing the samples at the lowest concentration (5 µg/ml).

Specificity/Selectivity

Selectivity is achieved by separating the peak of the active substance from other peaks [26]. This was confirmed through a comparative analysis of the HPLC chromatograms of the blank and the loaded microemulsion.

Preparation of Microemulsion and Content Uniformity Analysis

As in our previous study, the microemulsion was prepared by titration method using ethyl oleate, propylene glycol, ethanol, Cremophor EL, and distilled water, and then, it was loaded with benzocaine (2%) and fusidic acid (2%) [6]. To quantify benzocaine and fusidic acid in the microemulsion, a certain amount of the formulation (500 mg) was weighed into a beaker, and 100 ml of acetonitrile was added. It was stirred for one hour. Then, one ml sample was taken and filtered. In the study conducted in three repetitions, the samples were analyzed at 210 nm by HPLC.

RESULT AND DISCUSSION

One of the most used devices for determining the number of active substances in a drug delivery system is HPLC. The determination and quantification analysis is performed in all processes of the drug synthesis, preformulation, formulation, and stability. Validation of the method developed for analysis against various factors such as device, instrument, and personnel is also an important requirement for the reliability of the method [21]. According to the International Organization for Standardization (ISO), "verification, where the specified requirements are appropriate for an intended use" [27]. The development of a successful analytical method depends on the precision and validation [28]. To this end, the HPLC method is validated in terms of parameters such as accuracy, precision, and selectivity [21].

The appropriate wavelength for the active substances was achieved through the execution of absorption measurements using a UV spectrophotometer. It was studied at 210 nm [19], 235 nm [29] wavelengths for fusidic acid, 285 nm [30], 254 nm [31], and 322 nm [20] wavelengths for benzocaine in the quantification analyses conducted by HPLC-UV device. The spectra of the substances are shown in Figure 1. Depending on these results, HPLC analyses were performed at a wavelength of 210 nm.

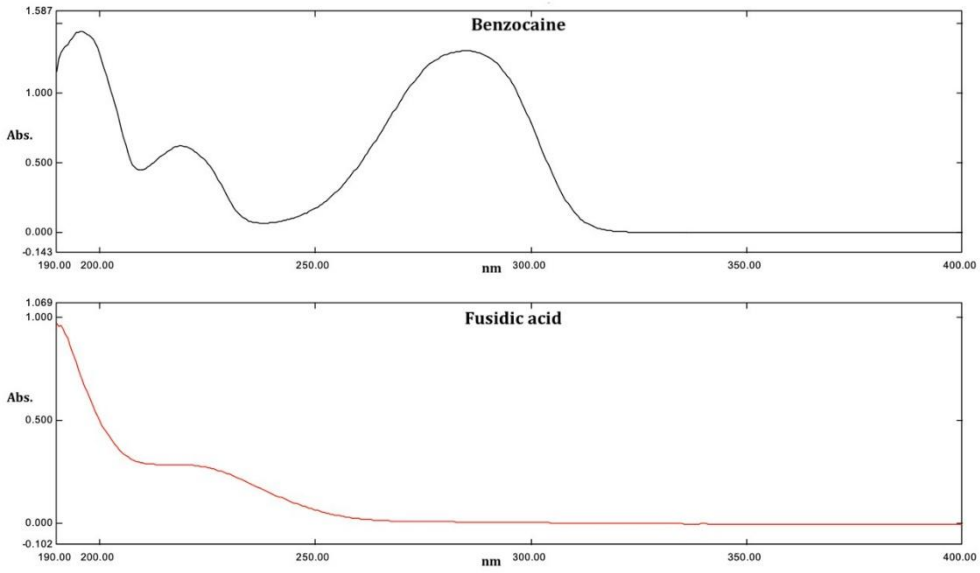


Figure 1. UV spectrum of benzocaine and fusidic acid solutions (10 µg/ml)

The samples prepared by diluting from stock solutions were analyzed using the HPLC device. A linearity graph showing the concentration against the peak areas on the chromatograms was drawn, the

equation of the curve was created, and the correlation coefficient (R^2) was calculated. The R^2 values for benzocaine and fusidic acid were determined as 0.9999 and 0.9999, respectively (Figure 2). Additionally, the system suitability data is shown in Table 1.

Table 1. System suitability results of the developed HPLC method

Active substances	Resolution	Capacity factor	Tailing factor	Assimetry factor	HETP	Theoretical plates
Benzocaine	13.33	0.93	1.03	1.05	0.01	11209
Fusidic acid	20.30	3.29	1.05	1.09	0.01	19663

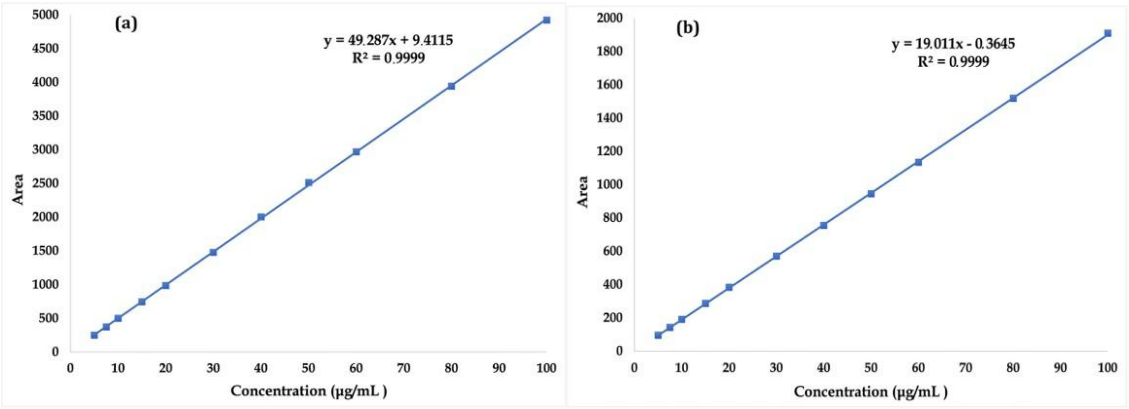


Figure 2. Linearity curves created by HPLC data. (a) Benzocaine. (b) Fusidic acid

Table 2. Accuracy and recovery study results of benzocaine and fusidic acid samples

Benzocaine				Fusidic acid			
C_t	Area	C_p	Recovery (%)	C_t	Area	C_p	Recovery (%)
5	252	4.92	98.44	5	96	5.07	101.38
	251	4.90	98.03		94	4.98	99.59
	250	4.88	97.63		96	5.05	100.96
	256	5.00	100.06		95	5.03	100.64
	256	5.00	100.06		97	5.12	102.43
		Mean	98.84			**Mean**	101.00
		SD	1.15			**SD**	1.04
		RSD (%)	1.16			**RSD (%)**	1.03
40	2009	40.59	101.45	40	761	40.05	100.12
	2008	40.54	101.36		767	40.36	100.91
	1989	40.16	100.40		765	40.26	100.65
	1996	40.31	100.78		757	39.84	99.60
	2006	40.51	101.27		766	40.31	100.78
		Mean	101.05			**Mean**	100.41
		SD	0.45			**SD**	0.55
		RSD (%)	0.44			**RSD (%)**	0.54
100	4952	100.28	100.28	100	1909	100.44	100.43
	4934	99.92	99.92		1890	99.44	99.44

Table 2 *(continue).* Accuracy and recovery study results of benzocaine and fusidic acid samples

Benzocaine				Fusidic acid			
C_t	**Area**	**C_p**	**Recovery (%)**	**C_t**	**Area**	**C_p**	**Recovery (%)**
100	4996	101.18	101.17	100	1920	101.01	101.01
	4953	100.30	100.30		1911	100.54	100.54
	4876	98.74	98.74		1905	100.22	100.22
		Mean	100.08			**Mean**	100.33
		SD	0.88			**SD**	0.58
		RSD (%)	0.88			**RSD (%)**	0.58

C_p: Practical (measurement) concentration. C_t: Theoretical (Nominal) concentration. SD: Standard deviation. RSD: Relative standard deviation (variation coefficient)

The precision results are shown in Table 3. The mean, standard deviation, and variation coefficient (%) of the obtained data were calculated. It was found that the variation coefficients for both substances were lower than 2%, and the standard deviations were low. These data showed that the analytical technique was accurate and reproducible.

Table 3. Precision study results of benzocaine and fusidic acid solutions

Benzocaine				Fusidic acid			
No	**C_t (µg/ml)**	**Area**	**C_p (µg/ml)**	**No**	**C_t (µg/ml)**	**Area**	**C_p (µg/ml)**
1	30	1498	30.20	1	30	567	29.84
2		1481	29.86	2		562	29.58
3		1481	29.86	3		568	29.90
4		1477	29.80	4		562	29.58
5		1478	29.80	5		570	30.00
6		1476	29.76	6		563	29.63
7		1477	29.78	7		572	30.11
8		1478	29.80	8		564	29.69
9		1478	29.80	9		570	30.00
10		1477	29.78	10		563	29.63
		Mean	29.84			**Mean**	29.80
		SD	0.12			**SD**	0.19
		RSD (%)	0.42			**RSD (%)**	0.63

C_p: Practical (measurement) concentration. C_t: Theoretical (Nominal) concentration. SD: Standard deviation. RSD: Relative standard deviation (variation coefficient)

Table 4. Intermediate precision results performed interday of benzocaine and fusidic acid samples

Benzocaine						Fusidic acid					
Theoretical concentration – 30 µg/ml						**Theoretical concentration – 30 µg/ml**					
1. day			**2. day**			**1. day**			**2. day**		
No	**Area**	**C_p (µg/ml)**	**No**	**Area**	**C_p (µg/ml)**	**No**	**Area**	**C_p (µg/ml)**	**No**	**Area**	**C_p (µg/ml)**
R-1-1	1476	29.76	R-2-1	1472	29.68	R-1-1	563	29.63	R-2-1	568	29.90
R-1-2	1477	29.78	R-2-2	1468	29.59	R-1-2	572	30.11	R-2-2	570	30.00
R-1-3	1478	29.80	R-2-3	1484	29.92	R-1-3	564	29.69	R-2-3	574	30.21
R-1-4	1478	29.80	R-2-4	1478	29.80	R-1-4	570	30.00	R-2-4	571	30.05
R-1-5	1477	29.78	R-2-5	1477	29.78	R-1-5	563	29.63	R-2-5	564	29.69
R-1-6	1478	29.80	R-2-6	1483	29.90	R-1-6	567	29.84	R-2-6	574	30.21

Table 4 ***(continue).*** Intermediate precision results performed interday of benzocaine and fusidic acid samples

Benzocaine						**Fusidic acid**					
Theoretical concentration – 30 µg/ml						**Theoretical concentration – 30 µg/ml**					
1. day			**2. day**			**1. day**			**2. day**		
No	**Area**	**C_p (µg/ml)**	**No**	**Area**	**C_p (µg/ml)**	**No**	**Area**	**C_p (µg/ml)**	**No**	**Area**	**C_p (µg/ml)**
R-1-7	1497	29.18	R-2-7	1478	29.80	R-1-7	562	29.58	R-2-7	568	29.90
R-1-8	1481	29.86	R-2-8	1471	29.66	R-1-8	568	29.90	R-2-8	571	30.05
R-1-9	1481	29.86	R-2-9	1484	29.92	R-1-9	562	29.58	R-2-9	572	30.11
R-1-10	1477	29.78	R-2-10	1484	29.92	R-1-10	570	30.00	R-2-10	565	29.74
	Mean	29.84		**Mean**	29.79		**Mean**	29.80		**Mean**	29.99
	SD	0.13		**SD**	0.12		**SD**	0.20		**SD**	0.18
	RSD (%)	0.44		**RSD (%)**	0.41		**RSD (%)**	0.66		**RSD (%)**	0.60

C_p: Practical (measurement) concentration. SD: Standard deviation. RSD: Relative standard deviation (variation coefficient)

The intermediate precision performed interday was determined by analyzing the samples of 30 µg/ml over two days. The data are shown in Table 4. The alterations between the practical concentration values were not statistically significant for both benzocaine and fusidic acid ($p>0.05$), and the variation coefficient was found to be lower than 2%. The data obtained from studies conducted by two analysts on the same day under the same conditions are shown in Table 5. The differences in the peak areas were not statistically significant for both active substances ($p>0.05$). The data obtained in the intermediate precision studies proved that the system was highly reproducible and that there was no difference between the data obtained in the studies conducted by different analysts.

Table 5. Intermediate precision test results performed by two analysts of benzocaine and fusidic acid samples

Benzocaine						**Fusidic acid**					
Theoretical concentration – 5 µg/ml						**Theoretical concentration – 5 µg/ml**					
1. Analyst			**2. Analyst**			**1. Analyst**			**2. Analyst**		
No	**Area**	**C_p (µg/ml)**	**No**	**Area**	**C_p (µg/ml)**	**No**	**Area**	**C_p (µg/ml)**	**No**	**Area**	**C_p (µg/ml)**
1	251	4.90	1	250	4.88	1	95.8	5.06	1	92.4	4.88
2	247	4.82	2	252	4.92	2	96.0	5.07	2	94.2	4.97
3	255	4.98	3	247	4.82	3	97.6	5.15	3	92.8	4.90
4	256	5.00	4	250	4.88	4	94.3	4.98	4	94.7	5.00
5	254	4.96	5	251	4.90	5	97.8	5.16	5	97.2	5.13
6	253	4.94	6	247	4.82	6	95.6	5.05	6	95.1	5.02
	Mean	4.94		**Mean**	4.87		**Mean**	5.08		**Mean**	4.99
	SD	0.07		**SD**	0.04		**SD**	0.06		**SD**	0.08
	RSD (%)	1.34		**RSD (%)**	0.86		**RSD (%)**	1.25		**RSD (%)**	1.67

C_p: Practical (measurement) concentration. SD: Standard deviation. RSD: Relative standard deviation (variation coefficient)

To determine the stability of benzocaine and fusidic acid solutions prepared by dissolving in acetonitrile, the samples (30 µg/ml) were analyzed by HPLC at 15, 30, 60, 120 min, 24, and 48 h. The analysis revealed that there was no significant difference in the concentrations of the active substances (Table 6). These results showed that the prepared solutions were stable throughout the validation process.

Table 6. Stability test results of benzocaine and fusidic acid samples (30 µg/ml)

Benzocaine				Fusidic acid			
Time	**Area**	**C_p (µg/ml)**	**Recovery (%)**	**Time**	**Area**	**C_p (µg/ml)**	**Recovery (%)**
0th min	1477	29.78	99.26	**0th min**	567	29.84	99.48
15th min	1480	29.84	99.46	**15th min**	566	29.79	99.31
30th min	1479	29.82	99.39	**30th min**	565	29.74	99.13
60th min	1474	29.72	99.05	**60th min**	569	29.95	99.83
120th min	1474	29.72	99.05	**120th min**	567	29.84	99.48
24th h	1475	29.74	99.12	**24th h**	569	29.95	99.83
48th h	1487	29.98	99.93	**48th h**	572	30.11	100.36
	Mean	29.80	99.32		**Mean**	29.89	99.63
	SD	0.09	0.29		**SD**	0.11	0.38

C_p: Practical (measurement) concentration. SD: Standard deviation

Table 7. The robustness results of the method

Condition	Value	Benzocaine			Fusidic acid		
		Peak area	**Retention time**	**Recovery (%)**	**Peak area**	**Retention time**	**Recovery (%)**
		Change (%)	**Change (%)**		**Change (%)**	**Change (%)**	
Flow rate (ml·min^{-1})	0.9	5.05	14.77	102.18	6.36	14.73	104.66
	1.1	-13.48	-5.58	84.12	-12.23	-5.53	86.37
Percent of organic phase (%)	65	-4.52	14.58	92.84	-4.38	35.59	94.09
	75	-4.75	-3.88	92.62	-2.74	-16.21	95.70
Column temperature (°C)	30	-3.68	1.94	93.67	-3.19	1.23	95.26
	35	-4.84	0.28	92.54	-3.65	-0.85	94.81

The investigation into robustness was conducted by analyzing the solutions of the substances, which were prepared to introduce the changes in the organic component ratio of the mobile phase, the flow rate, and the column temperature. An assessment of the change in parameters (e.g., retention time and peak area) was conducted (Table 7). In particular, it was evaluated that the change in the flow rate and the mobile phase ratio affected the retention time. In addition, it was found that fusidic acid was more sensitive to changes in conditions in terms of retention time, while benzocaine was more sensitive with respect to peak area. However, the recovery values obtained indicated the robustness of the method against minor alterations (<5%) in the conditions.

To demonstrate the specificity and selectivity of the method for the substances, the blank and the loaded microemulsion were analyzed. The retention time of benzocaine and fusidic acid was determined as 2.3-2.5 min (Figure 3a) and 5.2-5.5 min (Figure 3b), respectively. No impurities were observed in the chromatogram of either active substance (Figure 3a and 3b). While certain peaks attributable to excipients were observed in the blank microemulsion chromatogram (Figure 3c), it was detected that these peaks were not coincident with those of the active substances (Figure 3d). As stated in our previous study findings, the drug content uniformities of benzocaine and fusidic acid were also determined to be 99.40±1.62% and 99.53±0.97%, respectively [6]. While the LOD and LOQ values for benzocaine were calculated as 0.127 and 0.384 µg/ml, respectively, the LOD and LOQ values for fusidic acid were calculated as 0.209 and 0.633 µg/ml, respectively.

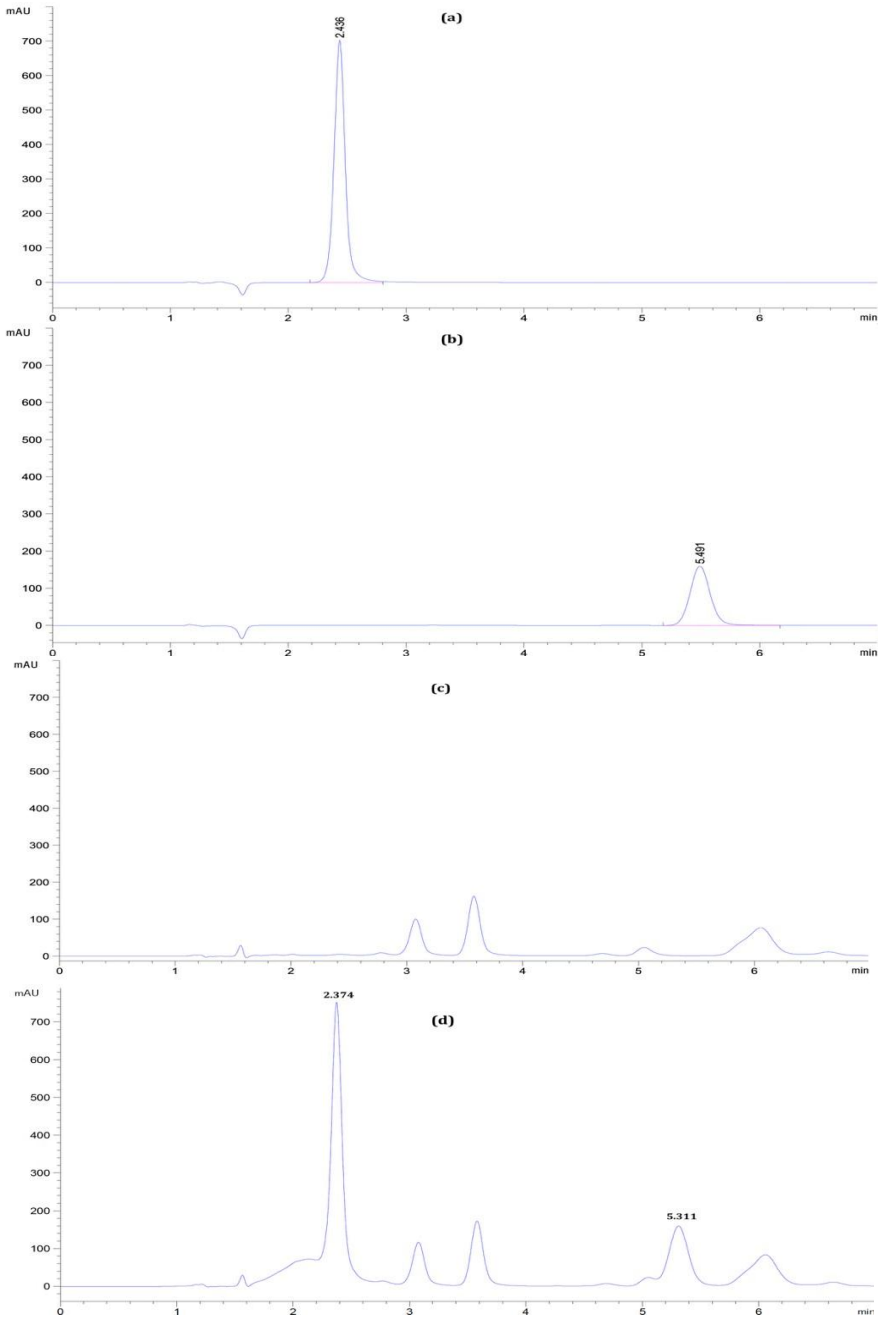


Figure 3. (a) HPLC chromatogram of benzocaine (100 µg/ml); (b) HPLC chromatogram of fusidic acid (100 µg/ml); (c) HPLC chromatogram of the blank microemulsion; (d) HPLC chromatogram of benzocaine- and fusidic acid-loaded microemulsion

To the best of our knowledge, no HPLC method currently exists that enables the simultaneous analysis of both benzocaine and fusidic acid using a single wavelength. However, separate methods have been developed for these substances contained in some marketed products. Curbete et al. [19] utilized a pre-column combined with a C18 column to quantify fusidic acid in a cream formulation, reporting a retention time of approximately 8 min. Yaseen *et al.* [33] employed a mobile phase consisting of acetonitrile and 0.001 M acetic acid (80:20, v/v), achieving a retention time of around 6 min for fusidic acid. Hirpara et al. [20] used acetonitrile and acetic acid (65:35, v/v) as the mobile phase and a detection wavelength of 243 nm in their method for the quantification of benzocaine, achieving a

retention time of approximately 4 min. On the other hand, the method developed in this study offered several advantages over existing methods, including the elimination of the need for a pre-column, the reduction of mobile phase consumption due to shorter retention times, and the capability to operate using a single wavelength.

An inexpensive, simple, and easily applicable HPLC method was developed for the determination and quantification of both active substances in fusidic acid and benzocaine-loaded microemulsions. The validation of the developed method demonstrated a very high correlation coefficient (0.9999 for both substances), high accuracy and recovery (98.84%, 101.05%, and 100.08% for benzocaine; 101.00%, 100.41%, and 100.33% for fusidic acid), and low variation coefficient for intermediate precision (<1.67). The results confirmed that this method is unique and highly reproducible for the quantification of both active substances.

ACKNOWLEDGEMENTS

The authors thank Berko İlaç for kindly providing fusidic acid.

AUTHOR CONTRIBUTIONS

Concept: M.D.A., E.Ş.Ç., N.Ü.O.; Design: M.D.A., E.Ş.Ç., N.Ü.O.; Control: M.D.A., E.Ş.C., N.Ü.O.; Sources: M.D.A., E.Ş.Ç., N.Ü.O.; Materials: M.D.A., E.Ş.Ç., N.Ü.O.; Data Collection and/or Processing: M.D.A., E.Ş.Ç., N.Ü.O.; Analysis and/or Interpretation: M.D.A., E.Ş.Ç., N.Ü.O.; Literature Review: M.D.A., N.Ü.O.; Manuscript Writing: M.D.A., N.Ü.O.; Critical Review: M.D.A., N.Ü.O.; Other: -

CONFLICT OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

The authors declare that the ethics committee approval is not required for this study.

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J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 49(3): 672-680, 2025
Doi: 10.33483/jfpau.1608315

ORIGINAL ARTICLE / ÖZGÜN MAKALE

ZERO-ORDER AND FIRST-DERIVATIVE SPECTROPHOTOMETRY FOR THE DETERMINATION OF SPIRONOLACTONE IN PHARMACEUTICAL TABLETS

FARMASÖTİK TABLETLERDEKİ SPİRONOLAKTONUN TAYİNİ İÇİN SIFIR DERECEDEN VE BİRİNCİ TÜREV SPEKTROFOTOMETRİ

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ABSTRACT

Objective: *Spironolactone, a potassium-sparing diuretic drug on the WHO's Essential Medicines List, is primarily used to treat fluid retention resulting from heart failure, kidney disease, and liver scarring. In this research article, zero-order, and first-derivative spectrophotometric approaches were applied to the quantity of spironolactone in pharmaceutical tablet formulation without any separation procedure.*
Material and Method: *Spironolactone compound was obtained from Sigma-Aldrich (Steinheim, Germany). As a solvent methanol (J.T. Baker, Netherlands, HPLC grade) was used for UV-visible spectrophotometric assays. In the implementation of the zero-order and first-derivative approaches, the quantification of Spironolactone was carried out using UV spectrophotometric measurements in the 200-300 nm wavelength range (with a 2 nm increment) and 1 cm quartz cells. These approaches were tested by analyzing spironolactone in independent test samples and spiked samples. The tablet analyses of spironolactone were performed on ALDACTONE®-A 25 mg Tablet supplied by Aris Ali Raif Pharmaceuticals Industry, Başakşehir, İstanbul, Türkiye.*
Result and Discussion: *The analysis of spironolactone was conducted using zero-order values measured at 239 nm and first-derivative values measured at 250.4 nm (n=3). The calibration graphs created at these wavelengths showed linearity within the 6.0 to 20.0 µg/ml concentration range, with correlation coefficients of r = 0.9996 for the zero-order method and r = 0.9997 for the first-order derivative method. The validity of the methods was proved by analyzing spironolactone in independent test samples and spiked samples. These methods were then applied to the quantitation of spironolactone in commercial tablets and a good agreement was reported between label claim assay results.*
Keywords: *Antihyypertensive drug, determination of spironolactone, first derivative spectrophotometry, tablet analysis, zero-order spectrophotometry*

ÖZ

Amaç: *WHO'nun Temel İlaçlar Listesi'nde yer alan potasyum tutucu bir diüretik ilaç olan spironolakton, öncelikli olarak kalp yetmezliği, böbrek hastalığı ve karaciğer skarlaşmasından*

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Submitted / Gönderilme : 27.12.2024
Accepted / Kabul : 15.04.2025
Published / Yayınlanma : 19.09.2025

kaynaklanan sıvı tutulumunu tedavi etmek için kullanılır. Bu araştırma makalesinde, herhangi bir ayırma prosedürü olmaksızın farmasötik tablet formülasyonundaki spironolakton miktarına sıfırıncı mertebeden ve birinci türev spektrofotometrik yaklaşımlar uygulandı.

Gereç ve Yöntem: *Spironolakton bileşiği Sigma-Aldrich'ten (Steinheim, Almanya) elde edildi. Çözücü olarak metanol (J.T. Baker, Hollanda, HPLC sınıfı) UV-görünür spektrofotometrik analizler için kullanıldı. Sıfırıncı mertebe ve birinci türev yaklaşımlarının uygulanmasında, Spironolaktonun kantifikasyonu 200-300 nm dalga boyu aralığında (2 nm artışla) ve 1 cm kuvars hücrelerde UV spektrofotometrik ölçümler kullanılarak gerçekleştirildi. Bu yaklaşımlar, spironolaktonun bağımsız test örneklerinde ve spike edilmiş örneklerde analiz edilmesiyle test edildi. Spironolaktonun tablet analizleri, Aris Ali Raif İlaç Sanayi, Başakşehir, İstanbul, Türkiye tarafından tedarik edilen ALDACTONE®-A 25 mg Tablet üzerinde gerçekleştirildi.*

Sonuç ve Tartışma: *Spironolakton analizi, 239 nm'de ölçülen sıfırıncı mertebeden değerler ve 250,4 nm'de ölçülen birinci türev değerleri kullanılarak gerçekleştirildi (n=3). Bu dalga boylarında oluşturulan kalibrasyon grafikleri, sıfırıncı mertebeden yöntem için r = 0,9996 ve birinci mertebeden türev yöntemi için r = 0,9997 korelasyon katsayılarıyla 6,0 ila 20,0 µg/ml konsantrasyon aralığında doğrusallık gösterdi. Yöntemlerin geçerliliği, bağımsız test örneklerinde ve güçlendirilmiş örneklerde spironolaktonun analiz edilmesiyle kanıtlandı. Daha sonra bu yöntemler ticari tabletlerdeki spironolaktonun kantifikasyonuna uygulandı ve etiket iddiası deney sonuçları arasında iyi bir uyum bildirildi.*

Anahtar Kelimeler: *Antihipertansif ilaç, birinci türev spektrofotometri, sıfırıncı mertebe spektrofotometri, spironolakton tayini, tablet analizi*

INTRODUCTION

Cardiovascular diseases, which are among the most common chronic and non-communicable diseases in the world, are diseases with high social and economic costs that can result in organ damage, stroke, and even death if left untreated [1-5]. The initial pharmacological treatment for hypertension includes calcium channel blockers, thiazide diuretics, angiotensin-converting enzyme inhibitors, and angiotensin receptor blockers, or a combination of two of these agents [6-7]. Spironolactone, a potassium-sparing diuretic drug on the WHO's Essential Medicines List, is primarily used to treat fluid retention resulting from heart failure, kidney disease, and liver scarring [8-9]. In addition, it is also used in the treatment of precocious puberty (for boys), excessive hirsutism, and acne (for women), and as part of transgender hormone therapy (for transgender women) [10-12]. The chemical name of spironolactone, whose chemical structure is given in Figure 1, is 7α-acetylthio-3-oxo-17α-pregn-4-ene 21,17β-carbolactone.

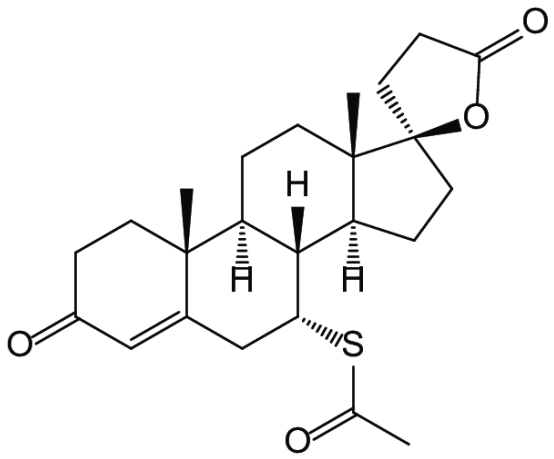


Figure 1. Chemical construction of spironolactone

The literature review stated various analytical methods for determining spironolactone alone or in combination with other active pharmaceutical ingredients, including chromatographic [13-17],

voltammetric [18-20], and spectrophotometric [21-25] techniques. This indicates that there are no reported analytical methods for quantitatively estimating spironolactone using zero-order and first-derivative spectrophotometry. Due to the possibility of derivatizing zero-order spectra by computer programs, there is a need for more accurate and sensitive derivative spectrophotometry, which allows valuable analytical treatments. Derivative spectrophotometry eliminates excipients' effect in commercial products, matrix effects from sample turbidity, and noise peaks from instruments and provides an adequate resolution for complex systems when the analysis conditions are suitable [26].

First-derivative spectrophotometry calculates and plots a derivative of a spectral curve, providing an alternative approach for pharmaceutical analysis. Therefore, this article aimed to develop and validate a new, straightforward, rapid, accurate, sensitive, and precise zero-order and first derivative spectrophotometric approach for the quality control and routine analysis of spironolactone in pharmaceutical tablet form.

MATERIAL AND METHOD

Apparatus and Software

UV visible spectrophotometric records were gathered using a Shimadzu double-beam UV-Visible spectrophotometer (UV-2550, Kyoto, Japan) featuring a fixed slit width (2 nm). Absorbance measurements were taken using a 1 cm quartz cuvette, in the wavelength range of 200-300 nm, and at 0.2 nm intervals. After transferring the data to Microsoft Excel (Microsoft, USA), it was used for processing the spectra and conducting statistical and regression analysis. Additionally, all graphical representations were generated.

Chemicals and Reagents

Spironolactone compound was obtained from Sigma-Aldrich (Steinheim, Germany). As a solvent methanol (J.T. Baker, Netherlands, HPLC grade) was used for UV-visible spectrophotometric assays. Working solutions were freshly and daily prepared and stored in the dark and the refrigerator throughout the analysis.

Preparation of Standard, Calibration, and Validation Solutions

A stock solution of spironolactone was prepared by accurately weighing 10.0 mg of the compound and dissolving it in methanol. The resulting solution was quantitatively transferred to a 100 ml volumetric flask and diluted to volume with methanol to obtain the final stock solution. This stock solution was subsequently utilized to prepare both standard solutions for calibration and independent validation solutions. For the preparation of standard solutions, a series of dilutions were performed to achieve concentrations in the range of 6.0 to 20.0 µg/ml. These solutions were used to establish calibration curves, employing both the zero-order absorption and first-derivative spectrophotometric methods. Calibration curves were constructed by plotting absorbance against concentration for each method. To ensure the accuracy and linearity of the calibration, multiple concentration levels were included in this range. For method validation, independent test samples were prepared at specific concentrations of 6.0, 14.0, and 20.0 µg/ml following the same procedure as used for the calibration standards. These validation solutions were subjected to analysis using the same spectrophotometric methods to verify the precision, accuracy, and reproducibility of the analytical method.

In the standard addition method, known quantities of spironolactone were spiked into a sample of the pharmaceutical tablet solution to account for any potential matrix effects. Specifically, spironolactone standard solutions at four different concentration levels (0, 4, 8, and 12 µg/ml) were added to 0.6 ml of the pharmaceutical tablet solution, which was then diluted to 10 ml in a volumetric flask. This procedure was repeated for each concentration level, and all experiments—including the preparation of calibration standards, validation samples, and pharmaceutical tablet solutions—were conducted in triplicate to ensure consistency, precision, and reliability of the results.

By conducting the experiments in triplicate, statistical validation of the method was ensured, providing confidence in both the repeatability and robustness of the spectrophotometric determination

of spironolactone in pharmaceutical formulations.

Preparation of Tablet Samples

In the implementation of the zero-order spectrophotometric and first-derivative spectrophotometric methods in commercial pharmaceutical tablet analysis, 10 tablets of ALDACTONE®-A 25 mg Tablet (Aris Ali Raif İlaç Sanayi, Başakşehir, İstanbul, Türkiye) were weighed precisely and ground into powder in a mortar. After determining the amount equivalent to one-tenth of a tablet, it was dissolved in methanol in a 25 ml volumetric flask. The solution was mixed using a mechanical stirrer for 30 minutes and then filtered through a 0.22 µm pore size PVDF filter (Isolab, Wertheim, Germany). For analysis, 1.8 ml of the filtered solution was transferred to a 10 ml volumetric flask, and the volume was made up to 10 ml with the same solvent.

RESULT AND DISCUSSION

When spectrophotometric methods are compared with other methods such as chromatography and electrophoresis, the widespread availability of instrumentation, the rapidity and simplicity of the procedures, and the high sensitivity and accuracy of the methods still make spectrophotometric methods attractive. In spectrophotometric methods, various techniques are applied to obtain qualitative and quantitative information from spectra consisting of unresolved bands. The most important of these is derivative spectrophotometry, which is obtained by applying the first or higher-order mathematical derivative ($dA/d\lambda$) of wavelength versus absorbance curves (zero-order spectra). This technique generally improves the resolution of bands, removes the influence of the matrix, and supplies more characterized fingerprints than direct absorbance spectra (zero absorption spectra), since it increases the detectability of minor spectral properties. In this context, we applied first derivative spectrophotometry to zero-order absorption spectra to directly quantify spironolactone in the pharmaceutical preparation. For this purpose, the suitable calibration range of spironolactone, complying with Beer's law, was determined as 6.0-20.0 µg/ml. In this calibration range, zero-order spectra were recorded in the wavelength range of 200-300 nm and the wavelength at which spironolactone gave maximum absorption was determined as 239 nm. The obtained spectra are presented in Figure 2.

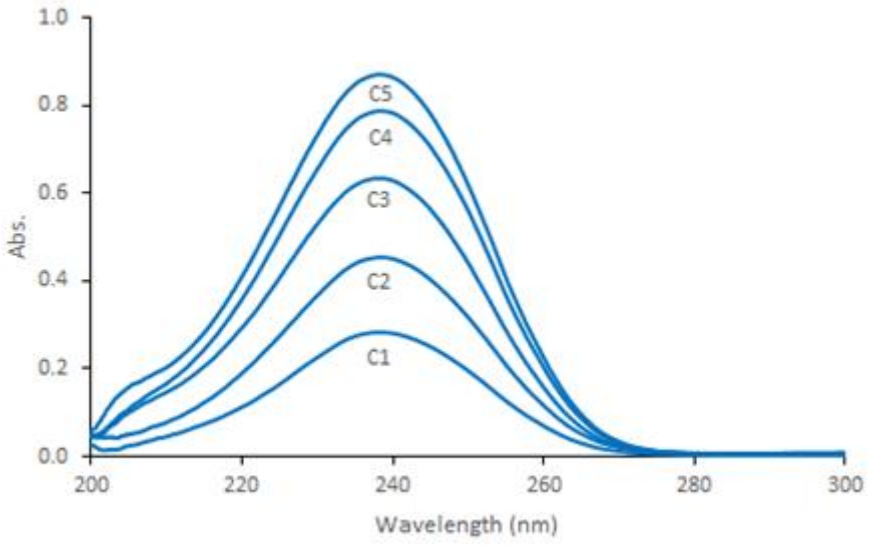


Figure 2. Zero-order spectra of spironolactone in the 6.0-20.0 µg/ml calibration range

The first derivative spectra of spironolactone were plotted with $\Delta\lambda$= 2 nm intervals and a scaling factor of 30 from the computed zero absorption spectra of the calibration samples obtained by measuring the $dA/d\lambda$ value at 250.4 nm as in Figure 3.

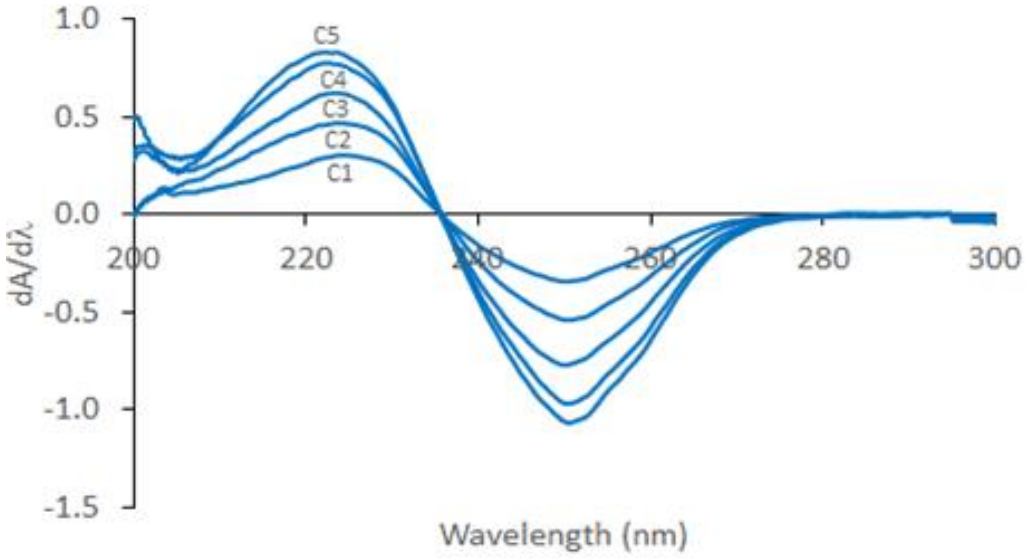


Figure 3. The first-derivative spectra of spironolactone in the 6.0-20.0 µg/ml calibration range

The calibration curves for spironolactone were obtained by measuring absorbances at the selected wavelength of 239 nm for zero-order spectra and 250.4 nm for first-derivative spectra, respectively. The statistical regression analysis results are given in Table 1. Then, spironolactone was estimated through the obtained calibration curves.

Table 1. Statistical overview: Results of the linear regression analysis

Parameter	Zero-order	First derivative
m	0.0417	0.0515
n	0.0382	0.0381
r	0.9996	0.9997
SE(m)	$7.13x10^{-4}$	$7.85x10^{-4}$
SE(n)	$1.04x10^{-2}$	$1.14x10^{-2}$
SE(r)	$8.17x10^{-3}$	$7.85x10^{-4}$
LOD (µg/ml)	0.75	0.66
LOQ (µg/ml)	2.48	2.22

m, n, and r are the regression equation's slope, intercept, and correlation coefficients. SE (m), SE (n), and SE (r) are the standard errors of slope, intercept, and correlation coefficient, respectively. LOD: Detection limit (µg/ml), LOQ: Quantification limit (µg/ml)

Zero-order spectrophotometric and first-derivative spectrophotometric methods were validated by analyzing the independent test samples and spiked samples (prepared by adding the standards to the pharmaceutical tablet solution). Validation parameters such as range, linearity, accuracy, precision, selectivity, the limit of detection (LOD), and the limit of quantitation (LOQ) were given in Table 1. Correlation coefficients higher than 0.9990 were obtained for zero-order and first-derivative methods. Limit of detection (LOD) and limit of quantification (LOQ) parameters were calculated using the calibration curves' standard deviation and slope values , and the results are presented in Table 1. The validity, precision, accuracy, and reproducibility of two different proposed methods were verified by analyzing independent test samples of spironolactone. The average of the three results, mean recovery, and relative standard deviation values are given in Table 2. As can be seen in this table, these methods achieved desirable precision and accuracy without requiring any preliminary separation steps for the analysis of spironolactone.

Table 2. Research on recovery and the outcomes achieved by applying methods to independent test samples

Added	**Found* (µg/ml)**		**Recovery (%)**	
(µg/ml)	Zero-order	First-derivative	Zero-order	First-derivative
	239 nm	250.4 nm		
6	5.85	5.94	97.5	99.0
14	14.11	14.06	100.8	100.4
20	19.98	19.99	99.9	99.9
		Mean	99.40	99.77
		Standard Deviation	1.71	0.70
		Relative Standard Deviation	1.72	0.71

* Results were obtained by averaging the results of three assays for each concentration level

To show whether the excipients in the tablet affect the determination of the spironolactone, spiked samples were prepared at 4, 8, and 12 µg/ml concentration levels and analyzed with the proposed zero-order and first-derivative methods. Then added recovery results for each drug were calculated. Their results (recovery value and standard deviation) are given in Table 3. The results of the experiments were calculated as the average of triplicates for each concentration level. As can be seen from the results in Table 3, it was observed that the sample matrix did not affect the analysis of spironolactone. In other words, the results were found to be satisfactory in terms of the selectivity of the proposed analytical methods.

Table 3. Recovery results obtained by implementing the methods in spiked samples

Added (µg/ml)	**Tablet**	**6**	**6**	**6**
	Standard	4.00	8.00	12.00
Found (µg/ml)	Zero-order	3.98	8.18	12.09
	First derivative	3.86	8.10	12.03
Recovery (%)	Zero-order	99.50	102.20	100.70
	First derivative	96.50	101.20	100.20
RSD (%)	Zero-order	0.93	2.25	1.13
	First derivative	0.82	2.64	1.01

RSD: Relative standard deviation
The added amount of the tablet is approximately 6 µg/ml

Zero-order spectrophotometric and first-derivative spectrophotometric methods were used for the quantity of pharmaceutical tablet formulations containing spironolactone. Table 4 shows the assay results of spironolactone in commercial tablets. The results provided a good agreement with the reported label claim of the commercial sample. The standard deviation and relative standard deviation given in Table 4 confirm the applicability and performance of the proposed methods.

A statistical analysis was conducted on the experimental results obtained from using the zero-order and first-derivative methods to determine the concentration of spironolactone in a commercial tablet. This analysis was carried out using Microsoft Excel software. A paired t-test was performed at a significance level of $p = 0.05$ to compare the assay results of both methods. The statistical results indicated no significant difference between the outcomes of the zero-order and first-derivative methods. Therefore, it was concluded that both proposed methods yield comparable results, as demonstrated in Table 4.

Table 4. Quantitation results of spironolactone in tablet samples using zero-order and first-derivative methods

Experiment	mg/ tablet	
Number	**Zero-order**	**First derivative**
1	24.87	24.91
2	25.09	25.23
3	24.65	24.66
4	24.73	24.83
5	24.77	24.97
Mean	24.82	24.92
Standard deviation	0.17	0.21
Relative standard deviation	0.68	0.83
	t-calculated= 2.306	t-tabulated=2.571
	p=0.438	p=0.05

Label amount: 25 mg spironolactone per tablet

Conclusion

In analytical procedures, it is crucial to quantify compounds in matrix systems without any separation processes. As it is known, methods such as chromatography and capillary electrophoresis, which use separation techniques that require expensive and complex devices, are tedious due to the difficulties in optimizing experimental conditions in the analysis processes, long analysis times, and in some cases not yield successful results, forcing analytical chemists to search for faster, more easily applicable and more economical methods. In this research article, zero-order and first derivative methods were successfully implemented for the assay of spironolactone from pharmaceutic preparations because they are easier to apply, faster and more economical, and provide reproducible, sensitive, accurate, and reliable results. It can be easily used for routine analysis of spironolactone in scientific studies and industry.

ACKNOWLEDGEMENTS

This work was carried out at the Chemometric Laboratory of Ankara University, Faculty of Pharmacy funded by the scientific research project number 10A3336001.

AUTHOR CONTRIBUTIONS

Concept: A.Ü., E.D., Ö.Ü.; Design: A.Ü., E.D., Ö.Ü.; Control: A.Ü., E.D., Ö.Ü.; Sources: A.Ü., Ö.Ü.; Materials: N.B., E.D.; Data Collection and/or Processing: A.Ü., E.D.; Analysis and/or Interpretation: A.Ü., E.D., Ö.Ü.; Literature Review: A.Ü., N.B.; Manuscript Writing: A.Ü., E.D., Ö.Ü.; Critical Review: A.Ü., N.B., E.D., Ö.Ü.; Other: -

CONFLICTS OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

The authors declare that the ethics committee approval is not required for this study.

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J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 49(3): 681-688, 2025
Doi: 10.33483/jfpau.1647572

ORIGINAL ARTICLE / ÖZGÜN MAKALE

ELEVATED ENDOGLIN LEVELS AND THEIR LINK TO THE INFLAMMATORY TUMOR MICROENVIRONMENT IN COLORECTAL CANCER

KOLOREKTAL KANSERDE ARTMIŞ ENDOGLİN DÜZEYİ İLE İNFLAMATUVAR TÜMÖR MİKROÇEVRE İLİŞKİSİ

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ABSTRACT

Objective: *Colorectal cancer (CRC) is one of the most common cancers worldwide and is one of the leading causes of cancer-related deaths. The causal link between inflammation and cancer has been proposed based on the observation that tumors often develop in a chronic inflammatory environment and that inflammatory cells are present in tumor biopsy specimens. Epidemiological studies have revealed that chronic inflammation provides a basis for different types of cancer. There is a great deal of evidence to suggest that chronic inflammation can support cancer development and that tumor-induced inflammation maintains tumor progression by creating a snowball effect. Endoglin is a transmembrane glycoprotein identified in vascular endothelial cells and has been shown to be associated with angiogenesis and inflammation in various diseases. This study aimed to investigate the relationship between endoglin and the inflammatory microenvironment in colorectal cancer patients.*

Material and Method: *50 patients diagnosed with colorectal cancer and 50 healthy volunteers were included in the study. In plasma samples, endoglin and commonly known inflammation markers such as $sPLA_2$, $cPLA_2$, NfκB, and $TGF\beta_1$ levels were measured by ELISA (enzyme-linked immunosorbent assay) method. In addition, endoglin and PLA_2 mRNA expression were determined by Real-Time PCR.*

Result and Discussion: *In colorectal cancer patients, plasma endoglin, NfκB, $TGF\beta_1$ levels were found to be significantly higher than in the control group, while no significant difference was found between the groups in $cPLA_2$ and $sPLA_2$ levels. A significant positive correlation was found between plasma endoglin levels and NFκB in the colorectal cancer group. Gene expression analyses showed that endoglin and PLA_2 mRNA expression levels were significantly higher in the colorectal cancer group than in the control group. In conclusion, this study showed that the increase in endoglin in colorectal cancer may be associated with the development of inflammation and may play a role in poor prognosis associated with the inflammatory microenvironment.*

Keywords: *Colorectal cancer, Endoglin, NFκB, PLA_2, $TGF\beta_1$*

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Submitted / Gönderilme : 27.02.2025
Accepted / Kabul : 15.04.2025
Published / Yayınlanma : 19.09.2025

ÖZ

Amaç: *Kolorektal kanser (CRC) dünyada en yaygın görülen kanserlerden biri olup, kansere bağlı ölümlerin önde gelen nedenleri arasındadır. Tümörlerin sıklıkla kronik enflamasyon ortamında geliştiği ve tümör biyopsi örneklerinde enflamatuvar hücrelerin mevcut olduğu gözlemine dayanarak, enflamasyon ve kanser arasında nedensel bir bağlantı öne sürülmüştür. Epidemiyolojik çalışmalar, kronik enflamasyonun farklı kanser türlerine zemin hazırladığını ortaya çıkarmıştır. Kronik inflamasyonun kanser gelişimini destekleyebildiğini ve tümör kaynaklı enflamasyonun kartopu etkisi yaratarak tümör progresyonunu sürdürdüğünü gösteren çok sayıda kanıt bulunmaktadır. Endoglin, vasküler endotel hücrelerinde tanımlanmış bir transmembran glikoprotein olup, endoglinin çeşitli hastalıklarda anjiyogenez ve inflamasyon gelişimi ile ilişkili olduğu gösterilmiştir. Bu çalışmada, kolorektal kanser hastalarında endoglin ile inflamatuvar mikroçevre ilişkisinin araştırılması amaçlandı.*

Gereç ve Yöntem: *Kolorektal kanser tanısı almış 50 hasta ile 50 sağlıklı gönüllü çalışmaya dahil edildi. Plazma örneklerinde, endoglin ve yaygın olarak bilinen enflamasyon belirteçlerinden olan $sPLA_2$, $cPLA_2$, NfκB, TGF-β_1 düzeyleri ELISA (enzyme-linked immunosorbent assay) yöntemi ile ölçüldü. Ayrıca, endoglin ve PLA_2 mRNA ekspresyon düzeyleri Real-Time PCR ile belirlendi.*

Sonuç ve Tartışma: *Kolorektal kanser hastalarında plazma endoglin, NfκB, TGFβ_1 düzeylerinin, kontrol grubuna göre anlamlı olarak yüksek olduğu saptanırken, $cPLA_2$ ve $sPLA_2$ düzeylerinde gruplar arasında anlamlı bir farklılık bulunmadı. Kolorektal kanser grubuna ait plazma endoglin düzeyleri ile NFκB arasında anlamlı pozitif korelasyon saptandı. Gen ekspresyon analizi bulguları, kolorektal kanser grubunda endoglin ve PLA_2 mRNA ekspresyon düzeylerinin, kontrol grubuna göre anlamlı olarak yüksek olduğunu gösterdi. Sonuç olarak, bu çalışma endoglinin kolorektal kanserdeki artışının inflamasyon gelişimi ile ilişki olabileceğini ve inflamatuvar mikroçevre ile ilişkili kötü prognozda rol oynayabileceğini göstermiştir.*

Anahtar Kelimeler: *Endoglin, Kolorektal Kanser, NFκB, PLA_2, TGFβ_1*

INTRODUCTION

Colorectal cancer used to be the 4th leading cause of cancer-related death in humans, but today it has risen to the first in men and the second in women [1]. According to recent research findings, CRC accounts for 13% of all malignant tumors and is considered the most common malignant tumor in the gastrointestinal system. Less than 20% of CRC cases are inherited, with some being linked to specific syndromes like hereditary nonpolyposis colorectal cancer (lynch syndrome) and familial adenomatous polyposis [2]. It has been discovered that environmental factors, rather than inherited genetic alterations, are responsible for the majority of CRC cases. Colorectal cancer risk factors include some intestinal infections, environmental and food-derived mutagens, and persistent intestinal inflammation that precedes tumor growth [3].

It is well known that inflammation contributes to cancer development through various mechanisms. These include the direct carcinogenic effects of infectious agents [4], the promotion of chronic inflammation by immune-mediated diseases [5], subclinical inflammation conditions such as obesity [6], and exposure to environmental carcinogens [7].

With recent studies focusing on antibody-based therapeutic strategies in cancers, various potential antigens have been characterized. Endoglin, also known as CD105, has been identified within this scope and has gained widespread popularity in recent years. Endoglin is classified as a co-receptor for TGFβ (transforming growth factor-beta), a pleiotropic cytokine, and has been shown to be expressed in endothelial cells [8]. Its expression is regulated in actively proliferating endothelial cells [9]. Studies suggest that endoglin could serve as a suitable marker for tumor-associated inflammation and angiogenesis [10,11].

This study aims to investigate the relationship between endoglin levels, a transmembrane glycoprotein known to be upregulated in inflamed tissues, and the inflammatory microenvironment in CRC patients.

MATERIAL AND METHOD

Subjects

The study included 50 voluntary patients diagnosed with colorectal cancer who were receiving treatment at the Department of Medical Oncology, Faculty of Medicine, Ankara University. Blood samples collected from 50 healthy volunteers were used as the control group. To facilitate participant enrollment, informed consent forms and questionnaire forms were obtained from each participant, after attaining the necessary approval from the Clinical Research Ethics Committee. Considering these factors, collection of venous blood samples was carried out in EDTA-containing tubes, ensuring reliable sample extraction. Plasma and RNA samples obtained from these blood samples were used for analysis in this study.

Inclusion criteria included being diagnosed with colorectal cancer, not using anticoagulant medications, having no history of vascular disease or thromboembolic or hemorrhagic disorders, being an adult (20 years or older), and having no kinship relations among the participants. Volunteers who were related, using anticoagulant medications, or had a history of vascular disease, thromboembolic, hemorrhagic or inflammatory disorders were excluded from the study. No medications were used in this study.

ELISA Measurements

The plasma levels of endoglin and other inflammatory parameters were determined using commercially available enzyme-linked immunosorbent assay (ELISA) kits (BTLab, China). The analytical sensitivity of the kit was 0.16 ng/ml, and the detection range was 0.3-60 ng/ml. The analytical sensitivities of the ELISA kits used for measuring plasma levels of $cPLA_2$ and $sPLA_2$ were 0.01 ng/ml and 0.32 ng/ml, respectively, with detection ranges of 0.05-15 ng/ml and 0.5-200 ng/ml, respectively.

The analytical sensitivity of the kit used for detecting plasma NfκB levels was less than 0.01 ng/ml, while the detection range was 0.03-10 ng/ml. Plasma levels of TGFβ1 were also determined using a commercially available ELISA kit, with a sensitivity of 5.11 ng/ml and a detection range of 10-4000 ng/ml.

Gene Expression Analyses

Whole blood samples, collected in EDTA-containing tubes, underwent RNA isolation using the RNA isolation kit (Qiagen, Germany). Then, utilizing the Qiagen RotorGene PCR system and the corresponding cDNA synthesis kit (Qiagen, Germany), cDNA samples were efficiently generated. Subsequently, quantitative real-time PCR analysis was executed via the Qiagen RotorGene system (Germany). For accurate normalization, β-actin served as the primary housekeeping gene. To ensure reliability and compliance with established protocols, the PCR reaction mix was meticulously prepared according to the manufacturer's instructions. Moreover, samples were assayed in duplicate to heighten data precision. The forward and reverse primer sequences were as follows: forward: 5'-CTGTGTCCACTTCTCCTGACC-3', and reverse: 5'-ACACTGCTGTTACACTGAGG-3' for endoglin; forward: 5'-GTTCAGGAGTGGGTGTGGAG-3', reverse: 5'-CTTAGAGGGTAGGCGATGGG-3' for PLA_2. β-actin was used as the housekeeping gene in the experiments. The fold changes were calculated according to $2^{-\Delta\Delta Ct}$ equation.

Statistical Analysis

Statistical analyses were conducted using the GraphPad Prism (GraphPad Inc., Version 6) program. The data collected from demographic analysis and ELISA tests were presented as the mean ± standard deviation and compared with Mann-Whitney U test. The real-time PCR results were evaluated with one-way ANOVA test. The correlation between endoglin and other cytokines were analyzed with Spearman correlation test. A p-value of less than 0.05 was deemed statistically significant.

RESULT AND DISCUSSION

Colorectal cancer is one of the most prevalent gastrointestinal cancers globally and remains a significant cause of cancer-related deaths [12]. The functional relationship between inflammation and cancer has long been recognized. This relationship is based on observations such as tumors emerging in regions of chronic inflammation, the presence of inflammatory cells, chemokines, and cytokines in tumors, the overexpression of cytokines and chemokines inducing cancer, and the activation or inhibition of similar molecular targets and pathways in both inflammation and carcinogenesis [13].

In this study, we investigated the relationship between endoglin, a transmembrane glycoprotein known to be associated with angiogenesis and inflammation, and the inflammatory microenvironment in patients diagnosed with colorectal cancer. The demographic data of the patient and control groups included in the study are presented in Table 1.

Table 1. Demographic data of CRC and control groups

Variable	Control (n=50)	CRC (n=50)	P value
Age, Mean±SD	34.20±13.59	61.05±8.83	<0.0001
Male, %	45.83	46.67	0.857
Site			
Left colon, %	-	10.52	
Right colon, %	-	31.57	
Sigmoid, %	-	47.36	
Rectum, %	-	10.55	
Bowel perforation at diagnosis, %	-	ND	
Bowel obstruction at diagnosis, %	-	ND	
Adenocarcinoma, %	-	78.95	
Mucinous adenocarcinoma, %	-	21.05	
Histological differentiation	-	ND	
Grade 2, %	-	76.92	
Grade 3, %	-	23.08	
CEA	-	16.44±41.73 (0.94-183.36)	

Abbreviations: CEA, carcinoembriogenic antigen, ND, not determined

The findings of the ELISA analyses are presented in Table 2. According to the results, plasma endoglin levels in the colorectal cancer group (6.71±3.51 ng/ml) were significantly higher compared to the control group (3.90±2.48 ng/ml) ($p<0.05$). Similarly, plasma NfκB levels (1.08±0.51 ng/ml) and TGFβ1 levels (565.03±395.14 ng/ml) in CRC group were significantly higher than control (0.41±0.24 ng/ml and 132.61±82.73 ng/ml, respectively) ($p<0.05$). Barely, no significance was observed between the groups for $cPLA_2$ and $sPLA_2$ levels.

Table 2. Plasma levels of endoglin, NFκB, TGF-β1, $cPLA_2$, and $sPLA_2$ in CRC and control groups

	Control	CRC	P value
Endoglin (ng/ml)	3.90±2.48	6.71±3.51	0.0081
Nfκb (ng/ml)	0.41±0.24	1.08±0.51	<0.0001
TGFβ1 (ng/ml)	132.61±82.73	565.03±395.14	<0.0001
$cPLA_2$ (ng/ml)	3.84±1.28	4.80±2.57	0.3211
$sPLA_2$ (ng/ml)	43.59±32.50	55.94±30.13	0.3270

The correlation between the elevated endoglin levels detected in CRC patients and other inflammatory markers was analyzed, and the findings are presented in Figure 1 and Table 3. According to the results, a significant positive correlation was found between endoglin and NFκB levels.

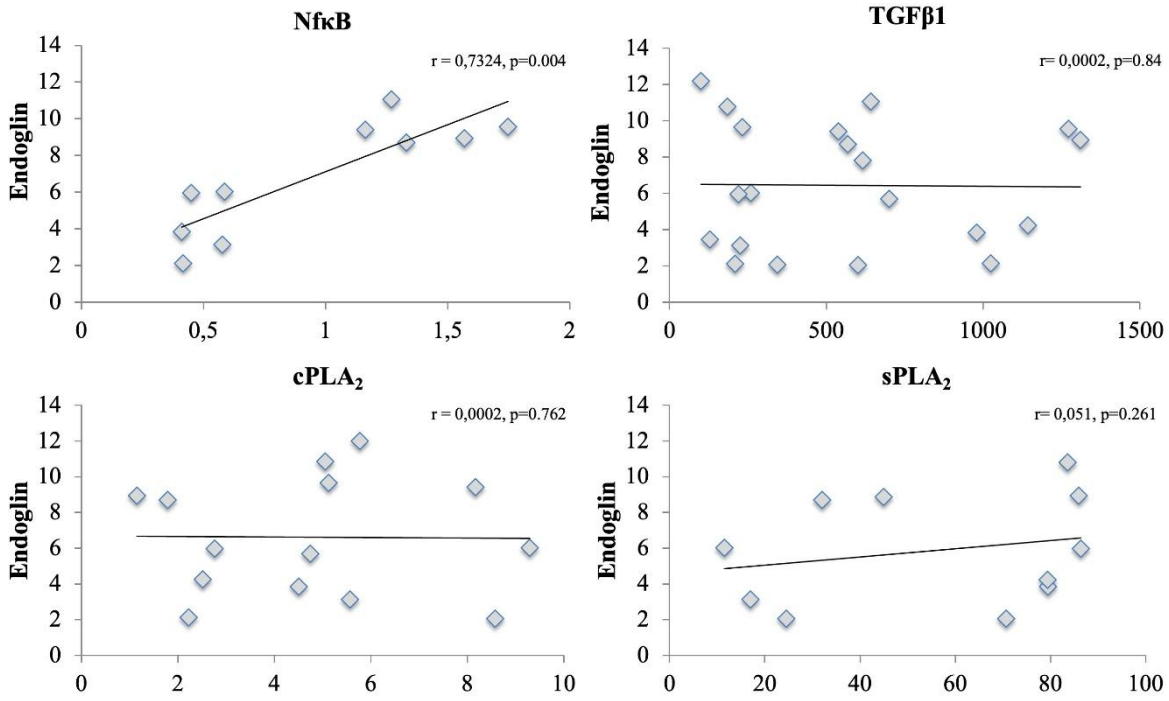


Figure 1. Correlation analyses of endoglin vs. other cytokines in CRC group

Table 3. The data of correlation analyses in CRC group

	Endoglin	
	r^2	**p value**
Nfκb	0.732	0.004
TGFβ1	0.00016	0.84
$cPLA_2$	0.00016	0.762
$sPLA_2$	0.0501	0.261

Recent research on antibody-based treatments for cancer has resulted in the identification of multiple potential targets for therapeutic intervention. Endoglin, also referred to as CD105, has been recognized within this context and has gained significant popularity in recent times. Endoglin as a TGFβ co-receptor, has been reported to be crucial for angiogenesis [14], since it is expressed in proliferating vasculature [15], and upregulated under hypoxic conditions [16]. It has been reported that endoglin is highly expressed in various tumors including lung, colorectal, and head and neck [15].

In our study, endoglin mRNA expression was measured using RT-PCR, and the results are presented in Table 4. According to our findings, endoglin mRNA expression levels in the CRC group (1.86±0.17) were significantly higher than control (1.58±0.08) ($p < 0.05$). Similarly, higher PLA_2 mRNA expression levels were measured in CRC group (1.92±0.08) vs. control (1.54±0.07) ($p < 0.05$).

Table 4. Fold increase in expression of endoglin and PLA2 mRNA in CRC and control groups

Fold increase	Control	CRC	P value
Endoglin	1.58±0.08	1.86±0.17	<0.0001
PLA_2	1.54±0.07	1.92±0.08	<0.0001

Patients with metastatic breast and colorectal tumors showed significantly elevated levels of serum endoglin compared to the control [17,18]. Serum endoglin levels have been observed to decrease as a result of chemotherapy [18]. When combined, these results indicate that measuring endoglin levels in the blood could help categorize patients with advanced cancer and those who are at risk of developing metastases. Additionally, endoglin has been reported to be useful for monitoring recurrence in cancer patients following chemotherapy [19]. Consistent with the literature, our study also found higher plasma and mRNA levels of endoglin.

Intestinal epithelial cells are in constant interaction with various gut microorganisms and other luminal components. NFκB coordinates immune system defense against external threats by maintaining barrier integrity and regulating the inflammatory response. Disruption of this homeostasis leads to susceptibility to inflammatory bowel disease and colorectal cancer [20]. It has been reported that plasma cytokine levels are frequently elevated in CRC patients [21]. NFκB, a major regulator of inflammation has been implicated in tumorigenesis and this signaling pathway is triggered by cytokines such as IL-1 and TNF, as well as T-cell receptors and B-cell receptors [22]. In colon cancer specifically, the activation of NFκB in intestinal epithelial cells and chronic inflammation is thought to have a role in tumorcigenesis [23]. NFκB activation has been reported to contribute to immune defense, particularly in acute inflammatory responses, while also exhibiting pro-inflammatory properties associated with pro-tumorigenic functions [24]. In our study, plasma NFκB levels were found to be significantly higher in the CRC group compared to controls.

Studies have shown that TGFβ1 expression is increased in colorectal cancer compared to benign adenomas and non-cancerous tissue [25]. High TGFβ levels have been observed in primary tumor tissue and plasma samples from CRC patients, correlating with metastasis and poor prognosis [26]. It's been reported that disruption of TGFβ signaling has important role in pathogenesis of variable molecular types of CRC [27]. Some studies, consistent with our findings, have reported that circulating TGFβ levels are elevated in patients with hematologic malignancies and solid tumors compared to controls [28,29]. Pathological TGFβ levels have been shown to impair both innate and adaptive cellular immunity in cancer patients [30].

Colonic tumors have been suggested to release free arachidonic acid and typically produce a higher quantity of prostaglandins (PGs) than normal mucosal tissue [31]. $cPLA_2$ is a crucial enzyme in the synthesis of PGs and its role in the development of intestinal tumors has been defined. In 1996, Soydan et al. [32] were the first to demonstrate that human colon tumors, contain high-molecular-weight $cPLA_2$. Other research indicated that $cPLA_2$ is overexpressed in 35-50% of colorectal cancers [33,34]. Additionally, another study revealed that $cPLA_2$ was overexpressed in CRC tissue samples, but showed no correlation with clinicopathological parameters, except for its association with VEGF expression [35]. Our findings indicated no significant difference in $cPLA_2$ and $sPLA_2$ levels between CRC and control groups.

In conclusion, this study alerts the existence of a CRC predictor based on endoglin, as the analysis of the relation between inflammatory microenvironment and endoglin in CRC patients and found that endoglin plasma and mRNA levels were significantly elevated in CRC patients. The correlation between NFκB and endoglin suggests that the increase in endoglin levels may be linked to the development of inflammation in CRC. Endoglin can therefore be implicated in the tumor associated inflammation acting in the colorectal cancer microenvironment.

ACKNOWLEDGEMENTS

This study was supported by Ankara University Research Foundation with 18L0237008 project number.

AUTHOR CONTRIBUTIONS

Concept: G.O., F.B.A.; Design: G.O., F.B.A.; Control: G.O., F.B.A.; Sources: G.O., D.M.K., F.B.A.; Materials: G.O., D.M.K., F.B.A.; Data Collection and/or Processing: G.O., D.M.K., F.B.A.; Analysis and/or Interpretation: G.O., F.B.A.; Literature Review: G.O., F.B.A.; Manuscript Writing: G.O., F.B.A.; Critical Review: G.O., D.M.K., F.B.A.; Other: -

CONFLICT OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

The study was conducted with the approval of the Clinical Research Ethics Committee of Ankara University with 04-225-18 number.

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J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 49(3): 689-713, 2025
Doi: 10.33483/jfpau.1479879

ORIGINAL ARTICLE / ÖZGÜN MAKALE

ACTIVE COMPOUNDS FROM MEDICINAL PLANTS AS NOVEL APPROACHES FOR ASTHMA THERAPY: *IN SILICO* DOCKING ANALYSIS

ASTIM TEDAVİSİNDE YENİ YAKLAŞIMLAR İÇİN TIBBİ BİTKİLERDEN ELDE EDİLEN AKTİF BİLEŞİKLERİN İN SİLİKO DOCKING ANALİZİ

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ABSTRACT

Objective: *Pharmaceutical molecules have historically drawn inspiration from natural substances. Nowadays, research on natural compounds is escalating, particularly on phytochemicals. In this context, asthma, characterized by airway constriction and inflammation, occupies a significant place in the search for effective herbal treatments. Nonetheless, the bronchodilatory efficacy of the bioactive phytochemicals remains ambiguous. This study explores plant-derived compounds proposed for asthma treatment, focusing on their bronchodilator effects mediated through β2-adrenergic and M3-muscarinic receptors. These receptors facilitate muscle relaxation respectively by increasing intracellular cAMP levels and reducing intracellular calcium release from the endoplasmic reticulum.*

Material and Method: *A comprehensive literature search was performed on phytochemicals tested on asthmatic animal models. A total of 898 articles were assessed in this manner. Following the elimination of duplicates, 100 bioactive plant compounds in 3D format, demonstrating potential bronchodilator effects, were assessed in silico for their impact on β2 and M3 receptors. The receptors were obtained from the Protein Data Bank. Compounds and references were docked to specific proteins by the PyRx program to facilitate the docking, and high-scored molecules were visualized by the DSV program. Besides, in silico pharmacokinetic parameters were evaluated via SwissADME tool and toxicity parameters were determined using ADMETLab 3.0 platform.*

Result and Discussion: *In the docking experiments, 34 out of the 100 compounds were demonstrated to have a potentially high affinity to β2 and/or M3 receptors. Several molecules like cryptotanshinone, paeoniflorin and rottlerin were found to have high binding affinities for the β2 receptor. On the other hand, it has been demonstrated that tetrandrine, fisetin, and cryptotanshinone have strong affinities for the M3 receptor. Considering the findings, these bioactive substances could benefit bronchodilation, particularly through the β2 and M3 receptor pathways. This in silico study highlighted the potential of plant compounds for bronchodilation in respiratory diseases and suggested avenues for future research and experimental validation in asthma therapy.*

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Submitted / Gönderilme : 07.05.2024
Accepted / Kabul : 17.04.2025
Published / Yayınlanma : 19.09.2025

Keywords: *Asthma, β2 adrenergic receptor, M3 muscarinic receptor, molecular docking, phytochemical*

ÖZ

Amaç: *İlaç molekülleri tarihsel olarak doğal maddelerden ilham alagelmektedir. Günümüzde doğal bileşikler ve özellikle fitokimyasallar üzerinde yapılan araştırmalar giderek artmaktadır. Bu bağlamda, solunum yolu daralması ve enflamasyonu ile karakterize astım hastalığı, etkili bitkisel tedavilere yönelik araştırmalarda önemli bir yer tutmaktadır. Bununla birlikte, biyoaktif fitokimyasalların bronkodilatör etkinliği belirsizliğini korumaktadır. Bu çalışmada astım tedavisi için önerilen bitkisel kaynaklı bileşiklerin, β2-adrenerjik ve M3-muskarinik reseptör aracılı bronkodilatör etkilerine odaklanılmıştır. Bu reseptörler, sırasıyla hücre içi cAMP seviyelerini artırarak ve endoplazmik retikulumdan hücre içi kalsiyum salınımını azaltarak kas gevşemesini sağlar.*

Gereç ve Yöntem: *Astımlı hayvan modelleri üzerinde çalışılmış bitkisel bileşikler hakkında kapsamlı bir literatür araştırması yapılmıştır. Toplamda 898 makale bu şekilde değerlendirmeye alınmıştır. Tekrar eden literatürlerin çıkarılmasının ardından, potansiyel bronkodilatör etkinlik gösteren üç boyutlu yapıdaki 100 farklı bitkisel bileşik, β2 ve M3 reseptörleri üzerindeki etkileri açısından in silico olarak değerlendirilmiştir. Reseptörler Protein Data Bank'tan elde edilmiştir. Bileşikler ve referanslar, kenetlenmeyi kolaylaştırmak için PyRx programı tarafından belirli proteinlere kenetlenmiş ve yüksek puanlı moleküller DSV programı tarafından görselleştirilmiştir. Ayrıca, in silico farmakokinetik parametreleri SwissADME aracılığıyla değerlendirilmiş ve toksisite parametreleri ADMETLab 3.0 platformu kullanılarak belirlenmiştir.*

Sonuç ve Tartışma: *Docking deneylerinde, 100 bileşikten 34'ünün β2 ve/veya M3 reseptörlerine potansiyel olarak yüksek afiniteye sahip olduğu gösterilmiştir. Kriptotanşinon, paeoniflorin ve rottlerin gibi birkaç molekülün β2 reseptörü için yüksek bağlanma afinitesine sahip olduğu bulunmuştur. Diğer taraftan, tetrandrin, fisetin ve kriptotanşinonun M3 reseptörü için güçlü afinitelere sahip olduğu gösterilmiştir. Bulgular göz önünde bulundurulduğunda, bu biyoaktif maddeler özellikle β2 ve M3 reseptör yolları aracılığıyla bronkodilatasyona fayda sağlayabilir. Bu in silico çalışma, solunum yolu hastalıklarında bronkodilatasyon için bitki bileşiklerinin potansiyelini göstermiştir. Astım tedavisinin gelecekteki araştırmaları ve deneysel çalışmaları için yollar göstermiştir.*

Anahtar Kelimeler: *Astım, β2 adrenerjik reseptör, bitkisel bileşik, M3 muskarinik reseptör, moleküler yerleştirme*

INTRODUCTION

Asthma has become more prevalent and severe worldwide, particularly over the past ten years. Th2-mediated immune response to environmental allergens causes allergic asthma having hallmarks such as chronic inflammation, airway aggression, mucus hypersecretion, airway eosinophilia, elevated IgE levels and airway hyperresponsiveness [1]. These result in reversible airway obstruction and airway remodeling accompanied by several symptoms such as coughing, wheezing, increased sputum production and sleep disturbance [2].

The mechanism of occurrence and development of asthma has not been exactly elucidated yet, as well as there is no definite cure [3,4]. However, currently available asthma treatments are used to alleviate symptoms and diminish the underlying airway inflammation [5]. These include β2 receptor agonists, M3 receptor antagonists, corticosteroids and xanthine with its derivatives [6].

β2 receptors are cell surface receptors that are clinically utilized in the treatment of bronchospasm in patients with bronchial asthma or other bronchospasm-related diseases [7].They are bound to heterotrimeric G protein, especially Gs protein. The activation of the G protein-coupled receptors by an agonist promotes the dissociation of guanosine diphosphate (GDP) from the α subunit of the cognate G protein. Guanosine triphosphate (GTP) then binds to G protein, and the α subunit dissociates from the β-γ unit. Thus, α subunit stimulates adenylyl cyclase to catalyze the conversion of adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP) [8]. cAMP acts as a second messenger within the cell, and in turn, cAMP initiates processes leading to smooth muscle relaxation (Figure 1) [9]. β2 receptors are predominantly found in airway smooth muscle. Numerous β2 agonists are used to

treat bronchospasm caused by disorders like bronchial asthma, including albuterol [10]. New drug development studies on the β2 receptors are ongoing.

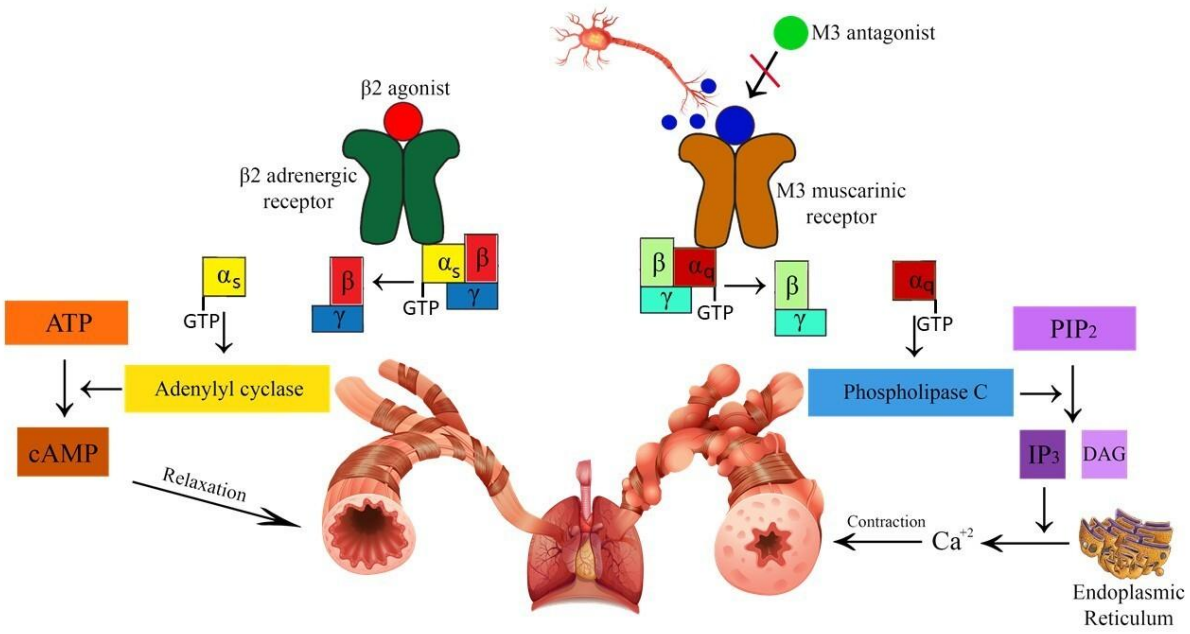


Figure 1. Mechanism of action of β2 agonists and M3 antagonists on bronchodilation
PIP_2: phosphatidylinositol 4,5- diphosphate, IP_3: inositol triphosphate, DAG: diacylglycerol

Through an asthmatic condition, elevated airway tone is seen primarily due to the activation of the vagus nerve that innervates the bronchi. The airway constriction is facilitated by the release of acetylcholine, which activates M3 receptors present throughout the bronchial system [11]. G proteins, specifically the Gq, are linked to the M3 receptors. Activation of the receptors by acetylcholine stimulates phospholipase C activity leading to membrane-derived phosphatidylinositol 4,5- diphosphate hydrolyzation. This results in the formation of inositol triphosphate (IP_3), which leads to the release of Ca^{+2} from the endoplasmic reticulum. That results in bronchial smooth muscle contraction and ultimately bronchoconstriction (Figure 1) [12]. Plants with anticholinergic effects, e.g. *Atropa belladonna*, have historically been used in the treatment of asthma, afterwards, their active compounds have led to therapeutics like muscarinic receptor antagonists, e.g. ipratropium bromide [13].

Molecular docking is an *in silico* structure-based method. Docking enables the identification of novel compounds of therapeutic interest, prediction of ligand-target interactions at the molecular level and determination of structure-activity relationships. Thus, docking has become an important component of the drug discovery process [14]. *In silico* technologies have had a tremendous influence on drug discovery, resulting in considerable savings in terms of time and cost. Utilization of cutting-edge technologies assists in selecting the appropriate chemicals for pre-clinical research [15]. Drug discovery frequently necessitates a comprehensive evaluation of numerous potential drug compounds on specific targets, in this regard, molecular docking serves as a broad screening approach to identify a wide array of potential compounds for therapeutic use [16].

Medicinal plants have been used worldwide for centuries, and in some cases, even for millennia, to promote and maintain good health, prevent diseases and treat various physical and mental ailments. These plants have garnered significant attention due to their substantial potential for addressing physiological disorders. Plants synthesize an extremely rich variety of secondary metabolites, including a large number of active or complementary compounds [17]. Although this diversity has many positive properties, it can also have certain undesirable consequences. The available empirical data regarding the

positive and negative impacts associated with the utilization of medicinal plants is currently insufficient. The field of ethnopharmacology has emerged to examine the effects of bioactive compounds derived from medicinal plants on biological systems, leading to further scientific research [6]. Indeed, many drugs in the market are derived from natural products. The application of plant extracts with hundreds of chemicals for medicinal purposes is growing day by day, and the mechanisms of action of their active compounds are being further investigated.

Numerous bioactive compounds extracted from medicinally important plants have been empirically demonstrated to possess therapeutic properties against asthma. Nonetheless, the precise mechanisms by which these substances exert their antiasthmatic effects remain ambiguous. The main objective of this study is to employ *in silico* analysis to assess the modulatory effects on β2 and M3 receptors of 100 plant-derived compounds experimentally shown to be potentially effective in treating asthma. The two receptors represent the most typically targeted molecules in the management of asthma.

MATERIAL AND METHOD

Construction of the Phytochemical Library

Plants have been employed as medicinal agents in traditional medicine for centuries. The pharmacological activities of the plants were generally attributed to the bioactive compounds, possessing a wide range of pharmacological activity. Therefore, a literature review was conducted to compile phytochemicals previously alleged to have anti-asthmatic effects. The search terms were chosen considering the sensitizing agents used to form/create asthma models on animals and the terms related to secondary metabolites since this research is focused on *in vivo* experimental studies and plant compounds. The keywords were as follows; ((asthma) OR (ovalbumin) OR (house dust mite)) AND ((medicinal plant) OR (phytotherapeutic) OR (herbal medicine) OR (bioactive compound) OR (phytochemistry) OR (essential oil) OR (secondary metabolite)).

For this review, PubMed, Scopus and Web of Science databases were searched for relevant articles in English published between 2013 and 2024. A total of 898 articles were retrieved from the databases. After removing duplicates, 653 studies remained. The primary selection criteria were that the experiments involved animals and employed a pure plant compound. The number of publications was reduced to 125 through implementing the requirements. 113 unique molecules were identified following the determination of the subjected molecules to the studies. Thirteen of these compounds lacked 3D structures in the PubChem database [18]. Ultimately, the *in silico* investigation encompassed 100 bioactive phytochemicals.

Protein Selection and Preparation

The X-ray crystallographic structures of the target proteins, i.e., M3 receptor (PDB code: 4U15) (resolution: 2.80 Å) and β2 receptor (PDB code: 6MXT) (resolution: 2.96 Å), were obtained from the Protein Data Bank (PDB) [19]. The 3D structures of two receptors were loaded to the chimera window and processed using the DockPrep tool in UCSF Chimera software. Water molecules were eliminated by the addition of polar hydrogens, and Gasteiger charges were accordingly calculated. In addition, all chains and residues except chain A were removed for simplicity and to ensure accuracy. Protein structures from X-ray crystallography may contain additional chains and residues due to crystal packing artefacts. These chains may not be biologically relevant in solution and can impede the docking investigations. Furthermore, the docking process is guaranteed to target the biologically relevant region by selecting the appropriate chain if the docking research focuses on a specific active site or interface. The PDBQT formats were ultimately obtained.

Ligand Preparation

Based on the comprehensive literature search, 100 of the natural compounds tested out *in vivo* to treat asthma were selected. Formoterol, a β2-receptor agonist, and tiotropium, an M3-receptor antagonist, were assigned as reference ligands based on their clinical use. These compounds were included in the docking process. Structures of the selected compounds were downloaded from PubChem in SDF format. The 3D SDF-formatted ligands were loaded into PyRx (software version 0.8) using the

OpenBabel graphical user interface [20]. The energy minimization of ligands was carried out using PyRx software. A total of 200 steps were performed using the conjugate gradient algorithm, with a step size of 1. The Universal Force Field (UFF) was employed during the minimization process, due to its well-suited for small-molecule ligand optimization. Following minimization, the ligands were converted to AutoDock-compatible PDBQT format using PyRx, ensuring the preservation of the minimized energy states for subsequent docking studies. Also, the protein was converted to PDBQT format using PyRx to prepare it for docking.

Molecular Docking

An *in silico* approach to ligand and receptor docking analysis has been used to investigate structural complexes. Therefore, PyRx virtual screening tool with Auto Dock Vina was employed in the current study. For each docking study, the grid box was determined based on the position of the ligand present in the X-ray structure. The grid box sizes were set to 24 × 24 × 24 and 25 × 25 × 25 for receptors PDB: 4U15 and PDB: 6MXT, respectively. Grid boxes were centered at x = 47.26, y = 92.48, z = 55.15 and x = -10.60, y = -3.93, z = 37.83 for the receptors, respectively. The other parameters were kept as default, besides, all of the bonds in the ligand were allowed to rotate freely, considering the receptor as rigid. The docking simulation was run with an exhaustiveness level of 8. The ligand with the most negative binding energy is meant to have the highest binding affinity [21]. The final visualization of the docked structures was performed using Discovery Studio Visualizer 3.0. The binding mode diagram between the target protein receptor and the compounds was drawn in 2D (two-dimensional).

The validation of the targeted active sites for both the β2-adrenergic receptor (6MXT) and the M3-muscarinic receptor (4U15) was performed by calculating the cluster Root-Mean-Square Deviation (RMSD) values. To ensure the reliability of the docking studies, the cluster RMSD values are required to fall within the range of 0–2 Å. For the validation of molecular docking, redocking was conducted within the predefined grid boxes created for the target regions of both 6MXT (including salmeterol) and 4U15 (including tiotropium). The cluster RMSD values for the ligand molecules, tested across 10 and 9 different conformations respectively, were determined to be 0.983 Å for 6MXT and 0.827 Å for 4U15. These results demonstrate that the ligand compounds docked to the designated target regions for both receptors exhibited RMSD values within the acceptable range, confirming the validity and consistency of the docking approach.

In silico ADMET

Pharmacokinetic parameters, including LogP, oral bioavailability, gastrointestinal absorption, blood-brain barrier permeability, skin permeation, cytochrome P450 enzyme inhibition, and drug-likeness (evaluated using the Lipinski Rule of Five), were calculated using the SwissADME tool [22]. Additionally, the potential toxicity of the substances was assessed utilizing the ADMETLab 3.0 platform [23].

RESULT AND DISCUSSION

Since β2 agonists and M3 antagonists are involved in the relaxation of airway bronchoconstriction as part of current therapeutic strategies, the receptors were identified as the targets for the plant-derived compounds [24].

Table 1 displays the docking study results for each of the receptors. The results are expressed as the docking scores (kcal/mol). Herein, low docking scores (kcal/mol) suggest high binding energies [25]. The reference compounds formoterol and tiotropium have docking scores of -9.297 and -9.312, respectively. It is noteworthy that plenty of the natural compounds have higher binding energies than the reference compounds like robustaflavone-4'-dimethyl ether, -10.5; cryptotanshinone, -10.2; rottlerin, -9.9 macelignan, -9.7; paeoniflorin, -9.7 for β2 receptor, and cryptotanshinone, -9.9; tetrandrine, -9.8; fisetin, -9.7; ovatodiolide, -9.5; columbianadin, -9.5 for M3 receptor.

Table 1. Assessment of the antiasthmatic activity of selected active compounds by molecular docking on β2 and M3 receptors

No	Compound's name	IUPAC name	Docking score (-) (kcal/mol)		References
			β2	M3	
1	(2'S,7'S)-O-(2-methylbutanoyl)-columbianetin (OMC)	(E)-3-(2-methylphenyl)prop-2-enamide	-7.8	-9.4	[26]
2	4-carvomenthenol	4-methyl-1-propan-2-ylcyclohex-3-en-1-ol	-6.6	-6.7	[27]
3	6-shogaol	(*E*)-1-(4-hydroxy-3-methoxyphenyl)dec-4-en-3-one	-8.8	-8.0	[28]
4	Acteoside	[(2*R*,3*R*,4*R*,5*R*,6*R*)-6-[2-(3,4-dihydroxyphenyl)ethoxy]-5-hydroxy-2-(hydroxymethyl)-4-[(2*S*,3*R*,4*R*,5*R*,6*S*)-3,4,5-trihydroxy-6-methyloxan-2-yl]oxyoxan-3-yl](*E*)-3-(3,4 dihydroxyphenyl)prop-2-enoate	-8.9	-8.5	[29]
5	Apigenin	5,7-dihydroxy-2-(4-hydroxyphenyl) chromen-4-one	-8.7	-9.0	[30]
6	Baicalein	5,6,7-trihydroxy-2-phenylchromen-4-one	-8.5	-9.2	[31]
7	Baicalin	(2*S*,3*S*,4*S*,5*R*,6*S*)-6-(5,6-dihydroxy-4-oxo-2-phenylchromen-7-yl)oxy-3,4,5-trihydroxyoxane-2-carboxylic acid	-9.4	-9.3	[32]
8	Berberine	16,17-dimethoxy-5,7-dioxa-13-azoniapentacyclo[11.8.0.0^{2,10}.0^{4,8}.0^{15,20}]henicosa-1(13),2,4(8),9,14,16,18,20-octaene	-9.6	-7.7	[33]
9	Bruceine D	(1R,2R,3R,6R,8S,12S,13S,14R,15R,16S,17R)-2,3,12,15,16-pentahydroxy-9,13,17-trimethyl-5,18-dioxapentacyclo[12.5.0.01,6.02,17.08,13]nonadec-9-ene-4,11-dione	-8.8	-9.3	[34]
10	Calcaratarin D	(3E,4S)-4-Hydroxy-3-{2-[(1S,4aS,8aS)-5,5,8a-trimethyl-2-methylenedecahydro-1-naphthalenyl]ethylidene}dihydro-2(3H)-furanone	-8.7	-9.2	[35]
11	Calycosin	7-hydroxy-3-(3-hydroxy-4-methoxyphenyl)chromen-4-one	-9.0	-8.8	[36]
12	Carvacrol	2-methyl-5-propan-2-ylphenol	-7.0	-7.0	[37]
13	Casticin	5-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-3,6,7-trimethoxychromen-4-one	-8.1	-7.2	[38]
14	Cedrol	(1*S*,2*R*,5*S*,7*R*,8*R*)-2,6,6,8-tetramethyltricyclo[5.3.1.0^{1,5}]undecan-8-ol	-7.5	-8.9	[39]
15	Celastrol	(2*R*,4*aS*,6*aR*,6*aS*,14*aS*,14*bR*)-10-hydroxy-2,4*a*,6*a*,6*a*,9,14*a*-hexamethyl-11-oxo-1,3,4,5,6,13,14,14*b*-octahydropicene-2-carboxylic acid	-5.5	-5.2	[40]
16	Chrysin	5,7-dihydroxy-2-phenylchromen-4-one	-8.8	-9.3	[41]
17	Columbianadin	2-[(8*S*)-2-oxo-8,9-dihydrofuro[2,3-h]chromen-8-yl]propan-2-yl (*Z*)-2-methylbut-2-enoate	-9.3	-9.5	[42]
18	Cryptotanshinone	(1*R*)-1,6,6-trimethyl-2,7,8,9-tetrahydro-1*H*-naphtho[1,2-g][1]benzofuran-10,11-dione	-10.2	-9.9	[43]
19	Cycloastragenol	(1*S*,3*R*,6*S*,8*R*,9*S*,11*S*,12*S*,14*S*,15*R*,16*R*)-15-[(2*R*,5*S*)-5-(2-hydroxypropan-2-yl)-2-methyloxolan-2-yl]-7,7,12,16-tetramethylpentacyclo[9.7.0.0^{1,3}.0^{3,8}.0^{12,16}]octadecane-6,9,14-triol	-8.7	-4.9	[44]
20	Daphnetin	7,8-dihydroxychromen-2-one	-8.3	-7.3	[45]
21	Dehydrodieugenol	2-(2-hydroxy-3-methoxy-5-prop-2-enylphenyl)-6-methoxy-4-prop-2-enylphenol	-7.3	-7.4	[46]
22	Dehydromatricarin	[(3*aR*,4*S*,6*S*,6*aS*,9*aR*,9*bR*)-6,9-dimethyl-3-methylidene-2,7-dioxo-4,5,6,6*a*,9*a*,9*b*-hexahydro-3*aH*-azuleno[4,5-b]furan-4-yl] acetate	-8.7	-9.1	[47]
23	Deoxyvasicine	1,2,3,9-tetrahydropyrrolo[2,1-b]quinazoline	6.5	7.4	[48]

Table 1 ***(continue).*** Assessment of the antiasthmatic activity of selected active compounds by molecular docking on β2 and M3 receptors

No	Compound's name	IUPAC name	Docking score (-) (kcal/mol)		References
			β2	M3	
24	Dihydromyricetin	(2*R*,3*R*)-3,5,7-trihydroxy-2-(3,4,5-trihydroxyphenyl)-2,3-dihydrochromen-4-one	-8.0	-8.3	[49]
25	Diosgenin	(1*S*,2*S*,4*S*,5'*R*,6*R*,7*S*,8*R*,9*S*,12*S*,13*R*,16*S*)-5',7,9,13-tetramethylspiro[5-oxapentacyclo[10.8.0.0^{2,9}.0^{4,8}.0^{13,18}]icos-18-ene-6,2'-oxane]-16-ol	-9.3	-3.6	[50]
26	Dmp-butenol	(E)-4-(3',4'-dimethoxyphenyl)but-3-en-1-ol	-6.6	-6.7	[51]
27	Ellagic acid	6,7,13,14-tetrahydroxy-2,9-dioxatetracyclo[6.6.2.0^{4,16}.0^{11,15}]hexadeca-1(15),4,6,8(16),11,13-hexaene-3,10-dione	-8.4	-8.5	[52]
28	Estragole	1-methoxy-4-prop-2-enylbenzene	-6.0	-6.1	[53]
29	Ethyl p-methoxycinnamate	ethyl (*E*)-3-(4-methoxyphenyl)prop-2-enoate	-6.8	-6.9	[54]
30	Eucalyptol	1,3,3-trimethyl-2-oxabicyclo[2.2.2]octane	-6.0	-6.6	[55]
31	Farnesol	(2*E*,6*E*)-3,7,11-trimethyldodeca-2,6,10-trien-1-ol	-7.6	-7.5	[56]
32	Fisetin	2-(3,4-dihydroxyphenyl)-3,7-dihydroxychromen-4-one	-8.2	-9.7	[57]
33	FLLL31	(1*E*,6*E*)-1,7-bis(3,4-dimethoxyphenyl)-4,4-dimethylhepta-1,6-diene-3,5-dione	-9.4	-8.1	[58]
34	Fucoidan	[(2*S*,3*S*,4*S*,5*S*,6*R*)-4-hydroxy-5-methoxy-2,6-dimethyloxan-3-yl] hydrogen sulfate	-5.7	-6.8	[59]
35	Fucoxanthin	(3S,3'S,5R,5'R,6S,6'R,8'R)-3,5'-Dihydroxy-8-oxo-6',7'-didehydro-5,5',6,6',7,8-hexahydro-5,6-epoxy-β,β-caroten-3'-yl acetate	-7.5	-0.7	[60]
36	Geniposide	methyl (1*S*,4*aS*,7*aS*)-7-(hydroxymethyl)-1-[(2*S*,3*R*,4*S*,5*S*,6*R*)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy-1,4*a*,5,7*a*-tetrahydrocyclopenta[c]pyran-4-carboxylate	-7.6	-7.6	[61]
37	Guggulsterone	(8*R*,9*S*,10*R*,13*S*,14*S*,17*Z*)-17-ethylidene-10,13-dimethyl-1,2,6,7,8,9,11,12,14,15-decahydrocyclopenta[a]phenanthrene-3,16-dione	-8.9	-8.9	[62]
38	Citronellol	3,7-dimethyloct-6-en-1-ol	-6.2	-5.8	[63]
39	Hypophyllanthin	(7*R*,8*R*,9*S*)-9-(3,4-dimethoxyphenyl)-4-methoxy-7,8-bis(methoxymethyl)-6,7,8,9-tetrahydrobenzo[g][1,3]benzodioxole	-8.0	-6.8	[64]
40	Icariin	5-hydroxy-2-(4-methoxyphenyl)-8-(3-methylbut-2-enyl)-7-[(2*S*,3*R*,4*S*,5*S*,6*R*)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy-3-[(2*S*,3*R*,4*R*,5*R*,6*S*)-3,4,5-trihydroxy-6-methyloxan-2-yl]oxychromen-4-one	-9.0	-5.8	[65]
41	Imperatorin	9-(3-methylbut-2-enoxy)furo[3,2-g] chromen-7-one	-8.2	-9.2	[66]
42	Isoliquiritigenin	(*E*)-1-(2,4-dihydroxyphenyl)-3-(4-hydroxyphenyl)prop-2-en-1-one	-8.7	-8.5	[67]
43	Isoquercitrin	2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-3-[(2*S*,3*R*,4*S*,5*S*,6*R*)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxychromen-4-one	-7.4	-8.8	[68]
44	LicochalconeA	(*E*)-3-[4-hydroxy-2-methoxy-5-(2-methylbut-3-en-2-yl)phenyl]-1-(4-hydroxyphenyl)prop-2-en-1-one	-8.2	-8.4	[69]
45	Ligustrazine	2,3,5,6-tetramethylpyrazine	-5.3	-5.8	[70]
46	Linalool	3,7-dimethylocta-1,6-dien-3-ol	-6.0	-6.0	[71]

Table 1 ***(continue).*** Assessment of the antiasthmatic activity of selected active compounds by molecular docking on β2 and M3 receptors

No	Compound's name	IUPAC name	Docking score (-) (kcal/mol)		References
			β2	M3	
47	Luteolin	2-(3,4-dihydroxyphenyl)-5,7-dihydroxychromen-4-one	-8.5	-9.3	[72]
48	Macelignan	4-[(2*S*,3*R*)-4-(1,3-benzodioxol-5-yl)-2,3-dimethylbutyl]-2-methoxyphenol	-9.7	-9.0	[73]
49	Magnolol	2-(2-hydroxy-5-prop-2-enylphenyl)-4-prop-2-enylphenol	-8.4	-8.4	[74]
50	Mangiferin	1,3,6,7-tetrahydroxy-2-[(2*S*,3*R*,4*R*,5*S*,6*R*)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]xanthen-9-one	-8.7	-6.4	[75]
51	Menthone	(2*S*,5*R*)-5-methyl-2-propan-2-ylcyclohexan-1-one	-6.0	-6.6	[76]
52	Morin	2-(2,4-dihydroxyphenyl)-3,5,7-trihydroxychromen-4-one	-8.2	-8.4	[77]
53	Myrtenol	(6,6-dimethyl-2-bicyclo[3.1.1]hept-2-enyl)methanol	-6.4	-6.6	[78]
54	Narciclasine	(2*S*,3*R*,4*S*,4*aR*)-2,3,4,7-tetrahydroxy-3,4,4*a*,5-tetrahydro-2*H*-[1,3]dioxolo[4,5-j]phenanthridin-6-one	-8.2	-8.4	[79]
55	Nepetin	2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-6-methoxychromen-4-one	-8.3	-8.4	[80]
56	Nujiangexanthone A	1,2,5,6-tetrahydroxy-3-methoxy-4,7,8-tris(3-methylbut-2-enyl)xanthen-9-one	-8.2	-5.3	[81]
57	Oleanolic acid	(4*aS*,6*aR*,6*aS*,6*bR*,8*aR*,10*S*,12*aR*,14*bS*)-10-hydroxy-2,2,6*a*,6*b*,9,9,12*a*-heptamethyl-1,3,4,5,6,6*a*,7,8,8*a*,10,11,12,13,14*b*-tetradecahydropicene-4*a*-carboxylic acid	-5.6	-0.9	[82]
58	Oleic acid	(*Z*)-octadec-9-enoic acid	-7.3	-7.1	[83]
59	Oroxylin A	5,7-dihydroxy-6-methoxy-2-phenylchromen-4-one	-8.0	-8.2	[84]
60	Osthole	7-methoxy-8-(3-methylbut-2-enyl)chromen-2-one	-7.4	-8.9	[85]
61	Ovatodiolide	(1*S*,3*E*,5*R*,9*S*,12*E*)-3,12-dimethyl-8-methylidene-6,18-dioxatricyclo[14.2.1.$0^{5,9}$]nonadeca-3,12,16(19)-triene-7,17-dione	-8.2	-9.5	[86]
62	Oxyresveratrol	4-[(*E*)-2-(3,5-dihydroxyphenyl)ethenyl]benzene-1,3-diol	-9.3	-8.2	[87]
63	Paeoniflorin	[(1*R*,2*S*,3*R*,5*R*,6*R*,8*S*)-6-hydroxy-8-methyl-3-[(2*S*,3*R*,4*S*,5*S*,6*R*)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy-9,10-dioxatetracyclo[4.3.1.$0^{2,5}$.$0^{3,8}$]decan-2-yl]methyl benzoate	-9.7	-9.0	[88]
64	Phyllanthin	4-[(2*S*,3*S*)-3-[(3,4-dimethoxyphenyl)methyl]-4-methoxy-2-(methoxymethyl)butyl]-1,2-dimethoxybenzene	-8.0	-6.8	[89]
65	Piscroside A	[(1*R*,4*R*,5*S*,6*R*,7*R*,8*S*,9*S*)-5-chloro-3-hydroxy-9-[(2*S*,3*R*,4*S*,5*S*,6*R*)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy-2,10-dioxatricyclo[5.3.1.$0^{4,8}$]undecan-6-yl] 4-hydroxy-3-methoxybenzoate	-9.2	-9.2	[90]
66	Praeruptorin E	[(9*S*,10*S*)-8,8-dimethyl-10-(3-methylbutanoyloxy)-2-oxo-9,10-dihydropyrano[2,3-f]chromen-9-yl] (*Z*)-2-methylbut-2-enoate	-9.0	-8.3	[91]
67	Protocatechuic acid	3,4-dihydroxybenzoic acid	-7.1	-6.3	[92]
68	Protostemonine	(5*Z*)-4-methoxy-3-methyl-5-[(1*S*,2*R*,3*S*,6*R*,11*S*)-3-methyl-11-[(2*S*,4*S*)-4-methyl-5-oxooxolan-2-yl]-5-oxa-10-azatricyclo[8.3.0.$0^{2,6}$]tridecan-4-ylidene]furan-2-one	-8.8	-2.9	[93]
69	Puerarin	7-hydroxy-3-(4-hydroxyphenyl)-8-[(2*S*,3*R*,4*R*,5*S*,6*R*)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]chromen-4-one	-8.9	-7.2	[94]

Table 1 ***(continue).*** Assessment of the antiasthmatic activity of selected active compounds by molecular docking on β2 and M3 receptors

No	Compound's name	IUPAC name	Docking score (-) (kcal/mol)		References
			β2	M3	
70	Pyrroloquinoline quinone	4,5-dioxo-1*H*-pyrrolo[2,3-f]quinoline-2,7,9-tricarboxylic acid	-8.8	-8.3	[95]
71	Quercetin	2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxychromen-4-one	-8.2	-8.6	[96]
72	Robustaflavone-7,4'-dimethyl ether	5,7-dihydroxy-6-[5-(5-hydroxy-7-methoxy-4-oxochromen-2-yl)-2-methoxyphenyl]-2-(4-hydroxyphenyl)chromen-4-one	-10.5	-2.5	[97]
73	Roseoside	(4*S*)-4-hydroxy-3,5,5-trimethyl-4-[(*E*,3*R*)-3-[(2*R*,3*R*,4*S*,5*S*,6*R*)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxybut-1-enyl]cyclohex-2-en-1-one	-8.4	-8.1	[98]
74	Rosmarinic acid	(2*R*)-3-(3,4-dihydroxyphenyl)-2-[(*E*)-3-(3,4-dihydroxyphenyl)prop-2-enoyl]oxypropanoic acid	-9.2	-8.9	[99]
75	Rottlerin	(*E*)-1-[6-[(3-acetyl-2,4,6-trihydroxy-5-methylphenyl)methyl]-5,7-dihydroxy-2,2-dimethylchromen-8-yl]-3-phenylprop-2-en-1-one	-9.9	-6.9	[100]
76	S-allyl cysteine	(2*R*)-2-amino-3-prop-2-enylsulfanylpropanoic acid	-5.1	-4.8	[101]
77	Safranal	2,6,6-trimethylcyclohexa-1,3-diene-1-carbaldehyde	-6.6	-6.6	[102]
78	Sarsasapogenin	(1*R*,2*S*,4*S*,5'*S*,6*R*,7*S*,8*R*,9*S*,12*S*,13*S*,16*S*,18*R*)-5',7,9,13-tetramethylspiro[5-oxapentacyclo[10.8.0.0^{2,9}.0^{4,8}.0^{13,18}]icosane-6,2'-oxane]-16-ol	-7.9	-4.0	[103]
79	Schisandrin B	3,4,5,19-tetramethoxy-9,10-dimethyl-15,17-dioxatetracyclo[10.7.0.0^{2,7}.0^{14,18}]nonadeca-1(19),2,4,6,12,14(18)-hexaene	-7.2	-7.6	[104]
80	Schisandrol A	3,4,5,14,15,16-hexamethoxy-9,10-dimethyltricyclo[10.4.0.0^{2,7}]hexadeca-1(16),2,4,6,12,14-hexaen-9-ol	-7.0	-6.5	[105]
81	Sesamol	1,3-benzodioxol-5-ol	-6.4	-5.7	[106]
82	Silymarin	3,5,7-trihydroxy-2-[3-(4-hydroxy-3-methoxyphenyl)-2-(hydroxymethyl)-2,3-dihydro-1,4-benzodioxin-6-yl]-2,3-dihydrochromen-4-one	-9.2	-9.3	[107]
83	Sinapic acid	(*E*)-3-(4-hydroxy-3,5-dimethoxyphenyl)prop-2-enoic acid	-7.4	-7.1	[108]
84	Sinigrin	potassium;[(*E*)-1-[(2*S*,3*R*,4*S*,5*S*,6*R*)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]sulfanylbut-3-enylideneamino] sulfate	-6.3	-6.9	[109]
85	Solasodine	(1*S*,2*S*,4*S*,5'*R*,6*R*,7*S*,8*R*,9*S*,12*S*,13*R*,16*S*)-5',7,9,13-tetramethylspiro[5-oxapentacyclo[10.8.0.0^{2,9}.0^{4,8}.0^{13,18}]icos-18-ene-6,2'-piperidine]-16-ol	-9.0	-3.6	[110]
86	Sophoraflavanone G	(2*S*)-2-(2,4-dihydroxyphenyl)-5,7-dihydroxy-8-[(2*R*)-5-methyl-2-prop-1-en-2-ylhex-4-enyl]-2,3-dihydrochromen-4-one	-8.5	-9.0	[111]
87	Sophoricoside	5,7-dihydroxy-3-[4-[(2*S*,3*R*,4*S*,5*S*,6*R*)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxyphenyl]chromen-4-one	-9.6	-8.2	[112]
88	Spilanthol (SP)	(2*E*,6*Z*,8*E*)-*N*-(2-methylpropyl)deca-2,6,8-trienamide	-7.4	-7.2	[113]
89	Synephrine	4-[1-hydroxy-2-(methylamino)ethyl]phenol	-7.0	-6.2	[114]
90	Syringin	(2*R*,3*S*,4*S*,5*R*,6*S*)-2-(hydroxymethyl)-6-[4-[(*E*)-3-hydroxyprop-1-enyl]-2,6-dimethoxyphenoxy]oxane-3,4,5-triol	-7.4	-6.9	[115]
91	Tetrandrine	(1*S*,14*S*)-9,20,21,25-tetramethoxy-15,30-dimethyl-7,23-dioxa-15,30-diazaheptacyclo[22.6.2.2^{3,6}.1^{8,12}.1^{14,18}.0^{27,31}.0^{22,33}]hexatriaconta-3(36),4,6(35),8,10,12(34),18,20,22(33),24,26,31-dodecaene	-4.8	-9.8	[116]

Table 1 ***(continue).*** Assessment of the antiasthmatic activity of selected active compounds by molecular docking on β2 and M3 receptors

No	Compound's name	IUPAC name	Docking score (-) (kcal/mol)		References
			β2	M3	
92	Thymol	5-methyl-2-propan-2-ylphenol	-6.4	-6.8	[117]
93	Tomatidine	(1*R*,2*S*,4*S*,5'*S*,6*S*,7*S*,8*R*,9*S*,12*S*,13*S*,16*S*,18*S*)-5',7,9,13-tetramethylspiro[5-oxapentacyclo[10.8.0.0^{2,9}.0^{4,8}.0^{13,18}]icosane-6,2'-piperidine]-16-ol	-8.4	-4.8	[118]
94	Trans-anethole	1-methoxy-4-[(*E*)-prop-1-enyl]benzene	-6.2	-6.2	[119]
95	Vasicine	(3*S*)-1,2,3,9-tetrahydropyrrolo[2,1-b]quinazolin-3-ol	-6.6	-8.0	[120]
96	Vasicinone	(3*S*)-3-hydroxy-2,3-dihydro-1*H*-pyrrolo[2,1-b]quinazolin-9-one	-7.2	-8.2	[120]
97	Vicenin-2	5,7-dihydroxy-2-(4-hydroxyphenyl)-6,8-bis[(2*S*,3*R*,4*R*,5*S*,6*R*)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]chromen-4-one	-8.3	-1.5	[121]
98	Vitexin	5,7-dihydroxy-2-(4-hydroxyphenyl)-8-[(2*S*,3*R*,4*R*,5*S*,6*R*)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]chromen-4-one	-8.5	-8.7	[122]
99	Withaferin A	(1*S*,2*R*,6*S*,7*R*,9*R*,11*S*,12*S*,15*R*,16*S*)-6-hydroxy-15-[(1*S*)-1-[(2*R*)-5-(hydroxymethyl)-4-methyl-6-oxo-2,3-dihydropyran-2-yl]ethyl]-2,16-dimethyl-8-oxapentacyclo[9.7.0.0^{2,7}.0^{7,9}.0^{12,16}]octadec-4-en-3-one	-9.3	-5.7	[123]
100	Wogonin	5,7-dihydroxy-8-methoxy-2-phenylchromen-4-one	-8.9	-9.2	[124]

The intra-receptor positions of the three significant compounds with the highest binding affinities in the molecular docking for each target protein are shown in Figures 2 and 3. Obtained 2D diagrams via Discovery Studio Visualization regarding the amino acids present in the ligand binding sites and chemical bonds are also given in the figures. The chemical bonds include carbon-hydrogen bonds, π-sigma bonds, alkyl bonds and van der Waals forces. Among these, hydrogen bonds in particular contribute far more to the strength of the interaction.

Inevitably, there is presently no radical treatment for every disease including asthma. However, there exist some approaches to alleviating the symptoms of asthma by medical therapy [125].

One of the strategies is the activation of β2 receptors that promote the relaxation of smooth muscle. Although the mechanism of smooth muscle relaxation is uncertain, it may involve the phosphorylation of myosin light-chain kinase to an inactive form [8]. On the other hand, the release of acetylcholine by the vagus nerve activates M3 receptors, triggering an increase in intracellular Ca^{+2} concentration. Eventually, smooth muscle contraction occurs and airway tone increases. Thereby, β2 agonists and M3 antagonists contribute to relaxing airway bronchoconstriction (Figure 1) [6]. Apart from bronchoconstriction, asthma also possesses an immunological involvement. Even though the inflammatory basis of asthma is not entirely understood, the Th-2-mediated immune response is known to be associated. Thus, either lessening inflammation or bronchodilation are among the most valuable therapeutic strategies in asthma [5].

In addition to their therapeutic benefits, pharmaceuticals prescribed for asthma entail a variety of negative side effects. Hence, novel effective natural alternatives with minimal side effects are required. In this context, the effects of many plant-derived substances on the treatment of asthma have already been investigated in previous studies [126–130]. Once evaluating the effects of plant compounds on the immunopathological aspects, it has been depicted that the phytochemicals inhibit certain mediators involved particularly in asthma, such as Ig E, IL-4, IL-5 and eosinophils [126,127]. On the other hand, not much is known about the capacity of phytochemicals to alleviate bronchoconstriction. That is why,

the current study is focused on B2 and M3 receptors due to their involvement in bronchodilation, which aids asthma treatment.

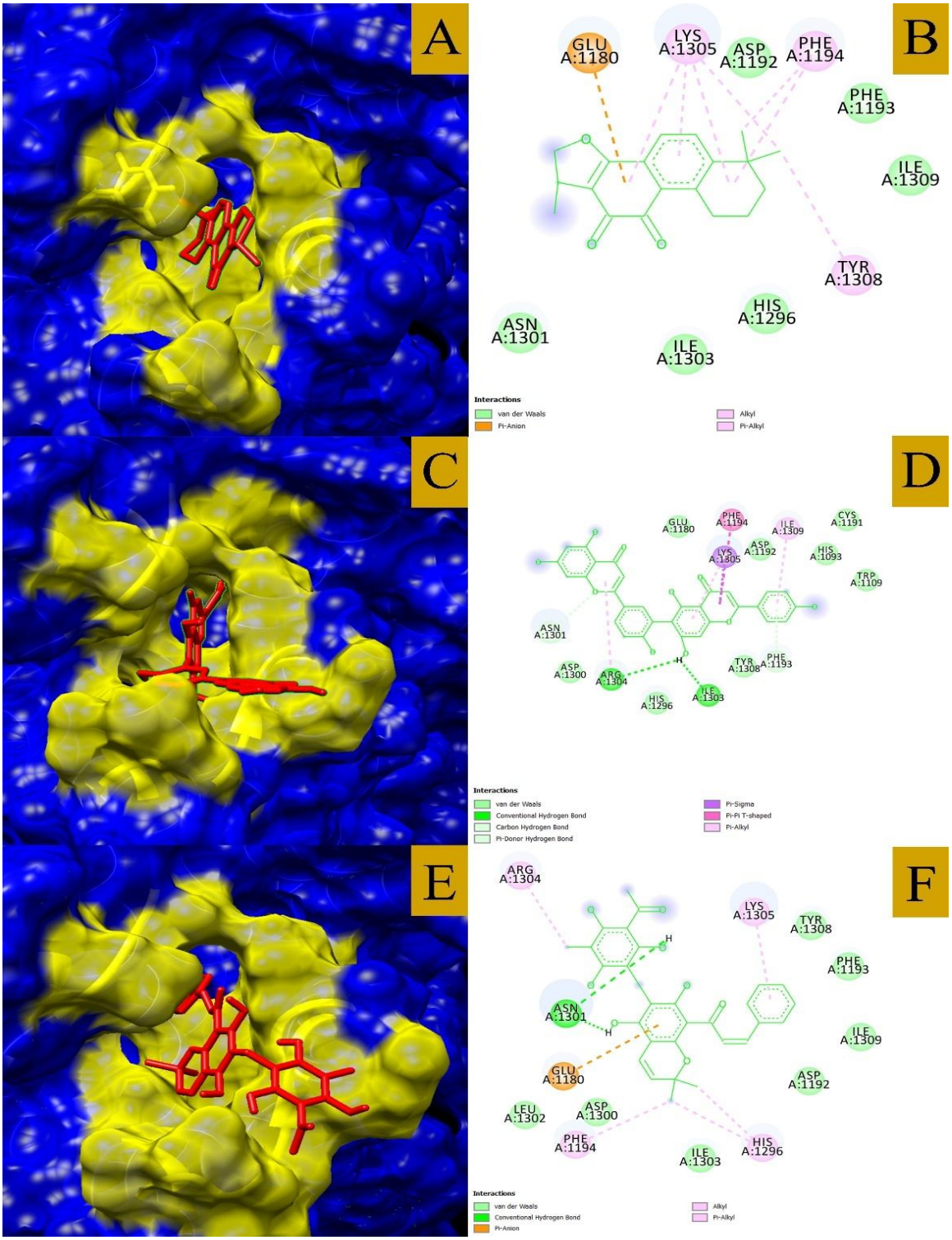


Figure 2. Molecular docking diagram of chemical composition to 6MXT (β2 receptor) and their 2D diagram of ligand binding site atoms of cryptotanshinone (A, B), robustaflavone-4'-dimethyl ether (C, D), rottlerin (E, F)

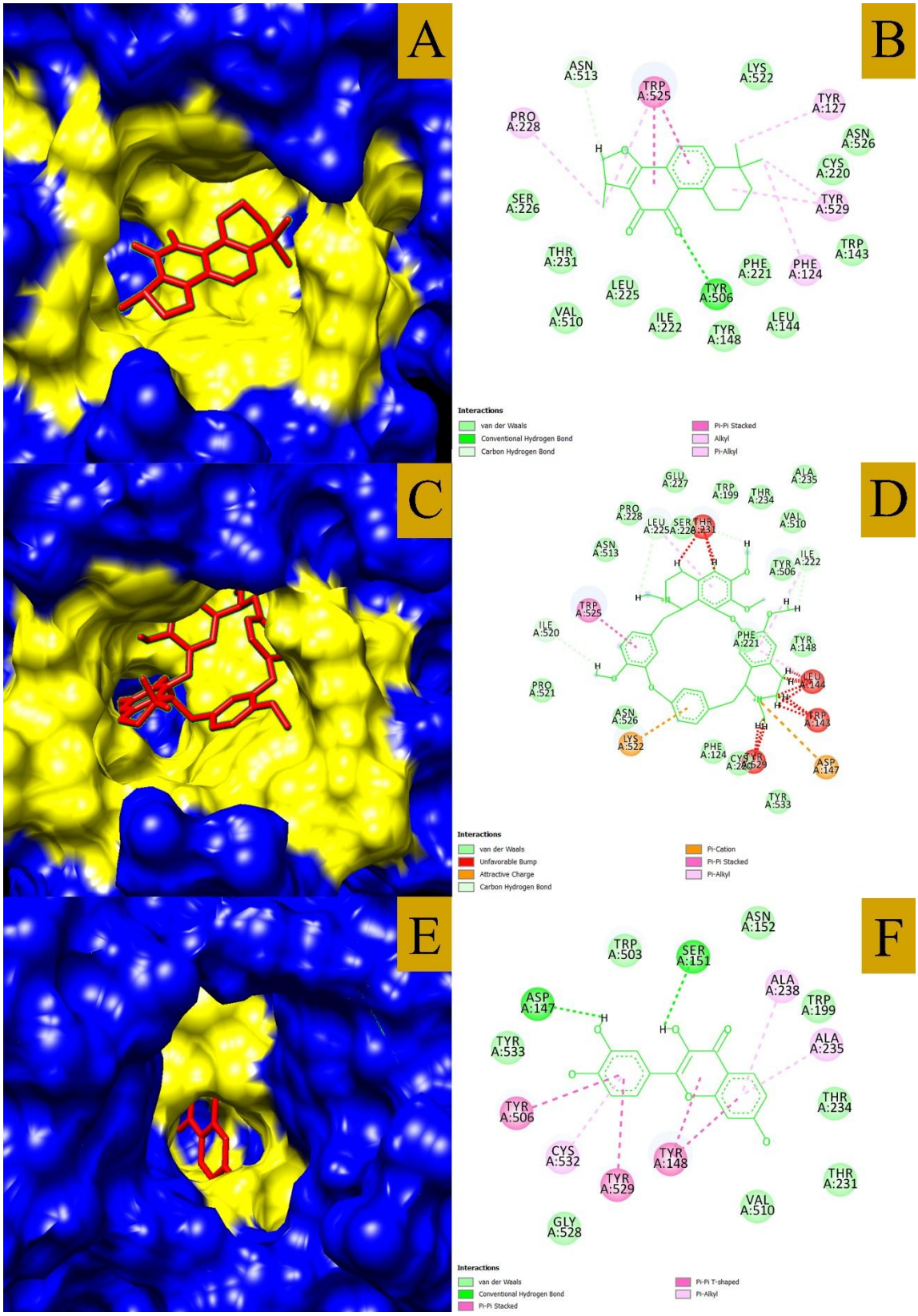


Figure 3. Molecular docking diagram of chemical composition to 4U15 (M3 receptor) and their 2D diagram of ligand binding site atoms of cryptotanshinone (A, B), tetrandrine (C, D), fisetin (E, F)

The docking score obtained is inversely proportional to the affinity of a molecule to a receptor. With the current *in silico* studies, comprehending the mode of action of 100 compounds that exhibit potential efficacy in asthma management was aimed. According to the results, the compounds have different affinities for β2 and M3 receptors. Considering the docking scores of formoterol (-9.297) and tiotropium (-9.312) -the established ligands for the target molecules β2 and M3 receptors, respectively- compounds with lower docking scores are encouraging.

Table 2. Molecular docking results of compounds with docking scores of -9 and above against each protein (β2 and M3 receptor)

Compounds	β2 receptor	M3 receptor	H-bond form with binding β2's residues	H-bond form with binding M3's residues
Baicalin	-9.4	-9.3	His1296, Ile1303, Asn1301, Phe1193	Tyr148, Ile222, Tyr506, Ala235
Berberine	-9.6	-7.7	Asn1301, Lys1305, Asp1192	Ser226, Pro228, Val510
Calycosin	-9.0	-8.8	Tyr1308, Cys1191	Asp147, Tyr529, Cys532
Columbianadin	-9.3	-9.5	Phe1193	Tyr539, Tyr148, Ala235
Cryptotanshinone	-10.2	-9.9	0	Tyr506, Asn513
Diosgenin	-9.3	-3.6	Phe1193, Cys1191	Tyr506
Flll31	-9.4	-8.1	Ser1207	Lys522, Cys220, Tyr127, Leu144, Thr234
Icariin	-9.0	-5.8	Asn1312, Thr1195, His1296, Val1297, Asn1301	Tyr148, Asn513,
Macelignan	-9.7	-9.0	Ser1207, Asn1293, Ser1203, His1093	Asp147
Oxyresveratrol	-9.3	-8.2	Thr1118, Ser1203, Asn1293, Val1114, Ser1204, Thr1110	Thr234, Asp147, Ala235
Paeoniflorin	-9.7	-9.0	His1296, Ile1303	Trp525, Tyr506, Leu225, Thr231
Piscroside A	-9.2	-9.2	Phe1193	Tyr506, Tyr148, Ile222, Asn526, Thr231
Praeruptorin E	-9.0	-8.3	Lys1305, His1296	Lys522,
Robustaflavone-4'-dimethyl ether	-10.5	-2.5	Arg1304, Ile1303, Asn1301, Phe1193	Leu225, Tyr148, Tyr506, Asn152, Asn526
Rosmarinic acid	-9.2	-8.9	Asn1312, Tyr1316, Trp1313, Phe1193, His1093	Trp199, Asn152, Ser151, Thr231, Asn507, Tyr529
Rottlerin	-9.9	-6.9	Asn1301	Asn507, Ile222
Silymarin	-9.2	-9.3	Asp1113, His1093, Asp1192, Lys1305, His1296, Asp1192	Trp525, Tyr506, Leu225, Thr231, Ala235
Solasodine	-9.0	-3.6	Cys1191	Tyr506
Sophoricoside	-9.6	-8.2	Gly1090, Asn1312, Phe1193	Ala238, Ile222, Tyr148, Asn152
Withaferin A	-9.3	-5.7	Asn1312, Asn1301	Ile222, Ser151
Omc	-7.8	-9.4	Phe1193	Ser151, Asn152
Apigenin	-8.7	-9.0	Tyr1316, Gly1090, Phe1193	Tyr529, Thr231
Baicalein	-8.5	-9.2	Tyr1316, Trp1313, Gly1090, Phe1193	Asn507, Thr231
Bruceine D	-8.8	-9.3	Ile1303, Glu1180, Phe1194	Tyr506
Calcaratarin D	-8.7	-9.2	His1296	Asp147
Chrysin	-8.8	-9.3	Phe1193, Trp1313	Asn507, Ala235
Dehydromatricarin	-8.7	-9.1	His1296, Asn1301	Lys522, Ile222, Phe221
Fisetin	-8.2	-9.7	Phe1193, Asp1113, Tyr1316,	Asp147, Ser151
Imperatorin	-8.2	-9.2	Asp1113, Phe1193, Trp1313	Tyr506
Luteolin	-8.5	-9.3	Ser1207, Val1114	Asp147, Ala235
Ovatodiolide	-8.2	-9.5	0	Tyr529, Tyr506
Sophoraflavanone G	-8.5	-9.0	Phe1193, Thr1195, Phe1194	Ser226, Leu225, Tyr529
Tetrandrine	-4.8	-9.8	Asn1301, Asp1192, Asp1300	Ile520, Leu225, Thr231, Ile222
Wogonin	-8.9	-9.2	Tyr1308, Thr1195, Val1114	Asn507, Tyr148, Thr234

The following compounds have superior docking scores according to the reference value of -9.297 on the β2 receptors: robustaflavone-4'-dimethyl ether, -10.5; cryptotanshinone, -10.2; rottlerin, -9.9; paeoniflorin, -9.7; macelignan, -9.7; sophoricoside, -9.6; berberine, -9.6; FLLL31, -9.4; baicalin, -9.4; columbianadin, -9.3; diosgenin, -9.3; oxyresveratrol, -9.3; withaferin A, -9.3; rosmarinic acid, -9.2. Among them, paeoniflorin has been demonstrated to relax the airway via B2 receptors [127-133]. Besides, β2 receptor interaction of rosmarinic acid has been evidenced both *in vitro* and by molecular docking [134]. Consistency with the aforementioned literature data suggests that the other substances that have not been experimentally proven yet are likewise associated with the β2 receptors. On the other hand, experimental studies with berberine conducted in an organ bath indicates that the bronchodilation is not correlated with the β2 receptor [135,136]. This could be as a result of the fact that studies on molecular docking do not always accurately depict the practice. Another promising phytochemical rottlerin has been shown to be associated with the K channel, however, its association with β2 receptor has not been investigated [137]. Due to the fact that a molecule may have multiple mechanisms of action, hence, additional research is required to explore the relationship between the compound and the β2 receptors.

The molecules that yielded docking scores of -9.282 and lower on the M3 receptor are as follows: cryptotanshinone, -9.9; tetrandrine, -9.8; fisetin, -9.7; columbianadin, -9.5; ovatodiolide, -9.5; (2'S,7'S)-O-(2-methylbutanoyl)-columbianetin (OMC), -9.4; baicalin, -9.3; bruceine D, -9.3; chrysin, -9.3; luteolin, 9.3; silymarin, -9.3; paeoniflorin, -9.0. According to the literature, fisetin is involved in the relaxation of acetylcholine-precontracted airway smooth muscle by dual inhibition of PLCβ and PDE4 [138]. PLCβ is indirectly associated with the M3 receptor, suggesting that the efficiency of fisetin may be M3-related. Another active compound, luteolin relaxes the bronchi probably by inhibiting PDE activity and decreasing the amount of Ca^{+2} in the trachealis [139]. Although paeoniflorin has been documented to be active via β2, the effect on the M3 receptor has not been evaluated [133]. A comparison of the affinity of these compounds for β2 and M3 is displayed (Table 2). It should be highlighted that several compounds appear to have an affinity for both proteins, necessitating further research.

In silico approaches have been established as an efficient component of various drug development programs, from lead discovery to optimization. A variety of methodologies, including computational screening based on ligands or targets, are frequently implemented in this type of investigation. Docking results provide information about the binding energy and the interaction between the ligand and macromolecule. However, they do not state whether or not this is an agonist/antagonist interaction [140]. While image analysis allows us partially make predictions in this regard, it is not a quantitative approach. By conducting molecular dynamics, more trustworthy data may be obtained about the type of interaction.

X-ray crystallography was preferred in selecting the target protein since X-ray crystallography is an efficient process that yields high-resolution structural data at the atomic level. What is more, resolution rates were maintained low suggesting the study was trustworthy. The adequate length of the amino acid chain of the protein was also taken into consideration. The existence of a small molecule in the X-ray structure that represents the receptor's ligand, which was utilized as a reference to establish the grid box, was an important factor in the protein selection process. Despite the fact that the X-ray structure of the β2 receptor was derived from human cells, X-ray structure of the M3 receptor could be acquired only from rat cells due to the lack of literature. For the sake of data validity, comparability, and particularly public health, it would be ideal if both protein structures were of human origin.

The evaluation of drug-like properties is essential in drug discovery and development. Key parameters include molecular weight (MW), which should ideally be ≤500 g/mol to satisfy Lipinski's rule of five; hydrogen bond acceptors (HBA) and donors (HBD), with thresholds of ≤10 and ≤5, respectively. MLogP, representing lipophilicity, should be ≤5 for optimal bioavailability. Gastrointestinal (GI) absorption indicates the likelihood of intestinal uptake, while BBB permeability predicts a compound's ability to cross the blood-brain barrier. P-glycoprotein (P-gp) substrate status and CYP450 enzyme inhibition assess potential drug efflux and metabolism-related interactions, respectively.

Table 3. The pharmacokinetic properties of the 34 promising substances (the SwissADME platform)

	MW (g/mol)	H-Bond Acceptor/ Donor	MLogP	GI Absorption	BBB Permeant/ P-gp Substrate	CYP Enzymes inhibitor*	Skin Permeation (Log Kp. cm/s)	Druglikeness Lipinski Filter	Oral Bioavailability Score (F%)
Apigenin	270.24	5/3	1.89	High	No/No	a, d, e	-5.80	Yes	0.55
Baicalein	270.24	5/3	2.43	High	No/No	a, d, e	-5.70	Yes	0.55
Baicalin	446.36	11/6	1.75	Low	No/Yes		-8.23	No	0.11
Berberine	336.36	4/0	0	High	Yes/Yes	a, d, e	-5.78	Yes	0.55
Bruceine D	410.42	9/5	-0.49	Low	No/Yes		-10.52	Yes	0.55
Calcaratarin D	318.45	3/1	3.23	High	Yes/No	b, c	-5.27	Yes	0.55
Calycosin	284.26	5/2	2.40	High	No/No	a, d, e	-6.30	Yes	0.55
Chrysin	254.24	4/2	2.27	High	Yes/No	a, d, e	-5.35	Yes	0.55
Columbianadin	328.36	5/0	3.51	High	Yes/No	a, b, c, e	-5.68	Yes	0.55
Cryptotanshinone	296.36	3/0	2.81	High	Yes/Yes	a, b, c, e	-5.41	Yes	0.85
Dehydromatricarin	304.34	5/0	2.15	High	Yes/Yes		7.06	Yes	0.55
Diosgenin	414.62	3/1	4.49	High	Yes/No		-4.80	Yes	0.55
Fisetin	286.24	6/4	1.50	High	No/No	a, d, e	-6.65	Yes	0.55
Flll31	424.49	6/0	4.05	High	Yes/No	c, d, e	-5.50	Yes	0.55
Icariin	676.66	15/8	3.78	Low	No/Yes		-9.25	No	0.17
Imperatorin	270.28	4/0	3.05	High	Yes/No	a, b, c	-5.46	Yes	0.55
Luteolin	286.24	6/4	1.86	High	No/No	a, d, e	-6.25	Yes	0.55
Macelignan	328.40	4/1	3.82	High	Yes/No	b, c, d	-4.64	Yes	0.55
Omc	161.20	1/1	1.65	High	Yes/No		-6.09	Yes	0.55
Ovatodiolide	328.40	4/0	2.71	High	Yes/No	b, c	-6.61	Yes	0.55
Oxyresveratrol	244.24	4/4	1.44	High	No/No	a, c, e	-5.82	Yes	0.55
Paeoniflorin	480.46	11/5	2.49	Low	No/Yes		-9.96	Yes	0.55
Piscroside A	548.92	13/6	1.97	Low	No/No		-9.79	No	0.17
Praeruptorin E	428.47	7/0	3.88	High	No/No	b, c, e	-5.78	Yes	0.55
Robustaflavone-4'-dimethyl ether	566.51	10/4	4.11	Low	No/No	c	-5.72	Yes	0.55
Rosmarinic acid	360.31	8/5	1.48	Low	No/No		-6.82	Yes	0.56
Rottlerin	516.54	8/5	3.57	Low	No/No	b	-5.25	Yes	0.55
Silymarin	482.44	10/5	2.79	Low	No/No	e	-7.89	Yes	0.55
Solasodine	413.64	3/2	4.26	High	Yes/Yes		-5.00	Yes	0.55
Sophoraflavanone G	424.49	6/4	3.14	High	No/No	c, e	-4.79	Yes	0.55
Sophoricoside	432.38	10/6	2.21	Low	No/No		-8.33	Yes	0.55
Tetrandrine	622.75	8/0	5.23	High	No/No		-5.37	Yes	0.55
Withaferin A	470.60	6/2	3.24	High	No/Yes		-6.45	Yes	0.55
Wogonin	284.26	5/2	2.55	High	No/No	a, c, d, e	-5.56	Yes	0.55

*CYP enzymes correspond to a-CYP1A2, b-CYP2C19, c-CYP2C9, d-CYP2D6, e-CYP3A4.

Table 4. The toxicological properties of the 34 promising substances (the ADMETLab 3.0 platform)

	hERG Blockers	Drug-Induced Liver Injury (DILI)	AMES Toxicity	Rat Oral Acute Toxicity	Skin Sensitization	Carcino-genicity	Respiratory	Human Hepato-toxicity	Drug-induced Nephro-toxicity	Drug-induced Neuro-toxicity	Oto-toxicity	Hemato-toxicity
Apigenin	(+)	(++)	(+)	(+)	(++)	(++)	(+)	(---)	(---)	(---)	(---)	(---)
Baicalein	(--)	(+++)	(++)	(+)	(+++)	(++)	(++)	(+)	(---)	(---)	(+)	(---)
Baicalin	(---)	(+++)	(+)	(---)	(+++)	(---)	(---)	(+)	(++)	(---)	(+)	(--)
Berberine	(-)	(---)	(+)	(+++)	(+++)	(++)	(+++)	(---)	(---)	(---)	(---)	(---)
Bruceine D	(---)	(+)	(++)	(+++)	(+++)	(---)	(+++)	(+)	(+++)	(---)	(+++)	(+)
Calcaratarin D	(--)	(-)	(+)	(+++)	(+++)	(+++)	(---)	(+++)	(+++)	(+)	(++)	(+)
Calycosin	(--)	(+)	(+)	(+)	(+)	(++)	(++)	(+)	(--)	(+)	(+)	(---)
Chrysin	(---)	(+++)	(+)	(+)	(+)	(++)	(++)	(+)	(---)	(---)	(---)	(---)
Columbianadin	(--)	(+++)	(+++)	(++)	(+++)	(+++)	(++)	(++)	(+)	(+)	(--)	(--)
Cryptotanshinone	(---)	(+++)	(+++)	(+)	(+++)	(+++)	(+)	(+++)	(+++)	(+++)	(+++)	(+++)
Dehydromatricarin	(---)	(+++)	(+++)	(-)	(+++)	(+++)	(-)	(+)	(+)	(-)	(-)	(+++)
Diosgenin	(--)	(++)	(---)	(---)	(+++)	(+++)	(+)	(++)	(+)	(---)	(+++)	(---)
Fisetin	(---)	(++)	(+)	(+)	(++)	(++)	(++)	(+)	(---)	(---)	(+)	(---)
Flll31	(-)	(+)	(+)	(---)	(++)	(++)	(+)	(++)	(+)	(+++)	(+)	(+)
Icariin	(---)	(+++)	(+++)	(---)	(+++)	(---)	(---)	(++)	(+++)	(---)	(+++)	(---)
Imperatorin	(--)	(++)	(+)	(+)	(---)	(++)	(+)	(+)	(+)	(+)	(++)	(+)
Luteolin	(---)	(+++)	(+)	(+)	(+++)	(++)	(++)	(---)	(---)	(---)	(+)	(---)
Macelignan	(-)	(+)	(+)	(---)	(+)	(++)	(++)	(++)	(++)	(++)	(+)	(++)
Omc	(--)	(+)	(++)	(---)	(++)	(++)	(++)	(++)	(---)	(++)	(---)	(---)
Ovatodiolide	(---)	(+++)	(+)	(+)	(+++)	(++)	(---)	(+++)	(+++)	(+++)	(+)	(+)
Oxyresveratrol	(--)	(---)	(+)	(+)	(+++)	(+)	(++)	(---)	(---)	(+)	(---)	(---)
Paeoniflorin	(---)	(--)	(+++)	(---)	(+++)	(---)	(---)	(++)	(+++)	(---)	(+++)	(+)
Piscroside A	(---)	(+++)	(+++)	(---)	(+++)	(---)	(---)	(++)	(++)	(---)	(+++)	(+)
Praeruptorin E	(---)	(+++)	(+)	(+)	(++)	(++)	(+)	(++)	(++)	(---)	(+)	(+)
Robustaflavone-4'-dimethyl ether	(--)	(+++)	(+++)	(+++)	(---)	(++)	(+++)	(++)	(---)	(---)	(---)	(---)
Rosmarinic acid	(---)	(+++)	(+)	(---)	(+++)	(---)	(---)	(+++)	(+)	(---)	(+++)	(---)
Rottlerin	(---)	(---)	(+)	(---)	(+++)	(---)	(+++)	(+++)	(+)	(---)	(--)	(---)
Silymarin	(---)	(---)	(+++)	(++)	(+)	(++)	(++)	(++)	(+)	(+)	(+++)	(---)
Solasodine	(--)	(---)	(---)	(---)	(+++)	(+++)	(+)	(++)	(+)	(---)	(+++)	(---)
Sophoraflavanone G	(---)	(+)	(+++)	(+++)	(+++)	(---)	(+++)	(++)	(++)	(+)	(++)	(---)
Sophoricoside	(---)	(+++)	(+++)	(---)	(+++)	(+)	(---)	(+++)	(+++)	(---)	(+++)	(---)
Tetrandrine	(+++)	(---)	(++)	(++)	(++)	(++)	(++)	(++)	(+)	(+++)	(+)	(---)
Withaferin A	(---)	(---)	(+)	(---)	(+++)	(+++)	(++)	(---)	(+++)	(---)	(++)	(++)
Wogonin	(---)	(+++)	(+)	(+)	(+)	(++)	(+++)	(++)	(---)	(---)	(---)	(+)

Skin permeation (Log Kp) measures transdermal absorption potential, and oral bioavailability score (F%) estimates the compound's suitability for oral administration. These parameters collectively aid in determining a compound's pharmacokinetic profile and drug-likeness. Among the substances with high docking scores for the β2-adrenergic receptor (as shown in Table 2, and detailed pharmacokinetic properties of the 34 key compounds are summarized in Table 3), cryptotanshinone and macelignan meet the fundamental pharmacokinetic criteria. However, the other substances, including paeoniflorin, rottlerin, and robustaflavone-4'-dimethyl ether, can be unsuitable as drug candidates due to their high molecular weights, which limit their drug-like potential. Similarly, for the M3 muscarinic receptor, compounds such as cryptotanshinone, fisetin, tetrandrine, ovatodiolide, and columbianadin demonstrate

promising docking scores. Upon further evaluation, all compounds except for tetrandrine may be considered as viable drug candidates, however, tetrandrine's high molecular weight renders it ineligible according to conventional drug-likeness standards.

The toxicity profiles of several molecules, as presented in Table 4, reveal diverse effects across different categories. Tetrandrine, for example, has been associated with hERG blockade, which can lead to arrhythmias, a potentially harmful effect in asthma patients. Similarly, substances such as robustaflavone-4'-dimethyl ether and rottlerin, which exhibit respiratory toxicity, may significantly impact individuals with asthma. While each drug can present distinct toxic effects, the selection of a therapeutic agent must be carefully evaluated in the context of the patient's specific condition. Considering the cardiovascular and pulmonary risks associated with asthma, drug candidates like cryptotanshinone, ovatodiolide, and paeoniflorin - which exhibit minimal or no detrimental effects on these systems - may offer valuable therapeutic options. Nonetheless, it is crucial to assess the patient's other medical conditions, as these factors may influence the selection of the therapeutic molecule. Ultimately, these findings underscore the variability in toxicity profiles and highlight the need for further research to ensure the safety and therapeutic viability of these molecules.

Asthma is a medical condition characterized by bronchoconstriction accompanied by an immunological base. In the treatment, therapeutic agents with anti-inflammatory or bronchodilator properties are preferred. Even while the primary focus of our research was the bronchodilator mechanism, it is noteworthy that nearly all compounds examined in silico have been demonstrated to have anti-inflammatory characteristics in previous experiments. The ability to show both a bronchodilator and the anti-inflammatory effect of a compound has significant potential regarding the enhancement of asthma therapy.

This study is the first to investigate *in silico* the effects of active compounds from medicinal plants on bronchodilator processes, which has been demonstrated experimentally to be potentially beneficial in the treatment of asthma. β2 and M3 receptors have already been the subject of attention in the treatment of asthma owing to their alleviating properties on airway constriction. Our results suggest that evaluated plant compounds might act as bronchodilators via activating β2 receptors and inhibiting M3 receptors. The compounds apparently show promise for the future of asthma treatment. Additional research is required to confirm the bronchodilator activity and therapeutic efficacy in the management of asthma. Accordingly, the findings might provide a basis for further research on the mechanisms of action of relevant compounds.

AUTHOR CONTRIBUTIONS

Concept: F.U., S.O.A.; Design: M.Z.Ç., S.O.A.; Control: F.U., S.O.A.; Sources: M.Z.Ç., A.C.A.; Materials: - ; Data Collection and/or Processing: M.Z.Ç., A.C.A.; Analysis and/or Interpretation: M.Z.Ç., N.H.T., S.O.A.; Literature Review: M.Z.Ç., F.U.; Manuscript Writing: M.Z.Ç., N.H.T.; Critical Review: M.Z.Ç, F.U., N.H.T., A.C.A., S.O.A.; Other: -

CONFLICT OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

The authors declare that the ethics committee approval is not required for this study.

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J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 49(3): 714-728, 2025
Doi: 10.33483/jfpau.1553867

ORIGINAL ARTICLE / ÖZGÜN MAKALE

IN SILICO AND *IN VITRO* ANTIDIABETIC ACTIVITY STUDIES ON THE ETHANOL EXTRACT OF *DESMANTHUS VIRGATUS* (L.) LEAVES

DESMANTHUS VİRGATUS (L.) YAPRAKLARININ ETANOL EKSTRESİ ÜZERİNE İN SİLİKO VE İN VİTRO ANTİDİYABETİK AKTİVİTE ÇALIŞMALARI

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ABSTRACT

Objective: *In vitro and in silico studies using α-glucosidase activity inhibition aimed at identifying the potential antidiabetic properties of ethanol extracts of D. virgatus leaves.*
Material and Method: *Compounds in the ethanol extract of D. virgatus leaves were identified by LC-MS. In vitro and in silico methods were used to determine the antidiabetic activity.*
Result and Discussion: *The protein codes (PDB ID) used were 3AJ7 and 3W37, then acarbose was used as a positive control. By using LC-MS, 160 compounds were identified from the extract. All of the identified compounds were tested in silico. There are 18 compounds (with 3AJ7 protein) and 10 compounds (with 3W37 protein) that fulfill Lipinski's rule with binding affinity values lower than acarbose, namely ≤ -8.2 kcal/mol for 3AJ7 protein and ≤ -8.1 kcal/mol for 3W37. In addition, α-glucosidase inhibition was used to assess the antidiabetic effect of the ethanol extract of D. virgatus leaves in vitro, resulting in IC_{50} values of 144.11 ppm for the extract and 3.78 ppm for acarbose. The IC_{50} values of the extract were included in the medium category.*
Keywords: *Antidiabetic, Desmanthus virgatus, ethanol extract, in silico, in vitro*

ÖZ

Amaç: *Bu çalışma, in siliko ve in vitro (α-glukozidaz inhibisyonu) yöntemlerle D. virgatus bitkisinin yapraklardan elde edilen etanol ekstresinin antidiyabetik etki potansiyelini belirlemeyi amaçlamaktadır.*
Gereç ve Yöntem: *LC-MS kullanılarak D. virgatus yapraklarının etanol ekstresindeki bileşikler tanımlanmıştır. Antidiyabetik aktivite in silico ve in vitro olarak belirlenmiştir.*
Sonuç ve Tartışma: *Kullanılan protein kodları (PDB ID) 3AJ7 ve 3W37 olup, akarbose pozitif kontrol olarak kullanılmıştır. LC-MS kullanılarak ekstrakttan 160 bileşik tanımlanmıştır. Tanımlanan bileşiklerin tümü silikoda test edilmiş. (3AJ7 proteini ile) 18 bileşik ve (3W37 proteini ile) 10 bileşik Lipinski kuralını karşılamakta ve bağlanma afinitesi değerleri akarbose'dan daha düşük olmak üzere, 3AJ7 için ≤ -8.2 kcal/mol ve 3W37 için ≤ -8.1 kcal/mol olarak bulunmuştur. Ayrıca, ekstre ve akarbozun α-glukozidaz inhibitörü etkisi in vitro olarak test edilmiştir, elde edilen IC_{50} değeri sırasıyla 144.11 ppm'dir ve 3.78 ppm'dir. Ekstrenin etkisi orta kategoriye sokulabilir.*

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Submitted / Gönderilme : 20.10.2024
Accepted / Kabul : 02.04.2025
Published / Yayınlanma : 19.09.2025

Anahtar Kelimeler: *Antidiyabetik, Desmanthus virgatus, etanol ekstresi, in siliko, in vitro*

INTRODUCTION

Chronic metabolic disease known as diabetes mellitus is indicated by increased blood sugar levels [1,2]. These chronic disorders have a negative effect on the metabolic system and are involved in the pathophysiology of diabetic complications [3]. The defining characteristic of diabetes mellitus (DM) is increased blood glucose levels brought on by either resistance to insulin or impaired insulin synthesis [4]. Various micro- and macro-vascular problems due to uncontrolled hyperglycemia lead to cardiovascular disease, retinopathy [5], neuropathy, nephropathy, and ulceration with high morbidity and mortality worldwide [6,7]. In the 10th edition Atlas at the end of 2021, the International Diabetes Federation (IDF), reported that one of the 21st century's most rapidly expanding international health emergencies is diabetes [8]. 537 million people all over the world had diabetes in 2021. By 2030 and 2045, there will be 643 million and 783 million more people in this number, respectively [9].

Type 1 diabetes mellitus (T1DM) and type 2 diabetes (T2DM) are the two kinds of diabetes [10]. T1DM is a metabolic disease brought on by apoptosis and an autoimmune or idiopathic mechanism that destroys pancreatic β-cells [11,12]. This causes the pancreas to not produce enough insulin or none at all so the patient has to rely on insulin from the outside [13]. In people with T2DM, the pancreas can still produce insulin, but the insulin produced is unable to incorporate glucose into the cells, causing blood glucose to rise [14]. T2DM can also be caused by insulin resistance [15] as well as pancreatic beta cell failure that contributes to impaired glucose tolerance [14].

Treatment of diabetes can use a therapeutic approach by inhibiting an enzyme responsible for glucose uptake such as α-glucosidase [16]. This strategy, which is present in pancreatic and salivary gland secretions, is crucial to the therapeutic process [17]. The α-D-(1,4)-glycosidic linkages in starch are broken down by this enzyme, which is an endoamylase family member, to produce monosaccharides. Inhibiting the enzyme is a useful method for reducing blood sugar [18].

Over the past few years, there have been significant developments in diabetes prevention. However, there is no curative drug that can reduce the risk of diabetes. Patients with diabetes have been treated with a variety of synthetic pharmaceuticals, including acarbose, biguanides, thiazolidinediones, and sulfonylureas [19]. Diabetes control is still a major issue despite significant advancements in the field of drug discovery and the availability of numerous synthetic medications of different kinds that efficiently combat hyperglycaemia. This is because synthetic substances have evident negative effects [6]. Continuous consumption of synthetic anti-diabetic drugs can cause side effects on the body and psychology of patients [20] such as gastrointestinal disturbances of nausea and diarrhea [21], liver disease [22], followed by an increased risk of hypoglycemia, and more serious side effects such as liver damage or cardiovascular problems [23]. The presence of these side effects necessitates the discovery of new drug entities that are relatively safer and cheaper derived from natural sources compared to synthetic drugs. One of the primary sources for finding new drugs that are helpful for treating a variety of disorders is traditional medicine. In addition, a variety of secondary metabolites found in plant-based traditional remedies act on a variety of targets and may provide additional benefits for the treatment of polygenic disorders like diabetes or obesity. The anti-hyperglycaemic properties of plant extracts and herbal medicine that may be employed as anti-diabetics have been supported by numerous research. Herbal remedies are frequently thought to have fewer adverse effects than manufactured medications [2]. Other than that, when it comes to human intake, traditional medications are comparatively safer than modern oral hypoglycaemic drugs [24].

One of the plants that has anti-diabetic potential is *Desmanthus virgatus* (L.). The absence of publications related to information on phytochemical contents and the use of *D. virgatus* leaves as anti-diabetics make this research important. Thus, this study aimed to conduct phytochemical analysis including phytochemical identification through LC-MS instruments, and determining the anti-diabetic potential of the leaves using *in vitro* and *in silico* methods.

MATERIAL AND METHOD

Materials

The study's materials included were *D. virgatus* (L.) leaves, ethanol (98%, Merck, Germany), structure protein receptor of α-glucosidase (PDB ID: 3AJ7 and 3W37) (retrieved from https://www.rcsb.org/), ligand conformers (retrieved from https://pubchem.ncbi.nlm.nih.gov/), α-glucosidase enzyme (Sigma Aldrich, USA), acarbose, phosphate buffer, 4-nitrophenyl β-D-glucopyranoside solution, and sodium carbonate (Sigma Aldrich, USA).

The leaves of the plant were collected from Sidoarjo, East Java, Indonesia. The plant materials were determined at Yayasan Generasi Biologi Indonesia, Cerme, Gresik Indonesia with specimen number BT 02/0352/24, March 22, 2024. The specimen was deposited in the Yayasan. In this study, the leaves were used because the leaves are often the part that is rich in secondary metabolites. As research conducted by Sembiring et al. (2018) on various parts of the *Caesalpinia bonduc* (L.) Roxb plant, which is still in the same family as *D. virgatus*, namely Fabaceae, reported that the results of phytochemical tests on the leaves of the plant contained secondary metabolites of flavonoids, saponins, steroids, and tannins. Then, the results of the total phenolic and flavonoid content tests in the leaves were reported at 31.05 ± 0.35 (mgQE/gram) and 146.64 ± 3.94 (mgGAE/gram) [25].

Instruments

The equipment used in this study were beaker glass, extraction chamber, spatula, Whatman filter paper, Buchner funnel, vacuum pump, Shimadzu LC-MS instrument (8040 Type, Shimadzu, Japan), micropipette, 96 well microplate, vacuum rotary evaporator (R-215, Buchi), microplate reader (Multiskan, Thermo Scientific), incubator (Memmert BE500), and Eppendorf tubes. The docking study was performed using Windows 10, Intel(R), 4,00 GB RAM, and a 64-bit operating system. The software used involved Discovery Studio Client 2019, PyRx-0.8, and pyMOL 3.0.

Plant Material Collection and Extraction

D. virgatus leaf samples were collected and air-dried for 3 days in the sun. After drying, the leaves were mashed with a grinder until they became powder, and weighed (3.8 kg). Then, the powdered sample was divided into 2 parts and each part (±1.9 kg) was macerated using 3.4 liters of ethanol by stirring several times, and the process was repeated 4 times. The rest is done in the same way. After that, filtering was carried out with a Buchner funnel assisted by a vacuum pump, and ethanol filtrate was obtained. The ethanol filtrate was evaporated using a vacuum rotary evaporator to obtain a thick greenish-brown extract (478 grams). A small amount of each extract (±0.5 mg) was used for identification of phytochemical content in LC-MS analysis. Ethanol solvent is used because ethanol is a solvent that is safe for human consumption as a solvent for natural ingredients for both food and natural medicines [26]. In addition, ethanol is a semi-polar compound that can dissolve polar and non-polar compounds effectively. This can allow it to extract various secondary metabolites, including phenolics, flavonoids, and alkaloids that are important for antidiabetic activity [27].

Determination of Ethanol Extract's Secondary Metabolites Using LC-MS

Using an LC-MS, secondary metabolites of the ethanol extract of *D. virgatus* leaves were identified. Shimadzu 8040 Type was equipped with a Shimadzu Pack FC-ODS capillary column (2 mm × 150 mm id, 3 µm particle size). The injection volume was 1 µl. The apparatus is equipped with an Electrospray Ionization (ESI) source that has the following specifications: flow rate of 0.5 ml/min; ethanol solvent; focused ion mode MS type [M]+; ionization utilizing ESI; isocratic mobile phase; The eluents used were 0.1% formic acid in acetonitrile and 0.1% distilled water with an isocratic elution system. The isocratic elution system was performed at 0-1 min 95: 5 ratio, 0 min linear gradient elution A from 95% to 5%, 6-7 min isocratic elution 0:100 ratio, 6-7 min linear gradient elution solvent A from 0% to 100%, min 7.5-9 isocratic elution 95: 5 ratio, up to 7.5-9 min elution gradient linear A solvent from 95% to 5%; capillary voltage of 3.0 kv; and run time of 80 minutes. Using the NIST database library and the compounds' retention time and molecular mass spectra from the chromatograms, secondary metabolites present in the extracts were identified.

In Silico Study with Protein 3AJ7 and 3W37

Data generated from the LC-MS analysis were identified using the PubChem web database to determine the compound's name and chemical structure. Then, each of these compounds was tested for Lipinski Druglikeness and pharmacokinetics to determine its potential as a bioactive compound with Lipinski's five rules through the website http://www.scfbio-iitd.res.in/software/drugdesign/lipinski.jsp. Biocomputational molecular docking analysis was used to assess the antidiabetic activity of compounds that fulfilled the Druglikeness and pharmacokinetic tests. The receptor used *in silico* study was α-glucosidase with PPDB code 3AJ7 and 3W37. Preparation was done using PyMOL to obtain sterile protein. Furthermore, PyRx was used to carry out a molecular docking test and determine the binding affinity value. After molecular docking, the compound was interacted and visualized using PyMOL and Discovery Studio to determine the position and type of interaction formed.

In Vitro Study with α-Glucosidase Inhibition Assay

With minor modifications, the *in vitro* α-glucosidase inhibition of the ethanol extract was determined using Etsassala et al.'s (2020) method [28]. 50 µl 0.1 M phosphate buffer (100 mM, pH = 6.8), 10 µl α-glucosidase (1 U/ml), and 10 µl test samples (7.81; 15.63; 31.25; 62.50; 125.00; 250.00; and 500.00 ppm) were mixed, respectively and put into a 96-well plate. The entire mixture was incubated at 37 °C for 15 minutes. Afterward the pre-incubation phase, each well was filled with 20 µl of a 5 mM 4-nitrophenyl β-D-glucopyranoside solution, and the enzymatic reaction was activated. The plate was then incubated for 20 minutes at 37ºC. The reaction was halted after 20 minutes by adding 50 µl of 0.1 M sodium carbonate. Then, the absorbance was measured at a wavelength of 405 nm using a microplate reader. The positive control used in this assay is acarbose with various concentrations of 0.7813; 1.5625; 3.1250; 6.2500; 12.5000; 25.0000; and 50.0000 ppm. The formula was used to determine the level of α-glucosidase inhibition.

$$\text{The percentage of inhibitory activity (\%)} = (1 - A/B) \times 100$$

With A represents the sample absorbance and B represents the control absorbance.

Statistical Analysis

The results were reported as the mean IC_{50} value. Statistical analysis was performed using IBM SPSS Statistics 25. The IC_{50} differences were examined using parametric statistical methods with the One Way Anova test followed by the Games-Howell test. Results were considered significantly different when $p < 0.05$.

RESULT AND DISCUSSION

Determination of Ethanol Extract's Secondary Metabolites Using LC-MS

160 compounds were identified by LC-MS as the secondary metabolites of *D. virgatus* ethanol extract. The extract's LC-MS chromatogram is displayed in Figure 1.

Ethanol is semi-polar, which can attract compounds that are polar and non-polar [29]. The dominant compounds of ethanol extract are flavonoids including 7,4'-dihydroxyflavone, 5,7-dihydroxyisoflavone, demethyltorosaflavone C, calycosin, kaempferol, and naringenin. As known, flavonoids provide hypoglycemic effects in diabetics [30] by enhancing antioxidant status, lipid profile, and controlling glucose levels [31]. In addition, flavonoids have anti-cancer, anti-inflammatory, anti-oxidant, anti-bacterial, anti-thrombotic, anti-fungal, and anti-viral effects [32]. Furthermore, there are also phenylpropanoids, phenolic acids, glycosides, and their derivatives, besides aldehydes, ketones, terpenoids, alkaloids, and tannins in small amounts. Alkaloids are thought to be the primary inhibitors of the enzymes α-glucosidase and α-amylase, which are the best methods for reducing blood glucose levels [33]. On the other hand, tannins can lower hyperglycaemia after meals and stop or postpone the absorption of glucose by blocking α-glucosidase activity [34].

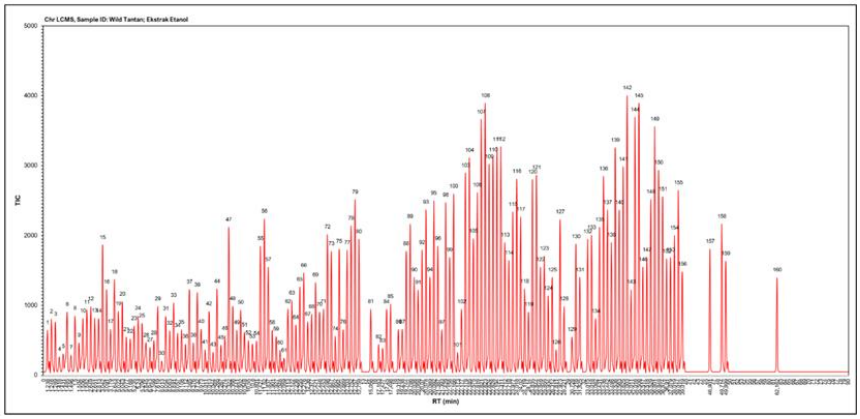


Figure 1. LC-MS chromatogram of the ethanol extract of *D. virgatus* leaves

In Silico Study with Protein 3AJ7 and 3W37

Of the 160 compounds from LC-MS, an evaluation of 5 Lipinski's rules was carried out and 65 compounds that fulfilled Lipinski's rules were obtained. Then, the 65 compounds that fulfill Lipinski's rules will be continued for *in silico* testing. According to the results of the *in silico* study, 18 compounds were determined that fulfill Lipinski's rule with binding affinity values lower than acarbose (as positive control) and have molecular interactions similar to the control. These compounds are 5-hydroxy-7-[2-(4-hydroxyphenyl)ethenyl]-2,2-dimethyl-2H-1 benzopyran, 7,4'-dihydroxyflavone, apigenin, 4-(3-methyl-but-1-enyl)-3,4',5-trihydroxystilbene, genistein, biochanin A, fustin, kaempferol, 2-(2,3-dihydroxy-3 methylbutyl)-5-(2-phenylethenyl)-1,3-benzenediol, artocarpanone, (2*S*)-5,7-dimethoxy-3',4'-methylenedioxyflavanone, glyceollin II, tricin, quercetin 3,5,3'-trimethylether, caesalpinin K, neocaesalpin H, caesalpinin C, and bonducellpin C.

First, the physicochemical tests were carried out in *in silico* study. Based on Lipinski's rule of five, the following physicochemical parameters are determined: logP values less than 5, molar refractivity (MR) values between 40 and 130 cm^3/mol, molecular weights under 500 dalton, hydrogen bonds no less than 5, and acceptor bond donors no less than 10 [35]. LogP value less than 5 represents hydrophobicity [36] and can be used to quantify the degree of lipophilicity of substances ingested by the human body [37,38]. Due to the molar refractivity value varying between 40 to 130 cm^3/mol, the molecule will engage with receptors with ease and have good steric characteristics [39]. Then, the molecular weight of coumarin and its derivatives have met the rules where the molecular weights are less than 500 daltons. The results of the Lipinski test of 18 potential compounds are presented in Table 1.

After the physicochemical test is done, the docking simulation was carried out. Before doing that, it is necessary to prepare the receptor. In general, protein structures in the Protein Data Bank (PDB) contain solvent molecules in the form of water and other residues (Figure 2). Therefore, it is necessary to remove water molecules so as not to interfere when docking simulations are carried out and to ensure that what really interacts is the ligand and receptor. In addition, it is also necessary to add hydrogen. In order to get the docking condition closer to pH 7, hydrogen atoms are also added [40].



Figure 2. Receptor α-glucosidase (PDB code: 3AJ7)

Table 1. The results of the physicochemical test of 18 potential compounds

No	Compound name	Molecular mass (<500) Dalton	LogP (<5)	Hydrogen bond donor (<5)	Hydrogen bond acceptors (<10)	Molar refractivity (40-130) cm^3/mol
1.	7,4'-dihydroxyflavone	254	2.7140	2	4	69.1491
2.	5-hydroxy-7-[2-(4- hydroxyphenyl)ethenyl]-2,2-dimethyl-2H-1-benzopyran	294	4.4524	2	3	88.8826
3.	4-(3-methyl-but-1-enyl)-3,4',5-trihydroxystilbene	296	4.6230	3	3	90.6784
4.	Apigenin	270	2.4196	3	5	70.8139
5.	Genistein	270	2.4196	3	5	70.8139
6.	Biochanin A	284	2.7226	2	5	75.7011
7.	Fustin	288	1.4807	4	6	71.5847
8.	Kaempferol	286	2.3053	4	6	72.3857
9.	Artocarpanone	302	2.5185	3	6	76.7469
10.	(2*S*)-5,7-dimethoxy-3',4'-methylenedioxyflavanone	328	3.1390	0	6	84.4273
11.	2-(2,3-dihydroxy-3- methylbutyl)-5-(2-phenylethenyl)-1,3- benzenediol	314	2.9425	4	4	91.1502
12.	Glyceollin II	338	3.2901	2	5	91.0506
13.	Tricin	330	2.4368	3	7	83.9179
14.	Quercetin 3,5,3'-trimethyl ether	344	2.7053	2	7	88.2051
15.	Caesalpinin K	376	3.4253	2	5	100.0566
16.	Neocaesalpin H	350	3.2014	2	5	90.3346
17.	Caesalpinin C	416	3.6731	1	6	109.2898
18.	Bonducellpin C	420	2.3457	2	7	105.4656

Validation of the docking method is done by re-docking the natural ligand acarbose (code: 3AJ7) using the prepared α-glucosidase receptor. Root Mean Square Deviation (RMSD) statistics will appear during docking validation. The RMSD expresses the change of protein-ligand interaction in the crystal structure before and after the docking procedure to calculate the deviation values [41]. If the RMSD value is less than 2Å, the docking approach is considered legitimate and can be applied to compound docking tests. This indicates that the docking parameters utilized were appropriate [40].

Validation of α-glucosidase with acarbose receptor resulted in a binding affinity value of -8.2 kcal/mol with an RMSD value of 0.0 Å. This indicates that the docking method used is valid and the parameter settings used fulfill the validation criteria, so that the parameters can be used further for docking test of compounds. From this, validation will be used for docking simulation on potential compounds. To identify the areas of the ligand and receptor proteins that interact, docking analysis was used. Based on the principle of binding energy, this interaction becomes easier to bind the smaller the energy value [42]. Table 2 displays the binding affinity values for 18 candidate compounds.

In addition to binding energy, the interactions that occur between the ligand and the receptor also need to be considered to guarantee that the ligand is bound to the active site of the receptor surface. Discovery Studio 2019 was used to create the visualization needed to see this interaction. Table 3 displays the outcomes of the molecular interactions.

Based on the binding affinity values in Table 2, the test compound with the lowest binding affinity value is Glyceollin II with a value of -10.1. While the highest binding affinity value is Apigenin at -8.2. While other compounds are included in the range. Judging from the data above, 18 test compounds are considered potential in inhibiting α-glucosidase. This is reinforced by previous research, where in the research of Ali et al. (2024) kaempferol and quercetin act as multi-target antidiabetic agents, leading to glucose homeostasis; both compounds show their ability to inhibit and regulate several important targets in the development of diabetes [43].

Table 2. The results of the physicochemical test of 18 potential compounds from *in silico* test with 3AJ7 protein

No	Compound name	Smile	CAS Number	Binding affinity (kcal/mol)
1.	7,4'-dihydroxyflavone	C1=CC(=CC=C1C2=CC(=O)C3 =C(O2)C=C(C=C3)O)O	2196-14-7	-8.7
2.	5-hydroxy-7-[2-(4-hydroxyphenyl)ethenyl]-2,2-dimethyl-2H-1-benzopyran	CC3(C)/C=C\c2c(O)cc(/C=C/c 1ccc(O)cc1)cc2O3	1114548-08-1	-8.6
3.	4-(3-methyl-but-1-enyl)-3,4',5- trihydroxystilbene	CC(C)/C=C/c2c(O)cc(/C=C/c1ccc(O)cc1) cc2O	87320-15-8	-8.9
4.	Apigenin	C1=CC(=CC=C1C2=CC(=O)C3=C (C=C(C=C3O2)O)O)O	8002-66-2	-8.2
5.	Genistein	C1=CC(=CC=C1C2=COC3=CC(=C C(=C3C2=O)O)O)O	446-72-0	-8.4
6.	Biochanin A	COC1=CC=C(C=C1)C2=COC3=C C(=CC(=C3C2=O)O)O	491-80-5	-8.3
7.	Fustin	C1=CC(=C(C=C1[C@@H]2[C@H](C(=O)C 3=C(O2)C=C(C=C3)O)O)O)O	4382-36-9	-8.8
8.	Kaempferol	C1=CC(=CC=C1C2=C(C(=O)C3=C(C=C (C=C3O2)O)O)O)O	520-18-3	-8.3
9.	Artocarpanone	COC1=CC(=C2C(=O)C[C@H](OC2=C1)C 3=C(C=C(C=C3)O)O)O	520-25-2	-8.5
10.	(2*S*)-5,7-dimethoxy-3',4'-methylenedioxyflavanone	COC1=CC2=C(C(=O)C[C@H](O2)C3= CC4=C(C=C3)OCO4)C(=C1)OC	133464-45-6	-9.1
11.	2-(2,3-dihydroxy-3-methylbutyl)-5-(2-phenylethenyl)-1,3-benzenediol	[H][C@@](O)(Cc2c(O)cc(/C=C/c1ccccc1)cc2O)C(C)(C)O	1114548-07-0	-8.5
12.	Glyceollin II	CC1(C=CC2=CC3=C(C=C2O1)OC[C @@]4([C@H]3OC5=C4C=CC(=C5)O)O)C	67314-98-1	-10.1
13.	Tricin	COC1=CC(=CC(=C1O)OC)C2=CC (=O)C3=C(C=C(C=C3O2)O)O	520-32-1	-8.4
14.	Quercetin 3,5,3'-trimethyl ether	COC1=CC(=CC2=C1C(=O)C(=C (O2)C3=CC(=C(C=C3)O)OC)OC)O	13459-09-1	-8.4
15.	Caesalpinin K	[H][C@]3(OC(C)=O)CCC(C)(C)[C@]4(C)C [C@]([H])([O+2]#C)[C@]2([N+2](#C)#C)[C@] ([H])(Cc1occc1[C@@]2(C)[N+]#C)[C@@]34C	468733-36-0	-8.4
16.	Neocaesalpin H	[H][C@]3(C)/C1=C/C(=O)O[C@]1(O)C[C@]4([H])[C@@]25CCC[C@@](C)(C(=O)O)[C@]2([H])CC[C+]34([H])C5	538357-65-2	-9.3
17.	Caesalpinin C	CC(=O)O[C@H]1C[C@H](C([C@]2([C@]1([C@H]3CC4=C(C=CO4) C(=C)[C@@H]3CC2)C)O)(C)C)OC(=O)C	817167-15-0	-8.9
18.	Bonducellpin C	CC(=O)O[C@H]1CCC([C@]2([C@] 1([C@H]3CC4=C(C=CO4)[C@H]([C@@H]3[C@H](C2)O)C(=O)OC)C)O)(C)C	197781-84-3	-9.1

Furthermore, tricin, which belongs to the flavonoid class of secondary metabolites, showed a protective role against high glucose-induced heart damage in diabetic cardiomyopathy (DCM) cell models. By reducing oxidative stress and inflammation and inhibiting the TLR4-MYD88-NF-κB pathway, tricin showed significant therapeutic potential for the treatment of DCM [44].

Molecular docking analysis not only offered crucial details about the structural relationship between the enzyme and the metabolites, but also provides the binding energy so that certain compounds can be considered favorable α-glucosidase inhibitors [45]. The interaction visualization demonstrated that acarbose interacts with active site residues TYR 158, SER 240, GLU 332, PRO 312, and HIS 280 by conventional hydrogen bonding. Some potential compounds such as 7,4'-dihydroxyflavone, artocarpanone, (2S)-5,7 dimethoxy-3',4'- methylenedioxyflavanone, glyceollin II, tricin, caesalpinin,

and caesalpinin C showed the same interaction. Some ligands having the same Pi-Alkyl bond are TYR158, HIS 280, and LYS 156. Pi interactions are often associated with stability.

Table 3. Visualization of ligan-receptor interaction of potential compound

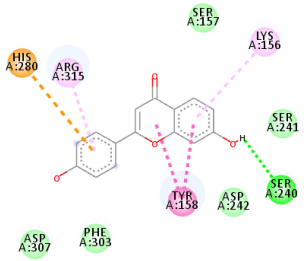
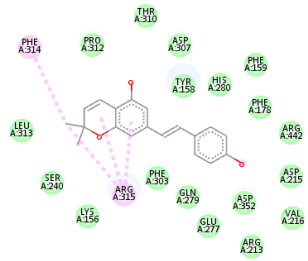
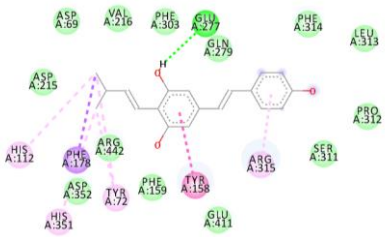
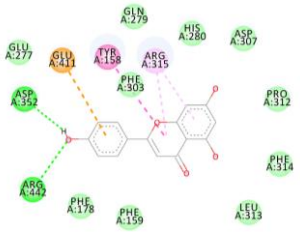
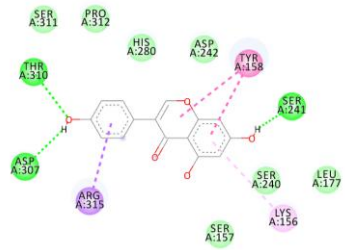
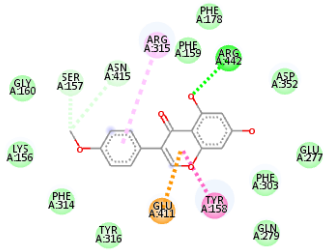
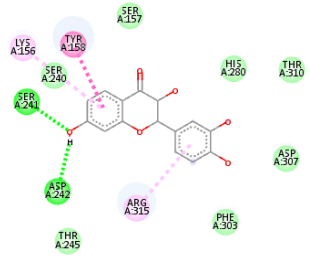
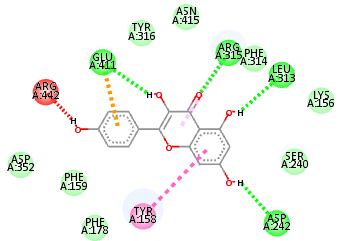
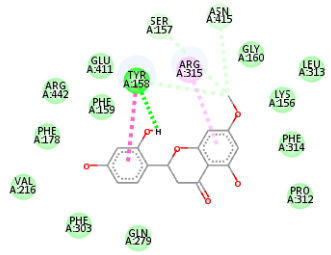
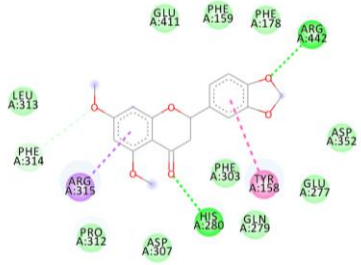
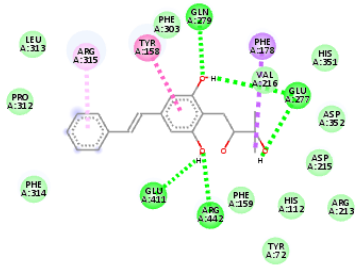
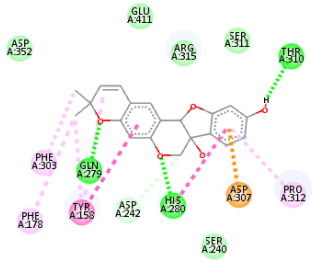
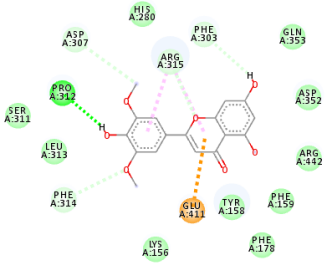
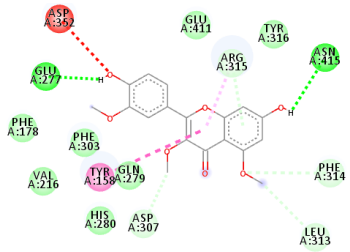
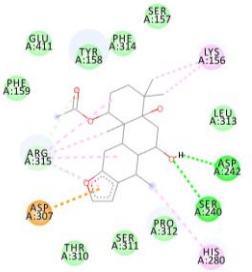
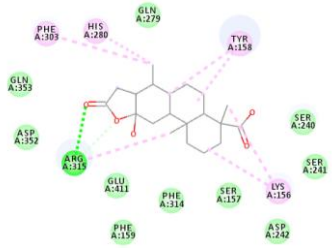
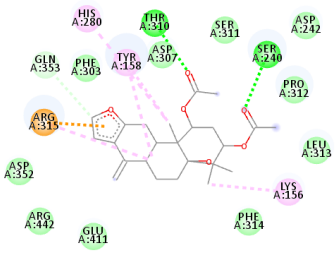
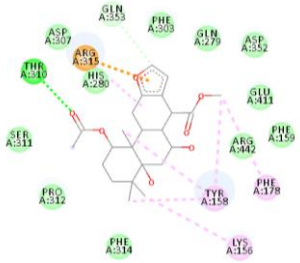
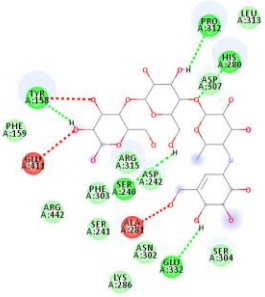
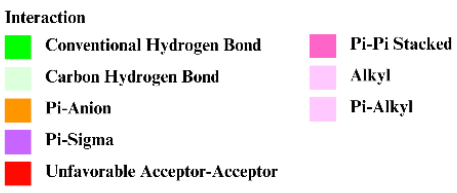
 7,4'-dihydroxyflavone	 5-hydroxy-7-[2-(4-hydroxyphenyl)ethenyl]-2,2- dimethyl-2H-1-benzopyran
 4-(3-methyl-but-1-enyl)-3,4',5- trihydroxystilbene	 Apigenin
 Genistein	 Biochanin A
 Fustin	 Kaempferol
 Artocarpanone	 (2*S*)-5,7-dimethoxy-3',4'- methylenedioxyflavanone

Table 3 *(continue)*. Visualization of ligan-receptor interaction of potential compound

 2-(2,3-dihydroxy-3- methylbutyl)-5-(2- phenylethenyl)-1,3- benzenediol	 Glyceollin II
 Tricin	 Quercetin 3,5,3'-trimethyl ether
 Caesalpinin K	 Neocaesalpin H
 Caesalpinin C	 Bonducellpin C
 Acarbose	

Charge transfer is involved in a lot of Pi-sigma (Pi-alkyl and Pi-sulfur) interactions, which aids in the drug's intercalation at the receptor binding site [46]. The way ligands attach to receptors is influenced by a variety of factors, including electrostatic and hydrophobic interactions as well as hydrogen bonding. The ligand-receptor complex's binding energy and strength are significantly influenced by these interactions [47].

In addition to *in silico* testing using protein 3AJ7, it was also carried out with protein 3W37 where the molecular docking results obtained 10 potential compounds that fulfill the Lipinski rule with a binding affinity value lower than acarbose (Table 4). Of the 10 compounds, there are 4 compounds that are the same in the test with protein 3AJ7 (Table 2). Variations in the three-dimensional (3D) structure can affect the active site and interaction with the ligand. In addition, amino acid residues around the binding site on proteins 3AJ7 and 3W37 can change the way the compound binds to the protein.

Table 4. The results of the physicochemical test of 10 potential compounds from *in silico* test with 3W37 protein

No	Compound name	Smile	CAS Number	Binding affinity (kcal/mol)
1.	5-hydroxy-7-[2-(4-hydroxyphenyl)ethenyl]-2,2-dimethyl-2H-1-benzopyran	CC3(C)/C=C\c2c(O)cc(/C=C/c1ccc(O)cc1)cc2O3	1114548-08-1	-8.3
2.	Luteolin	O=c2cc(c1ccc(O)c(O)c1)oc3cc(O)cc(O)c23	491-70-3	-8.2
3.	Fustin	C1=CC(=C(C=C1[C@@H]2[C@H](C(=O)C3=C(O2)C=C(C=C3)O)O)O)O	4382-36-9	-8.2
4.	Glyceollin I	O[C@]12[C@](C=3C(OC1)=C4C(=CC3)OC(C)(C)C=C4)(OC=5C2=CC=C(O)C5)[H]	57103-57-8	-8.9
5.	Glyceollin II	CC1(C=CC2=CC3=C(C=C2O1)OC[C@@]4([C@H]3OC5=C4C=CC(=C5)O)O)C	67314-98-1	-8.9
6.	Glyceofuran	CC(C)(O)c2cc1cc3c(cc1o2)OC[C@@]5(O)C[C@@H]3Oc4cc(O)ccc45	78873-52-6	-8.6
7.	Demethyltorosaflavone C	CC1=C(O)C(=O)C2OC3=CC4=C(C(=O)C=C(O4)C4=CC=C(O)C(O)=C4)C(O)=C3C12	145194-15-6	-8.5
8.	Neocaesalpin H	[H][C@]3(C)/C1=C/C(=O)O[C@]1(O)C[C@]4([H])[C@@]25CCC[C@@](C)(C(=O)O)[C@]2([H])CC[C+]34([H])C5	538357-65-2	-8.3
9.	Pulcherrin B	[H][C@@H]4C[C@@]2(O)C(C)(C)[C@@]([H])(OC(=O)c1ccccc1)CC[C@]2(C)[C@@]5([H])Cc3occc3[C+]6([H])(C)[C@]45[N+3]6(#C)#[O+]	1132772-55-4	-9.1
10.	Chebulagic acid	*=C6O[C@]%11(C)O[C@@H]3COC(=O)c1cc(O)c(O)c(O)c1c2c(O)c(O)c(O)cc2C(=O)[O+5]%12(#C)(C)O/C/5=C(O)\C[C@]\4%10/C=N/[C@]3(OC4=I/[C@@]9(O)C(=O)Oc8c(O)c(O)cc(C5=*\c7cc6cc(O)c7O)c8[C@@]9%10C=C)[C@H](C)[C@H]%11%12	23094-71-5	-9.6

In Vitro α-Glucosidase Inhibition Assay

Antidiabetic testing can generally use α-glucosidase and α-amylase inhibition. However, in this study, α-glucosidase inhibition was used. The use of α-glucosidase inhibitors is because it is more effective in controlling glucose levels compared to α-amylase. This is because α-glucosidase works at the final stage of carbohydrate digestion by hydrolysing oligosaccharides and disaccharides in the wall of the small intestine, thus helping to prevent a spike in glucose levels after a meal. By inhibiting this enzyme, glucose absorption can be significantly delayed [48]. In addition, research by Poongunran et al. (2016) showed that α-glucosidase inhibition has a lower IC_{50} value of 28.2 µg/ml compared to the α-amylase inhibitor of 39.9 µg/ml for the same extract fraction [49].

In vitro antidiabetic studies determined the levels of α-glucosidase activity inhibition by comparing the amount of p-nitrophenyl produced to the initial enzyme activity [50]. The primary enzyme involved in the digestion of carbohydrates, along with lipids, is called α-glucosidase [28]. It helps break down complex carbs into simpler, more readily absorbed forms [28] playing a role in the breakdown of complex carbohydrates into simpler and more easily absorbed carbohydrates [51]. Inhibition of this enzyme leads to decreased glucose uptake by the small intestine which results in decreased blood glucose levels [52]. People with diabetes have higher α-glucosidase levels than normal people [53]. In order to stop the post-prandial spike in blood sugar levels, the enzyme inhibitors delay the intestinal absorption of glucose [54]. Therefore, this enzyme is one of the target enzymes for the treatment of T2DM.

The α-glucosidase inhibitory activity was measured at 405 nm due to that's the wavelength at which p-nitrophenol absorbs the strongest (400–410 nm) [55]. Parameters of the α-glucosidase inhibition activity through *in vitro* study are the inhibition concentration (IC_{50}). This is the amount that can stop 50% of the activity of α-glucosidase [56]. The following are categories of several IC50 (ppm) values. IC50 values < 50 ppm, 50 < IC50 < 100 ppm, 100 < IC50 < 150 ppm, 150 < IC50 < 200 ppm, and IC50 > 200 ppm are categorized as very strong, strong, moderate, weak, and very weak, respectively [57]. This inhibition activity was tested by inhibiting α-glucosidase using *p*-NPG substrate and α glucosidase where α-glucosidase will hydrolyze *p*-NPG substrate into α-D-glucose and p-nitrophenol (yellow color). Using a microplate reader, the yellow color of the reaction served as an indicator of the enzyme inhibitory activity. If the amount of yellow color in the final solution is less than in the inhibitor-free solution, it means the inhibitor has a higher capacity to impede the α-glucosidase reaction [55].

Acarbose is the positive control that works by inhibiting the α-glucosidase activity [54]. It was reported that acarbose is a substrate that inhibits the α-glucosidase activity due to its structural similarity with oligosaccharides [58]. Acarbose is easily absorbed by the active side of the enzyme, which prevents the enzyme from doing its intended function [52]. The inhibitory potential against α-glucosidase enzyme is represented as IC_{50} value [59]. Percentage inhibition of α-glucosidase by acarbose and by the ethanol extract of *D. virgatus* leaves can be found in Tables 5 and 6, respectively.

Table 5. Percentage inhibition of α-glucosidase by acarbose and its IC_{50} value

Conc. (ppm)	Log[Conc.]	%Inhibition	IC_{50} (ppm)
0.7813	-0.1072	20.55 ± 6.72	3.78 ± 0.65
1.5625	0.1938	27.00 ± 5.76	
3.1250	0.4949	39.76 ± 6.40	
6.2500	0.7959	57.10 ± 2.11	
12.5000	1.0969	63.44 ± 3.03	
25.0000	1.3979	64.94 ± 0.65	
50.0000	1.6990	68.36 ± 3.78	

Table 6. Percentage inhibition of α-glucosidase by the ethanol extract of *D. virgatus* leaves and its IC_{50} value

Conc. (ppm)	Log[Conc.]	%Inhibition	IC_{50} (ppm)
7.81	0.89279	4.77 ± 1.76	144.11 ± 6.93
15.63	1.19382	15.30 ± 3.92	
31.25	1.49485	22.88 ± 4.38	
62.50	1.79588	35.66 ± 0.03	
125.00	2.09691	38.10 ± 0.70	
250.00	2.39794	59.38 ± 2.58	
500.00	2.69897	77.24 ± 1.11	

The data from Table 5 showed that the % inhibition value increased with increasing ethanol extract concentration. More inhibition of enzyme activity results from higher percentages of inhibition

resulting from each concentration [50]. From the results of %inhibition and log concentration, a graph was made as shown in Figure 3.

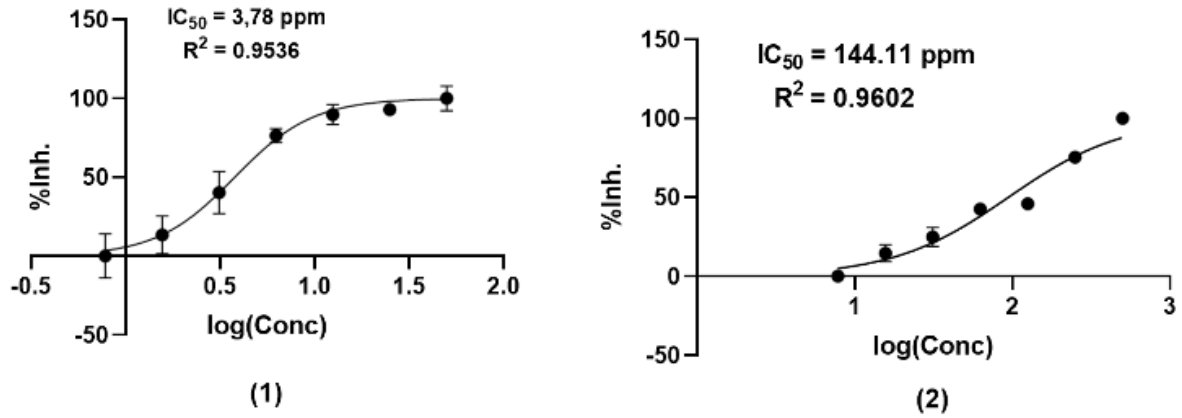


Figure 3. (1) graph of log concentration and % inhibition of acarbose (2) graph of log concentration and % inhibition of ethanol extract of *D. virgatus*

The IC_{50} value of 144.11 ppm was obtained from the testing of α-glucosidase inhibition from ethanol extract using a microplate reader. The ethanol extract of *D. virgatus* leaves can be categorized as medium based on the value obtained. This value is lower than acarbose whose IC_{50} value is 3.78 ppm. This is due to the fact that acarbose is one of the active substances that can successfully inhibit α-glucosidase's activity. In the meantime, it is evident from the ethanol extract sample of *D. virgatus* leaves that it contains a number of active compounds that enable competitive interactions amongst compounds, reducing the effect on the inhibition of α-glucosidase [50]. Based on statistical analysis using SPSS by comparing the IC_{50} value of acarbose with ethanol extract, there is a significant difference with a p value is 0.049 with a mean difference of 48.9065.

CONCLUSION

All of the identified compounds were tested *in silico*, using receptors (PDB ID: 3AJ7) 18 compounds and with (PDB ID: 3W37) 10 compounds were found to fulfill Lipinski's rule with a binding affinity value ≤ -8.2 for 3AJ7 protein and ≤ -8.1 for 3W37, which is similar to acarbose. Meanwhile, the antidiabetic test of the ethanol extract of *D. virgatus* (L.) leaves and acarbose *in vitro* study using α-glucosidase inhibition obtained IC_{50} values of 144.11 ppm and 3.78 ppm, respectively.

AUTHOR CONTRIBUTIONS

Concept: T.T., A.P.W., L.F., S.L.H., R.M.; Design: T.T.; Control: T.T., A.P.W.; Sources: T.T., A.P.W.; Materials: T.T., A.P.W.; Data Collection and/or Processing: L.F., S.L.H., R.M.; Analysis and/or Interpretation: L.F., S.L.H., R.M.; Literature Review: T.T., L.F., S.L.H., R.M.; Manuscript Writing: T.T., A.P.W., L.F.; Critical Review: T.T., A.P.W., L.F., S.L.H., R.M.; Other: -

CONFLICT OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

The authors declare that the ethics committee approval is not required for this study.

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J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 49(3): 729-742, 2025
Doi: 10.33483/jfpau.1636653

ORIGINAL ARTICLE / ÖZGÜN MAKALE

ASSESSMENT OF BIORELEVANT MEDIA TO ANTICIPATE FOOD EFFECT OF ORAL RITONAVIR AMORPHOUS SOLID DISPERSIONS

ORAL RİTONAVİR AMORF KATI DİSPERSİYONLARININ YİYECEK ETKİSİNİ TAHMİN ETMEK İÇİN BİYOUYUMLU ORTAMLARIN DEĞERLENDİRİLMESİ

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ABSTRACT

Objective: *The objective was to evaluate the polymers effects on solubility, solubilization capacity and dissolution of films in biorelevant media, thus, to understand the main mechanism underlaying RTN's negative food effect.*
Material and Method: *Amorphous films were prepared with various polymers via solvent-casting method, then solubility, solubilization capacity and dissolution studies were performed in biorelevant media (FeSSIF-V2 and FaSSIF-V2) and maleic acid buffers (pH 5.8 and 6.5).*
Result and Discussion: *Polymer rank-order to increase RTN solubility in FeSSIF-V2 was SoluPlus>EudragitS100>PVPVA=PEG6000>HPMCAS-L. For FaSSIF-V2, only EudragitS100, SoluPlus and PVP-VA increased RTN solubility. Solubilization capacity studies showed that RTN release was higher for 20% drug loaded films than for 40% for all polymers except for HPMCAS H in FeSSIF-V2, and for HPMCAS-H and HPMCAS-L:H in FaSSIF-V2. In FeSSIF-V2, dissolution studies showed that RTN films from HPMCAS-L and PVP-VA provided higher RTN release compared to other polymers in early time points. $AUC_{(0\text{-}120\ min)}$ in FaSSIF-V2 media was 9.0-fold, 8.3-fold, 5.9-fold, and 5.0-fold higher for SoluPlus, HPMCAS-L, PVPVA and HPMCAS-L:H, respectively, compared to crystalline RTN. Although, RTN's negative food effect, which was not replicated here in vitro, may be due to more complex interactions between drug, polymer, and food in vivo than simulated here using FeSSIF-V2 and FaSSIF-V2.*
Keywords: *Biorelevant media, film, food effect, ritonavir*

ÖZ

Amaç: *Polimerlerin, biyouyumlu ortamlarda filmlerin çözünürlüğü, çözünme kapasitesi ve çözünmesi üzerindeki etkilerinin değerlendirilmesi ve böylece yiyeceklerin RTN üzerindeki olumsuz etkisinin altında yatan temel mekanizmanın anlaşılması amaçlanmıştır.*
Gereç ve Yöntem: *Çeşitli polimerlerle çözücü-dökme yöntemi ile amorf filmler hazırlanmıştır, daha sonra biyouyumlu ortamlarda (FeSSIF-V2 ve FaSSIF-V2) ve maleik asit tamponlarında (pH 5.8 ve 6.5) çözünürlük, çözünme kapasitesi ve çözünme hızı çalışmaları gerçekleştirilmiştir.*
Sonuç ve Tartışma: *FeSSIF-V2'de RTN çözünürlüğünü artırmak için polimer sıralaması SoluPlus>Eudragit S100>PVPVA=PEG6000>HPMCAS-L'dir. FaSSIF-V2 için sadece Eudragit S100, SoluPlus ve PVPVA RTN çözünürlüğünü artırmıştır. Çözünürlük kapasitesi çalışmaları, RTN salınımının FeSSIF-V2'de HPMCAS-H hariç ve FaSSIF-V2'de HPMCAS-H ve HPMCAS-L:H hariç*

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Submitted / Gönderilme : 14.02.2025
Accepted / Kabul : 29.04.2025
Published / Yayınlanma : 19.09.2025

tüm polimerler için %20 ilaç yüklemeye sahip filmlerde %40'tan daha yüksek olduğunu göstermiştir. FeSSIF-V2'de çözünme çalışmaları, HPMCAS-L ve PVPVA'dan elde edilen RTN filmlerinin erken zaman noktalarında diğer polimerlere kıyasla daha yüksek RTN salınımı sağladığını göstermiştir. FaSSIF-V2 ortamındaki $AUC_{(0\text{-}120\,dk)}$ sırasıyla SoluPlus, HPMCAS-L, PVPVA ve HPMCAS-L:H için kristal RTN ile karşılaştırıldığında 9.0 kat, 8.3 kat, 5.9 kat ve 5.0 kat daha yüksek bulunmuştur. Bununla birlikte, bu çalışmada in vitro olarak görülmeyen RTN'nin negatif yiyecek etkisi, burada FeSSIF-V2 ve FaSSIF-V2 kullanılarak simüle edilenden daha karmaşık ilaç, polimer ve yiyecek etkileşimlerinden kaynaklanıyor olabilir.

Anahtar Kelimeler: *Biouyumlu ortam, film, ritonavir, yiyecek etkisi*

INTRODUCTION

Ritonavir (RTN) is an anti-HIV protease inhibitor and antiretroviral medication to treat human immunodeficiency virus / acquired immunodeficiency syndrome (HIV/AIDS) and especially Covid-19 infection. Norvir is the RTN marketed brand product in the form of tablets (100 mg strength), powder (100 mg strength) and solution (80 mg/ml). Norvir tablets and powder are amorphous solid dispersions (ASD) of RTN prepared via hot melt extrusion (HME) of RTN and the polymer polyvinylpyrrolidone/vinyl acetate (PVPVA) in the treatment of AIDS and HIV. Polymer-enabled drug supersaturation is generally a significant contributor to ASD-enhanced oral drug absorption [1,2]. The tablet and powder formulations are bioequivalent in the fasting state [3,4]. In several other tablet products, such as Paxlovid, RTN functions to boost other drugs via the inhibition of cytochrome P450 3A. All oral solid dosage forms of RTN employ PVPVA in ASD fabrication [5].

RTN is a high lipophilic, Biopharmaceutics Classification System (BCS) Class II drug [6]. Its solubility decreases with pH due to RTN being weakly basic. Its low solubility limits its intestinal absorption and oral bioavailability. After a single oral dose of RTV to healthy volunteers (600 mg), plasma half-life ($t_{1/2}$) is 3–5 h, and maximum plasma concentration (C_{max}) is 11.2 μg/ml. The fraction of dose absorbed from a 600 mg dose was estimated to be in the range of 60 to 80% [7,8].

In general, BCS Class II drugs with incomplete drug absorption in the fasted state can be expected to exhibit a positive food effect [9]. Indeed, RTN oral pharmacokinetics is affected by meal content [3]. However, interestingly, for both RTN powder and tablets, which are both amorphous formulations, there is about a 20% reduction in AUC and a 23−40% reduction in C_{max} in humans under the fed state as compared to the fasting state [10,11]. A slight difference in T_{max} is consistent with delayed gastric emptying following a meal [3]. While RTN drug substance in vitro solubility in the fed state is higher than the fasted state, its oral bioavailability is lower in fed state. This perhaps surprising negative food effect of Norvir tablets in humans has been attributed to the higher luminal fluid viscosity (and hence lower drug diffusivity) in the fed state, as well lower drug free fraction and hence lower gut wall permeation rate [12]. Alternatively, we hypothesize that the negative food effect is due to food reducing the PVPVA's ability to maintain RTN supersaturation in fed state compared to fasted state, but that other polymers are less prone to this effect.

The objective was to assess the impact of the fed state on RTN supersaturation from RTN ASD. Fed and fasted states were simulated in vitro using the biorelevant media Fed State Simulated Intestinal Fluid-Version 2 (FeSSIF-V2) and Fasted State Simulated Intestinal Fluid-Version 2 (FaSSIF-V2) [13]. RTN ASDs were fabricated as films, using a range of different polymers, including PVPVA as a control. In vitro dissolution of films and other tests were conducted in FeSSIF-V2 and FaSSIF-V2, in order to measure food effect on ASD supersaturation effect. This evaluation of multiple competing polymers was designed to identify a polymer that, unlike PVPVA, would not exhibit a negative food effect.

MATERIAL AND METHOD

Materials

RTN was obtained from ChemShuttle (Blue Current Inc., Hayward,CA). SoluPlus® and Kollidon®VA 64 (PVPVA) were provided by BASF (Ludwigshafen Germany). PEG-6000 (Polyethylene glycol-6000) was provided by Affymetrix (Cleveland, OH USA). HPMCAS L and

HPMCAS H (Hypromellose acetate succinate L and H grades) were provided by Ashland (Covington, KY USA). Eudragit S100 (Methyl methacrylate copolymer) was provided by Evonik (Piscataway, NJ USA). Methanol and dichloromethane (analytical grade) were purchased from Fischer Scientific (Fischer Scientific; Hampton, NH USA) and Sigma Aldrich (Sigma-Aldrich; St. Louis, MO USA).

Preparation of RTN-Containing Films

RTN films were prepared using seven different polymer systems (PEG-6000, PVPVA, SoluPlus, Eudragit S100, HPMCAS H, HPMCAS L and 1:1 combination (i.e. equal weight) of HPMCAS L: HPMCAS H). Polymers and RTN (to target 20% or 40% drug load) were solved in mixture of dichloromethane and methanol (2:1 w/w). Total solids content (i.e sum of RTN and polymer) of solutions was 10% w/w (i.e. 0.5 g RTN and polymer in 5 g solution), which aided films to have the same thickness. The solution (5 g) was poured into an aluminum dish (57 mm diameter). Solvent was evaporated at 25±2°C for 45 min. Film on dish was then placed into a desiccating cabinet (RH<5%) for 24 h. After 24 h, the dried films were triturated and milled into flakes [14]. Same solvent casting method was also applied on RTN, without polymer. Only the required amount of RTN (according to 20% or 40% drug load of films) were solved in the organic solvent without polymer. Then the effects of the polymers were evaluated compared to the no polymer. RTN was still in crystalline form after application of solvent casting.

Preparation of Biorelevant Media

The compositions of the biorelevant media are given in Table 1. FaSSIF-V2 and FeSSIF-V2 were prepared per vendor instructions [15]. To prepare 1.5 l of FeSSIF-V2 (pH 5.8), 4.905 g of NaOH pellets, 9.585 g of maleic acid and 11.00 g of NaCl were weighed as buffer components. Then, approximately 1.35 l of distilled water was added to the buffer components. After dissolving, the solution was adjusted to pH 5.8 with 1 M NaOH solution, and water was added to make up volume (1.5 l). Then, the 14.64 g of FeSSIF-V2 powder from Biorelevant.com was weighted and dissolved in the buffer component solution.

Table 1. FeSSIF-V2 and FaSSIF-V2 compositions

Component	FaSSIF-V2 Concentration (or attribute)	FeSSIF-V2 Concentration (or attribute)
Sodium taurocholate	3 mM	10 mM
Lecithin	0.2 mM	2 mM
Glyceryl monooleate	-	5 mM
Sodium oleate	-	0.8 mM
Maleic acid (MA)	19 mM	55.02 mM
NaOH	34 mM	81.65 mM
NaCl	69 mM	125.5 mM
HCl /NaOH conc. qs ad	pH 6.5	pH 5.8

To prepare the 0.25 l of FaSSIF-V2 (pH 6.5), 0.348 gr of NaOH pellets, 0.555 g of MA and 1.002 g of NaCl were weighted as buffer components. 0.225 l of distilled water was added and stirred until dissolved. pH was adjusted to 6.5, and then the volume was made up to 0.250 l with distilled water. This buffer solution was poured onto the weighted FaSSIF-V2 powder and stirred until dissolved. All media were stored at 4°C to equilibrate before using [16].

Solubility Studies

RTN solubility was measured in maleic acid buffer (pH 5.8), maleic acid buffer (pH 6.5), FaSSIF-V2 (pH 6.5), and FeSSIF-V2 (pH 5.8), each with and without 2 mg/ml of film polymer (i.e. each PEG-6000, PVPVA, SoluPlus, Eudragit S100, HPMCAS H, HPMCAS L and HPMCAS L: HPMCAS H). An excess amount of RTN (0.5mg/ml) was added to 50 mM maleic acid buffer (pH 5.8 or pH 6.5), FeSSIF-V2 (pH 5.8) and FaSSIF-V2 (pH 6.5), without and with 2 mg/ml polymer. Samples were equilibrated at

37°C for 72 hours in an orbitally shaken water bath. pH was checked after 72 h. After centrifugation, samples were filtered using a 0.22 µm membrane filter, and the amount of RTN was analyzed by HPLC.

Solubilization Capacity of Films

Prepared films with 20% (w/w) drug load (i.e. 20:80 ratio of drug:polymer; 12.5 mg which contained 2.5 mg of RTN) and films with 40% (w/w) drug load (i.e. 40:60 ratio drug:polymer; 12.5 mg which contained 5 mg of RTN) were incubated at 37°C for 1 h in 5 ml of FaSSIF-V2 or FeSSIF-V2. Sample was filtered via 0.22 µm membrane filter. The RTN solubilization capacity of the films, which differed in polymer component, was determined using HPLC.

Film Dissolution into Biorelevant Media

Films were subjected to USP II dissolution testing, with sample replacement. USP II apparatus (SR8PLUS, Hanson Research; Chatsworth CA) was used. USP II dissolution testing on each film formulation (n=3) used 900 ml of FeSSIF-V2 (pH 5.8) and FaSSIF-V2 (pH 6.5) at 37°C at 100 rpm for 6 h. Two ml samples were taken at the determined time points (0, 5, 10, 20, 30, 45, 60, 90, 120, 180, 240 and 360 min) and then filtered through a 0.45 mm membrane filter. Two ml of fresh dissolution media was added to the dissolution vessel to keep dissolution volume constant. USP II dissolution testing yielded RTN mass dissolved profile. Drug was quantified using HPLC method and the dissolution profiles were compared via comparing area under the dissolution curve (AUC) values.

Quantification of Ritonavir by HPLC

RTN concentration was determined via Waters 2489 HPLC system (Waters Corporation; Milford, MA USA). An isocratic mobile phase consisting of 53% phosphoric acid and 47% acetonitrile solution and a 4.6 × 150 mm Zorbax C18 5-µm HPLC column were used [17]. The injection volume and flow rate were 25.0 µl and 1 ml/min, respectively. The UV-vis detector was set to 240 nm [17,18]. The retention time of RTN was 9-10 min, with a 13 min run time. A calibration curve containing a range of 0.098-50 µg/ml RTN was run in triplicate with each analysis (r^2 = 0.9999). For solubility and solubilization capacity studies, the calibration curves were obtained for each media, which were maleic acid buffer (pH 5.8 and 6.5), FaSSIF-V2, and FeSSIF-V2, separately. The standard samples were obtained from the stock solution of RTN in methanol (1 mg/ml) by diluating 1:9 ratio with the solubility media. The standard solutions obtained for each media were diluted with mobile phase to prepare the 0.098-50 µg/ml concentrations.

Statistical Analysis

Data was analyzed using SPSS database Version 16 (Systat Software Inc., San Jose, CA USA). The statistical analysis of variance (ANOVA) was carried out at the 95% confidence level (n = 3). A comparison variance analysis ANOVA test followed by Tukey's post hoc testing was used to compare multiple groups. The t-test was used to compare two groups. Results were given as mean ± standard error of mean (SEM) (n = 3).

RESULT AND DISCUSSION

Evaluation of RTN Solubility

An increase in RTN solubility in both the fed and fasted states may be a way to mitigate the negative food effect of Norvir tablets and powder, which as RTN ASDs using PVPVA. To improve RTN solubility, the effect of different polymers (HPMCAS L, HPMCAS H, HPMCAS L:H, SoluPlus, PVPVA, PEG-6000 and Eudragit S100) was evaluated in FeSSIF-V2 (pH 5.8), and FaSSIF-V2 (pH 6.5). Moreover, to see the effect of the surfactant in FeSSIF-V2 (sodium taurocholate, lecithin and glyceryl monooleate, sodium oleate) and FaSSIF-V2 (sodium taurocholate, lecithin), biorelevant solubilities were compared to solubilities in each pH 5.8 and pH 6.5 buffers (Table 2 and Table 3).

Table 2 lists solubility results in buffer (pH 6.5) and FaSSIF-V2. RTN solubility was 2.99 µM (2.16±0.23 µg/ml) in maleic acid buffer (pH 6.5). Xu et al. found a similar solubility of about 2 µg/ml at pH 6.8 [10]. Table 3 lists the solubility results in buffer (pH 5.8) and FeSSIF-V2. RTN solubility in

the maleic acid buffer (pH 5.8) was 3.24 μM (2.34±0.02 μg/ml). RTN's lower solubility at higher pH reflects RTN being a weakly basic drug with pKa values of 1.8 and 2.6 [19-21]. RTN has a high aqueous solubility at pH < 1 and an extremely low solubility at pH 4−7 [10].

Table 2. RTN solubility in 50 mM maleic acid buffer (pH 6.5) and FaSSIF-V2 with and without polymer. When polymer present, polymer concentration was 2 mg/ml. pH = 6.5 in all media. Mean ± SE from n=3

Media (pH 6.5)	Solubility (μM)	Fold-enhancement compared to no polymer
Maleic acid buffer (MA) (no polymer)	2.99±0.33	-
MA (pH 6.5) + HPMCAS L	2.92±0.18	0.989±0.078
MA (pH 6.5) + HPMCAS H	1.34±0.33	0.481±0.173
MA (pH 6.5) + HPMCAS L:H (1:1)	1.08±0.05	0.365±0.030
MA (pH 6.5) + Soluplus	8.89±2.63	3.13±0.96
MA (pH 6.5) + PVPVA	2.96±0.11	1.00±0.074
MA (pH 6.5) + PEG-6000	2.75±0.13	0.931±0.064
MA (pH 6.5) + Eudragit S100	12.3±0.5	3.59±0.98
FaSSIF-V2 (no polymer)	4.25±0.24	-
FaSSIF-V2 + HPMCAS L	3.96±0.27	0.93±0.12
FaSSIF-V2 + HPMCAS H	1.35±0.05	0.315±0.030
FaSSIF-V2 + HPMCAS L:H (1:1)	1.20±0.13	0.284±0.049
FaSSIF-V2 + Soluplus	11.0±0.3	2.56±0.79
FaSSIF-V2 + PVPVA	4.40±0.19	1.03±0.11
FaSSIF-V2 + PEG-6000	4.17±0.25	0.98±0.12
FaSSIF-V2 + Eudragit S100	15.3±0.5	3.56±0.14

In FeSSIF-V2 and FaSSIF-V2, RTN solubility increased about 5-fold and 1.5-fold, respectively, compared to the buffer media. Solubility was higher in the fed state (15.8 μM; 11.37±0.42 μg/ml) than the fasted state (4.25 μM; 3.06±0.18 μg/ml), similar to literature [22,23]. Xu et al. found RTN solubility was 7.4±1.1 μg/ml in FaSSIF-V2 and 18.5±1.9 μg/ml in FeSSIF-V2 [10]. Kokott et al. reported RTN solubility in FaSSIF was 5.4±0.6 μg/ml [23].

The effects of polymers, which were used to prepare the amorphous films of RTN, on RTN solubility in buffer and biorelevant media were also evaluated. In buffer (pH 6.5), only SoluPlus (3.1-fold increase) and Eudragit S100 (3.6-fold increase) increased RTN solubility. In maleic acid buffer (pH 5.8), only SoluPlus materially impacted solubility, where RTN solubility increased about 5-fold with SoluPlus. In FaSSIF-V2, Soluplus and Eudragit S100 increased RTN solubility 2.6-fold and 3.6-fold, respectively, compared to no polymer. In FeSSIF-V2, SoluPlus and Eudragit S100 increased the solubility the most (i.e. 2.1-fold, and 1.7-fold, respectively) compared to no polymer.

Polymers tended to have more complex effects on drug solubilization in biorelevant media than in buffer, particularly in FaSSIF-V2. Most polymers slightly increased RTN solubility in FeSSIF-V2, where SoluPlus, Eudragit S100, and PVPVA performed the best across both biorelevant media. However, in FeSSIF-V2 and FaSSIF-V2, HPMCAS H and HPMCAS L:H (1:1) reduced RTN solubility.

Polymer rank-order to increase RTN solubility in FeSSIF-V2 was SoluPlus> Eudragit S100> PVPVA= PEG6000 > HPMCAS L. For FaSSIF-V2, only Eudragit S100, SoluPlus and PVPVA increased RTN solubility and the order was Eudragit S100 > SoluPlus >PVPVA. Similar results were previously obtained in media containing polyoxyethylene (10) lauryl ether (POE10), where SoluPlus performed the best [14]. SoluPlus may function as a surfactant to form RTN-loaded micelles to increase drug solubility [24]. Eudragit S100 is highly soluble at higher pH [25,26], so it was more effective here in pH 6.5 than pH 5.8. Interestingly, HPMCAS H (and HPMCAS L:H combination) decreased RTN solubility, in contrast with grade L's enhancement of solubility. Compared to HPMCAS L, HPMCAS H has a higher ratio of acetyl substitution to succinoyl substitution. Hence, HMPCAS H is more lipophilic and has less of an ionizable character than HPMCAS L [14,27].

Table 3. RTN solubility in 50 mM maleic acid buffer (pH 5.8) and FeSSIF-V2 with and without polymer. When polymer present, polymer concentration was 2mg/ml. pH = 5.8 in all media. Mean ± SE from n=3.

Media (pH 5.8)	Solubility (µM)	Fold-enhancement compared to no polymer	pH and Food effect (fed / fasted)
Maleic acid buffer (MA) (no polymer)	3.24±0.02	-	1.11±0.12
MA (pH 5.8) + HPMCAS L	4.24±0.14	1.31±0.04	1.46±0.01
MA (pH 5.8) + HPMCAS H	2.96±0.10	0.914±0.032	2.49±0.56
MA (pH 5.8) + HPMCAS L:H (1:1)	3.38±0.08	1.04±0.02	3.14±0.11
MA (pH 5.8) + Soluplus	15.6±1.1	4.74±0.31	2.13±0.79
MA (pH 5.8) + PVPVA	4.46±0.19	1.37±0.05	1.51±0.02
MA (pH 5.8) + PEG-6000	4.04±0.14	1.25±0.04	1.47±0.02
MA (pH 5.8) + Eudragit S100	3.23±0.14	0.994±0.032	0.360±0.103
FeSSIF-V2 (no polymer)	15.8±0.6	-	3.70±0.40
FeSSIF-V2 + HPMCAS L	20.2±0.9	1.28±0.04	5.12±0.15
FeSSIF-V2 + HPMCAS H	12.6±0.4	0.801±0.057	9.38±0.66
FeSSIF-V2 + HPMCAS L:H (1:1)	13.6±0.8	0.861±0.033	11.5±0.80
FeSSIF-V2 + Soluplus	32.9±7.3	2.08±0.44	3.20±0.36
FeSSIF-V2 + PVPVA	23.5±0.5	1.49±0.03	5.35±0.14
FeSSIF-V2 + PEG-6000	23.3±0.3	1.48±0.04	5.62±0.32
FeSSIF-V2 + Eudragit S100	26.8±2.8	1.71±0.20	1.74±0.14

In Table 3, pH and food effect results are listed. pH and food effect compare RTN solubility in Table 3 (i.e. fed state or pH 5.8 buffer, with or without polymer) against the corresponding RTN solubility in Table 2 (i.e. fasted state or pH 6.5 buffer, with or without polymer). A lower food effect was observed with SoluPlus (3.2-fold) and Eudragit S100 (1.7-fold), in part due to these polymers having provided greatest solubility enhancement in FaSSIF-V2. The food effect was about 5-6 fold with PVPVA, PEG-6000, HPMCAS L. The highest food effect was observed with HPMCAS H and HPMCAS L:H polymer, in part due to these polymers having reduced RTN solubility in FaSSIF-V2.

Evaluation of Solubilization Capacity

Figure 1 and 2 show the solubility capacity results of films having 20% and 40% drug loads in FeSSIF-V2 and FaSSIF-V2, respectively. The percentage RTN release from film after 1 h was assessed as the solubilization capacity of the film. In Figure 1, only about 0.6% of RTN was released in the absence of polymer (i.e. from crystalline RTN). Overall, in Figure 2, release in FeSSIF-V2 was higher for 20% drug load (panel a) than for 40% drug load (panel b) for all polymers except HPMCAS H. For 20% of drug loaded films, RTN % release was 38.4% with SoluPlus, 5.2% with HPMCAS L, 4.9% with HPMCAS L:H, 2.1% with PVPVA, 1.9% with PEG-6000, 0.6% with HPMCAS H, and 0.2% with Eudragit S100. So, there was 68-fold, 9.3-fold, 8.8-fold, 3.8-fold, and 3.4-fold increase with SoluPlus, HPMCAS L, HPMCAS L:H, PVPVA, and PEG-6000 compared to without polymer (i.e. 0.6% release). HPMCAS H and Eudragit S100 did not increase solubilization capacity (Table 4). RTN % release in FeSSIF-V2 from films with 40% drug load are shown in Figure 4 panel b. Percent release was 2.2% with SoluPlus, 1.8% with HPMCAS L, 2.0 % with HPMCAS L:H, 1.3% with PVPVA, 0.9% with PEG-6000, 1.9% with HPMCAS H, and 0.07% with Eudragit S100. So, there was 3.9-fold, 3.6-fold, 3.1-fold, 2.3-fold, 1.9-fold, and 1.6-fold increase with SoluPlus, HPMCAS L:H, HPMCAS L, PVPVA, HPMCAS H, and PEG-6000 compared to without polymer (i.e. 0.6% release). Only Eudragit S100 did not increase the solubilization capacity (Table 5).

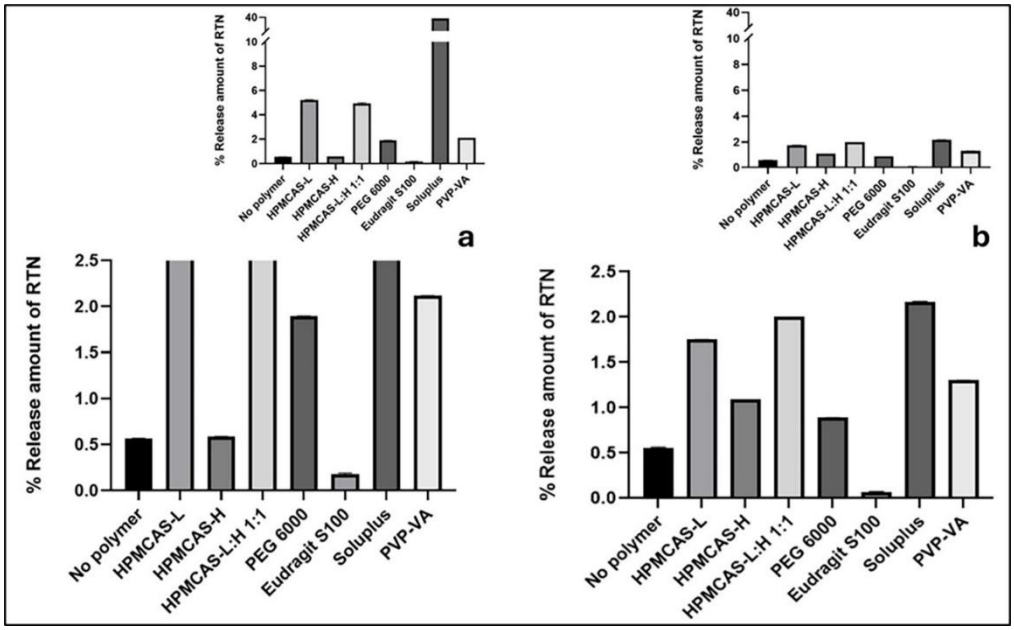


Figure 1. The solubilization capacity of the films contains 20% (a) and 40% (b) drug load of RTN films in FeSSIF-V2. Values are percent RTN dissolved after 1 h, out of 2.5 mg RTN (a) and out of 5 mg RTN (b). 12.5mg of film was tested. Each panel shows two plots with differing extents of RTN release, to facilitate comparisons across polymers and drug loads

Figure 2 plots the solubilization capacity of films in FaSSIF-V2. Only about 0.3% of RTN was released in the absence of polymer (i.e. from crystalline RTN). Like FeSSIF-V2, release was higher for 20% drug load (panel a) than for 40% drug load (panel b) for all polymers except HPMCAS H and HPMCAS L:H. For films having 20% drug load, RTN %release was 1.5% with SoluPlus, 1.0% with HPMCAS L (17.1±0.02 µg/ml), 0.4% with HPMCAS L:H, 2.3% with PVPVA (38.6±0.03 µg/ml), 1.4% with PEG-6000, 0.8% with HPMCAS H, and 0.09% with Eudragit S100.

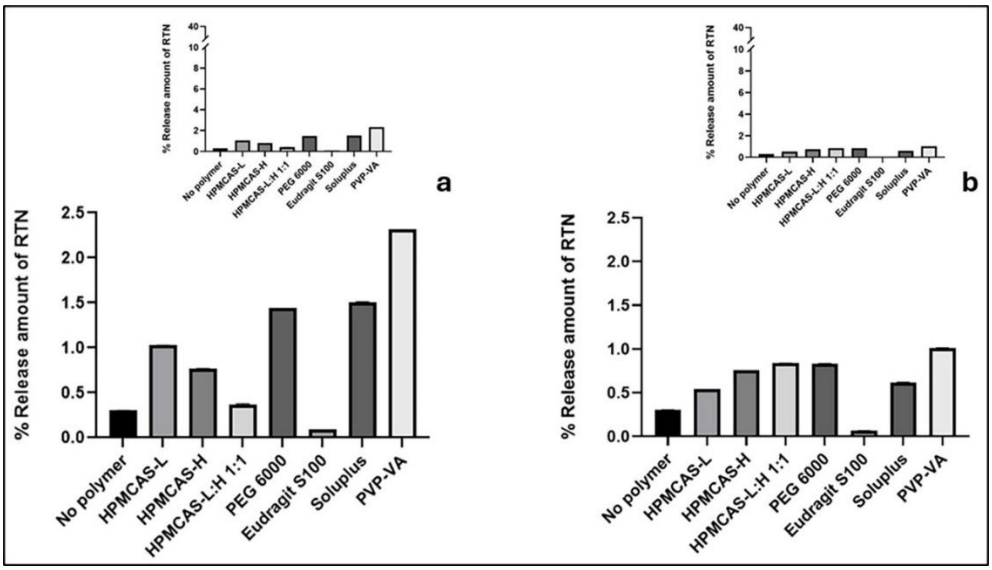


Figure 2. The solubilization capacity of films contains the 20% (a) and 40% (b) drug load of RTN films in FaSSIF-V2. Values are percent RTN dissolved after 1 h, out of 2.5 mg RTN (a) and out of 5 mg RTN (b). 12.5 mg of film was tested. Each panel shows two plots with differing extents of RTN release, to facilitate comparisons to FeSSIF-V2 in Figure 1

There was 7.7-fold, 5.0-fold, 4.8-fold 3.4-fold, 2.5-fold, and 1.2-fold increased with PVPVA, SoluPlus, PEG-6000, HPMCAS L, HPMCAS H, and HPMCAS L:H compared to without polymer (i.e. 0.3% release) (Table 4). Only Eudragit S100 did not increase solubilization capacity. Findings here were like literature, where RTN solubilization from 20% drug loaded ASDs was about 30-35 µg/ml with PVPVA and about 10-15 µg/ml with HPMCAS L, compared to 38.6±0.03 µg/ml for PVPVA and 17.1±0.02 µg/ml for HPMCAS L here [23].

Table 4. Food effect for solubilization capacity of 20% drug load RTN films. Media were FaSSIF-V2 (pH 6.5) and FeSSIF-V2 (pH 5.8), with and without polymer. When polymer present, polymer concentration was 2 mg/ml. Mean ± SE from n=3.

Media	Solubilization capacity (%release)	Fold-enhancement compared to no polymer	pH and Food effect (fed / fasted)
FaSSIF-V2 (no polymer)	0.302±0.001	-	-
FaSSIF-V2 + HPMCAS L	1.03±0.001	3.40±0.019	-
FaSSIF-V2 + HPMCAS H	0.769±0.002	2.54±0.02	-
FaSSIF-V2 + HPMCAS L:H (1:1)	0.373±0.005	1.23±0.02	-
FaSSIF-V2 + Soluplus	1.51±0.002	4.99±0.02	-
FaSSIF-V2 + PVPVA	2.32±0.002	7.66±0.04	-
FaSSIF-V2 + PEG-6000	1.44±0.001	4.77±0.02	-
FaSSIF-V2 + Eudragit S100	0.0876±0.0004	0.290±0.003	-
FeSSIF-V2 (no polymer)	0.562±0.004	-	1.86±0.01
FeSSIF-V2 + HPMCAS L	5.21±0.03	9.26±0.10	5.07±0.03
FeSSIF-V2 + HPMCAS H	0.583±0.003	1.04±0.01	0.759±0.002
FeSSIF-V2 + HPMCAS L:H (1:1)	4.92±0.08	8.75±0.19	1.57±0.02
FeSSIF-V2 + Soluplus	38.4±0.04	68.4±0.5	25.5±0.05
FeSSIF-V2 + PVPVA	2.12±0.003	3.76±0.02	1.63±0.01
FeSSIF-V2 + PEG-6000	1.89±0.003	3.365±0.03	2.33±0.01
FeSSIF-V2 + Eudragit S100	0.180±0.008	0.321±0.016	2.06±0.08

In Figure 2 panel b, RTN %release into FaSSIF-V2 was 0.6%, 0.5%, 0.8%, 1.0%, 0.8%, 0.8% and 0.07% with SoluPlus, HPMCAS L, HPMCAS L:H, PVPVA, PEG-6000, HPMCAS H and Eudragit S100, respectively. So, there was 3.4-fold, 2.8-fold, 2.8-fold, 2.5-fold, 2-fold, and 1.8-fold increase with PVPVA, PEG-6000, HPMCAS L:H, HPMCAS H, SoluPlus, and HPMCAS L compared to without polymer (i.e. 0.3% release). Like for 20% drug load, only Eudragit S100 did not increase solubilization capacity. By considering their collective results, SoluPlus, PVPVA, HPMCAS L and HPMCAS L:H are concluded to be preferred polymers for RTN ASD with higher solubilization capacity (Table 5).

Table 5. Food effect for solubilization capacity of 40% drug load RTN films. Media were FaSSIF-V2 (pH 6.5) and FeSSIF-V2 (pH 5.8), with and without polymer. When polymer present, polymer concentration was 2 mg/ml. Mean ± SE from n=3

Media	Solubilization capacity (%release)	Fold-enhancement compared to no polymer	pH and Food effect (fed / fasted)
FaSSIF-V2	0.302±0.001	-	-
FaSSIF-V2 + HPMCAS L	0.539±0.001	1.78±0.008	-
FaSSIF-V2 + HPMCAS H	0.759±0.0004	2.51±0.01	-
FaSSIF-V2 + HPMCAS L:H (1:1)	0.836±0.001	2.77±0.008	-
FaSSIF-V2 + Soluplus	0.618±0.001	2.05±0.01	-
FaSSIF-V2 + PVPVA	1.01±0.001	3.35±0.02	-
FaSSIF-V2 + PEG-6000	0.833±0.0003	2.76±0.01	-
FaSSIF-V2 + Eudragit S100	0.0648±0.001	0.214±0.002	-

Table 5 (*continue*). Food effect for solubilization capacity of 40% drug load RTN films. Media were FaSSIF-V2 (pH 6.5) and FeSSIF-V2 (pH 5.8), with and without polymer. When polymer present, polymer concentration was 2 mg/ml. Mean ± SE from n=3

Media	Solubilization capacity (%release)	Fold-enhancement compared to no polymer	pH and Food effect (fed / fasted)
FeSSIF-V2	0.562±0.004	-	1.86±0.01
FeSSIF-V2 + HPMCAS L	1.75±0.003	3.12±0.03	3.25±0.01
FeSSIF-V2 + HPMCAS H	1.94±0.01	1.94±0.01	1.44±0.001
FeSSIF-V2 + HPMCAS L:H (1:1)	2.00±0.001	3.56±0.02	2.39±0.005
FeSSIF-V2 + Soluplus	2.17±0.01	3.85±0.01	3.50±0.02
FeSSIF-V2 + PVPVA	1.30±0.01	2.31±0.01	1.28±0.002
FeSSIF-V2 + PEG-6000	1.58±0.01	1.58±0.01	1.07±0.001
FeSSIF-V2 + Eudragit S100	0.069±0.007	0.12±0.01	1.07±0.10

As expected, 20% drug load performed better than 40% of the drug load, similar to literature [23,28,29]. Simões et al. observed that drug release from polymeric blends decreased drastically with higher drug load [29]. Kokott et al. also performed small scale dissolution setup with RTN in FaSSIF-V2 and found that 40% drug load showed lower dissolution profile than 20% drug load for PVPVA and HPMCAS [23].

The food effect for solubilization capacity is also given in Table 4 and 5. For 20% drug load (Table 4), the lowest food effect was with HPMCAS H (0.759-fold) and highest was with SoluPlus (25.5-fold). There was a 2-fold increase with PVPVA, Eudragit S100, PEG-6000, and a 5-fold increase with HPMACS L. For 40% drug load, there was essentially no food effect with PEG-6000, Eudragit S100, PVPVA and HPMCAS H. There was 3-fold, 3.5-fold and 2.4-fold increase with SoluPlus, HPMCAS L, and HPMCAS L:H, respectively.

SoluPlus and PVPVA possessed good aqueous solubility, and their films improved RTN release compared to no polymer. SoluPlus has amphiphilic chemical structure, with a large number of hydroxyl groups, resulting in it serving as a good solubilizer of poorly soluble drugs in aqueous media [30]. HPMCAS L is more hydrophilic than HPMCAS H due to its lower ratio of acetyl substitution versus succinoyl substitution. HPMCAS L's enhancement of RTN solubility and solubilization capacity reflects the polymer's hydrophilicity. Adhikari et al. indicated that the dissolution performance of HPMCAS L was higher than the M and H grade contrasts with the solution stabilization and drug-polymer molecular interactions. By means of hydrophobicity of HPMCAS H and greater hydrophobic interactions between itraconazole and HPMCAS H provided retardation of itraconazole precipitation and higher stabilization of itraconazole [2]. Interestingly, Eudragit S100 did not provide an increase in solubilization capacity, although increased RTN solubility in both biorelevant media, perhaps reflecting to the precipitation or crystallization of RTN during the formation of films with Eudragit S100 [14]. The solubilization capacity results were confirmed by solubility enhancement in FeSSIF-V2 and FaSSIF-V2 by SoluPlus, PVPVA and HPMCAS L. Also, there was no increase with HPMCAS H. So, films with 20% drug load and fabricated from SoluPlus, HPMCAS L, HPMCAS L:H and PVPVA were selected for further comparative dissolution studies, similar to prior results of film dissolution into POE10 medium [14].

Evaluation of RTN Dissolution Profiles from Films into Biorelevant Media

Figure 3 and 4 plot RTN dissolution profiles from films of SoluPlus, HPMCAS L, PVPVA and HPMCAS L:H into FeSSIF-V2 and FaSSIF-V2, respectively. In FeSSIF-V2, the highest release was from HPMCAS L film. Early release was higher for PVPVA, but RTN precipitation occurred after 20 min. Meanwhile, HPMCAS L provided the highest, sustained supersaturation in FeSSIF-V2 for 6 h. Slower onset of dissolution of RTN from HPMCAS L compared to PVPVA agrees with the literature [23]. At 360 min, rank order release from films was HPMCAS L (88.3%) > PVPVA (53.7%) > SoluPlus (36.8%) > HPMCAS L:H (23.0%), which were higher than from RTN crystalline powder. Table 6 lists

AUC values over 120 min ($AUC_{0\text{-}120\ min}$). The highest AUC value was with HPMCAS L (2810 µg.min/ml). The lowest AUC was with HPMCAS L:H polymer (385 µg.min/ml). Rank order of $AUC_{(0\text{-}120\ min)}$ in FeSSIF-V2 was HPMCAS L > PVPVA > SoluPlus > HPMCAS L:H. While there was no increase in $AUC_{(0\text{-}120\ min)}$ with HPMCAS L:H compared to crystalline RTN powder (385 µg.min/ml), there was 2.31-fold, 7.31-fold and 6.55-fold increase with SoluPlus, HPMCAS L and PVPVA, respectively.

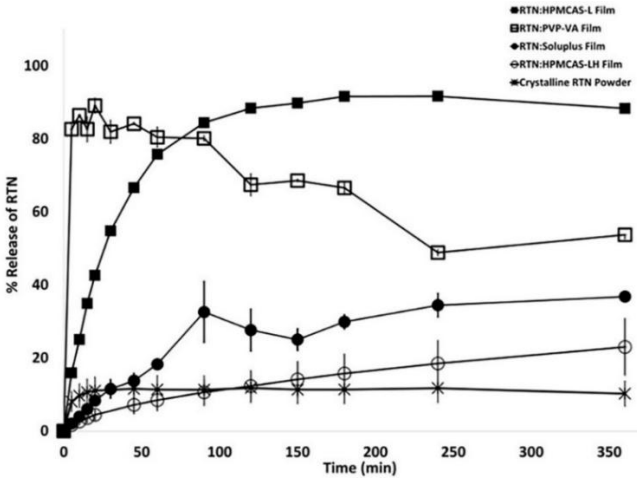


Figure 3. RTN dissolution profiles from films with 20% drug load into FeSSIF-V2. Best polymers were PVPVA, SoluPlus, HPMCAS L and HPMCAS L:H. Mean ± SE from n=3

Figure 4 plots dissolution from 20% drug loaded films containing SoluPlus, HPMCAS L, HPMCAS L:H and PVPVA in FaSSIF-V2, which has a higher pH than FeSSIF-V2. Dissolution was lower in FaSSIF-V2 (Figure 4) than into FeSSIF-V2 (Figure 3), similar to above solubility and solubilization capacity study findings where solubility and solubilization capacity were lower at higher pH condition, in agreement with literature [11]. Drug solubility is higher in low pH. This situation can be explained by the drug solubility was higher in lower pH [31]. Also, the increase in dissolution of HPMCAS L:H films in FaSSIF-V2 compared to FeSSIF-V2 can be related to the HPMCAS H polymer has higher solubility in pH 6.5 compared to the 5.8 [32].

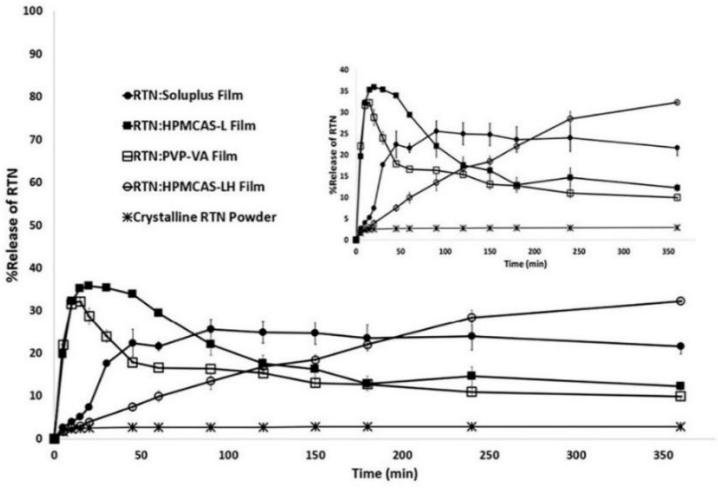


Figure 4. The dissolution profiles of the films contain the 20% drug load into FaSSIF-V2. Overall, RTN film dissolution into FaSSIF-V2 was lower than that into FeSSIF-V2. The graph shows an expanded view of the lower level of release. Mean ± SE from n=3

At 30 min, the highest RTN release was with HPMCAS L. Rank order release was HPMCAS L (35.4%) > PVPVA (24.0%) > SoluPlus (17.7%) > HPMCAS L:H (5.1%). Both HPMCAS L and PVPVA profiles showed drug precipitation, while HPMCAS L:H and SoluPlus profiles only increased with time. Hence, at 360 min, the rank order release was HPMCAS L:H >SoluPlus> HPMCAS L > PVPVA.

Table 6 lists AUC $_{(0\text{-}120\ min)}$. Overall, AUC $_{(0\text{-}120\ min)}$ in FaSSIF-V2 media was 9.0-fold, 8.3-fold, 5.9-fold, and 5.0-fold higher for SoluPlus, HPMCAS L, PVPVA and HPMCAS L:H, respectively, compared to crystalline RTN (2.9% release at 120 min). Food effect was also calculated by considering the ratio of AUC values in FeSSIF-V2 to FaSSIF-V2 and given in Table 6. While there was 3-fold enhancement for films containing HPMCAS L and PVPVA in FeSSIF-V2, there was no increase with SoluPlus and HPMCAS L:H polymers. 3-fold enhancement was also observed on the RTN crystalline powder in FeSSIF-V2. The films formulations and RTN powder showed the positive food effect, although there is about a 20% reduction in AUC under the fed state as compared to the fasting state in humans [10,11]. In the literature, there is higher in vitro dissolution of each Norvir tablet and Norvir oral powder in fed than fasted, using biorelevant dissolution media [10]. Likewise, Patel et al. also observed this same discordance with RTN in vivo negative food effect, as in vitro lipolysis profiles showed positive food effect [11]. The difference between the in vivo and in vitro may be related to the physiological parameters such as delayed gastric emptying, altered gastric pH, changes in regional distribution of bile salts, fluid volumes, viscosity, and blood flow [12,33,34].

Table 6. Dissolution profile AUC$_{(0\text{-}120\ min)}$ values of films containing SoluPlus, HPMCAS L, PVPVA, and HPMCAS L:H polymers in FeSSIF-V2 and FaSSIF-V2.

	FeSSIF-V2	**FaSSIF-V2**	**Food effect (fed / fasted)**
Film	**AUC $_{0\text{-}120}$ (µg.min/ml)**	**AUC $_{0\text{-}120}$ (µg.min/ml)**	
RTN-SoluPlus	889±442	1350±190	0.755±0.390
RTN- HPMCAS L	2810±160	1260±370	3.00±1.61
RTN- PVPVA	2520±1490	895±182	3.08±1.54
RTN- HPMCAS L:H	362±200	739±93	0.549±0.290
RTN crystalline powder	385±217	152±30	2.82±1.39

Drug release from ASD is determined in part by the dissolution of the polymeric carrier and not simply by the drug itself [29,35,36]. While solubility and solubilization capacity generally reflects dissolution results, there was some difference in the rank order of dissolution profiles, compared to rankings from solubility and solubilization capacity. These differences can be related to the dissolution test being a short-term test, while solubility reflected a longer-term assessment [37-39]. Overall, films from HPMCAS L and PVPVA provide higher RTN release compared to other polymers in early time points. Meanwhile, HPMCAS L maintained supersaturation for 6 hours in FeSSIF-V2 and provided 9-fold increase in RTN release in FaSSIF-V2, which has promised to remove the food effect of RTN oral dosing.

Conclusion

The films containing various polymer with different drug:polymer ratio was successfully prepared. To assess the effect of the various polymers, RTN solubility, solubilization capacity and dissolution profiles in biorelevant media were performed. By considering their collective favorable results of solubility and solubilization capacity studies, SoluPlus, PVPVA, HPMCAS L and HPMACS L:H preferred polymers for potential RTN films. 20% of the drug load performed better than 40% drug load in both FeSSIF-V2 and FeSSIF-V2. The highest in vitro dissolution AUC value was observed with HPMCAS L polymer in FeSSIF-V2, and it provided the highest supersaturation over 6 h. Also, RTN films containing HPMCAS L provided 9-fold increase in RTN % release in FaSSIF-V2, which can help to remove the food effect of RTN.

ACKNOWLEDGEMENTS

This study was supported by TUBITAK (The Scientific and Technological Research Council of Turkey) [BIDEB2219/Project code: 1059B192000617].

AUTHOR CONTRIBUTIONS

Concept: A.N.O.; Design: A.N.O.; Control: A.N.O., J.E.P.; Sources: A.N.O., J.E.P.; Materials: A.N.O., J.E.P.; Data Collection and/or Processing: A.N.O.; Analysis and/or Interpretation: A.N.O., J.E.P.; Literature Review: A.N.O., J.E.P.; Manuscript Writing, A.N.O., J.E.P.; Critical Review: A.N.O., J.E.P.; Other: -

CONFLICT OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

The authors declare that the ethics committee approval is not required for this study.

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J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 49(3): 743-755, 2025
Doi: 10.33483/jfpau.1595627

ORIGINAL ARTICLE / ÖZGÜN MAKALE

INVESTIGATION OF BIOCHEMICAL PROPERTIES, ANTIMICROBIAL, ANTIOXIDANT AND ANTIPROLIFERATIVE ACTIVITY OF THE *RHAMNUS CATHARTICA* L. EXTRACTS COLLECTED FROM KAZAKHSTAN

KAZAKİSTAN'DAN TOPLANAN RHAMNUS CATHARTİCA L. EKSTRAKTLARININ BİYOKİMYASAL ÖZELLİKLERİ, ANTİMIKROBİYAL, ANTİOKSİDAN VE ANTİPROLİFERATİF AKTİVİTELERİNİN ARAŞTIRILMASI

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ABSTRACT

Objectives: *This research evaluated the biochemical composition of hexane, ethyl acetate, and ethanol extracts and analyzed the antimicrobial, antioxidant and antiproliferative activities of the cortex, leaf, and fruit of the Rhamnus cathartica L. plant from Almaty, Kazakhstan.*
Material and Method: *The GC-MS method was used to evaluate the chemicals of Rhamnus cathartica L. cortex, leaf, and fruit extracts. The microdilution technique was used to evaluate the MIC values of plant extracts against pathogenic microorganisms. DPPH, FRAP, and Total Flavonoid Content were evaluated for antioxidant activity. The antiproliferative effect of the extracts on MCF-7, A549, C6 and WI-38 cells was measured by XTT assay.*
Result and Discussion: *According to GC-MS results, Trans-2-Decenal had the highest proportion at 2.88%. Eicosane is second at 1.71%. DPPH, FRAP and flavonoid values showed that the fruit ethyl acetate extract (F-EtAc) had the strongest antioxidant activity. F-EtAc extract had the strongest anticancer activity according to the selectivity index table (WI-38/MCF-7: 12.67, WI-38/C6: 31.38, WI-38/A549: 8.55). All plant extracts had high MIC values against Staphylococcus aureus based on reference source values. The MIC value of cortex and leaf in ethyl acetate was 0.078 mg/ml, cortex and leaf extracts had strong antimicrobial activity (0.312 mg/ml) for Bacillus cereus, while fruits had weakly effective MIC values (Significant=MIC <0.1 mg/ml). According to the results of this study, it can be said that Rhamnus cathartica L. plant extracts have an active content in terms of biological properties.*
Keywords: *Antimicrobial, antioxidant, antiproliferative, Rhamnus cathartica*

ÖZ

Amaç: *Bu araştırmada, Kazakistan'ın Almatı kentinden elde edilen Rhamnus cathartica L. bitkisinin*

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Submitted / Gönderilme : 23.12.2024
Accepted / Kabul : 02.05.2025
Published / Yayınlanma : 19.09.2025

korteks, yaprak ve meyvesinin hekzan, etil asetat ve etanol özütlerinin biyokimyasal bileşimi değerlendirilmiş ve antimikrobiyal, antioksidan ve antiproliferatif aktiviteleri analiz edilmiştir.

Gereç ve Yöntem: *Rhamnus cathartica L. korteks, yaprak ve meyve özütü kimyasallarını belirlemek için GC-MS yöntemi kullanıldı. Bitki ekstraktlarının patojen mikroorganizmalara karşı MİK değerlerini değerlendirmek için mikrodilüsyon yöntemi kullanılmıştır. Antioksidan aktivite tayini için DPPH, FRAP ve Toplam flavonoid içeriği belirlenmiştir. Ekstraktların MCF-7, A549, C6 ve WI-38 hücreleri üzerine antiproliferatif etkisi XTT testi ile ölçülmüştür.*

Sonuç ve Tartışma: *GC-MS sonuçlarına göre; Trans-2-Decenal %2.88 ile en yüksek orana sahiptir. Eicosane %1.71 ile ikinci sıradadır. DPPH, FRAP ve flavonoid değerleri meyve etil asetat ekstresinin (F-EtAc) en güçlü antioksidan aktiviteye sahip olduğunu göstermiştir. F-EtAc ekstresi, seçicilik indeksi tablosuna göre en güçlü antikanser aktivitesine sahiptir (WI-38/MCF-7: 12.67, WI-38/C6: 31.38, WI-38/A549: 8.55). Tüm bitki ekstraktları, referans kaynak değerlerine göre Staphylococcus aureus'a karşı anlamlı MİK değerlerine ulaşmıştır. Korteks ve yaprağın etil asetat içindeki MİK değeri 0.078 mg/ml, korteks ve yaprak ekstraktları Bacillus cereus için anlamlı antimikrobiyal aktiviteye (0.312 mg/ml) sahipken, meyveler zayıf etkili MİK değerlerine ulaşmıştır (Anlamlı=MİK <0.1 mg/ml). Bu çalışmanın sonuçlarna göre, Rhamnus cathartica L. bitki ekstraktlarının biyolojik özellikler açısından aktif bir içeriğe sahip olduğu söylenebilir.*

Anahtar Kelimeler: *Antimikrobiyal, antioksidan, antiproliferatif, Rhamnus cathartica*

INTRODUCTION

Rhamnaceae is a worldwide family with 50 genera and 900 species. Simple leaves, small flowers with four or five sepals that are valvate in bud, four or five stamens alternating with the sepals (obhaplostemony), anthers that are frequently enfolded by the hooded petal apices, ovaries that are usually two to three (four to five)-locular, an intrastaminal, nectariferous disc, and a tendency towards xeromorphism characterize them. On the inner side of the sepals, there is normally a fleshy layer that creates a keel and ends with a tubercle. This layer is histologically comparable to the nectariferous intrastaminal layer [1].

Genus *Rhamnus*; is one of the genera that is a member of the Rhamnaceae family, adapted to living in temperate and subtropical climates in the northern hemisphere [2].

Rhamnus cathartica is native to Europe, Northwest Africa, and Western Asia. It is a deciduous shrub or small tree; its seeds and leaves are considered toxic to humans and animals. It has been used as a folk remedy because of its purgative effect and good remedy against stomach cramps. It has also been used against various cancer diseases and as a diuretic in history [3].

In studies, antioxidant [4], antimicrobial [3,5], and anticancer [6,7] activities were determined in plant extracts of some *Rhamnus* species.

Numerous novel herbal medications have advanced to clinical trials, and medicinal plants are acknowledged as one of the possible sources for therapeutic development. To create novel antimicrobials from natural products, antibiofilm agents against different illnesses, antioxidant chemicals, and new medications against various diseases, including cancer, researchers are currently looking into the qualities of medicinal plants [8].

Due to the excessive use of antibiotics in human medicine, microbial antibiotic resistance is increasing. The problem of antibiotic resistance is not limited to just one continent, it is a global problem. Antimicrobial resistance is a hidden epidemic among us [9].

Infections caused by microorganisms with multi-drug resistance are a major problem, especially in intensive care units. In addition to being inexpensive to produce, using herbal medicines to treat human illnesses has several benefits, such as being easily accessible and biodegradable. Effective plant extracts can combat harmful germs in humans without causing harmful side effects or environmental risks [3].

The plant kingdom produces a wide variety of compounds with antioxidant properties that are thought to be able to protect against oxidative damage in the human organism. Multipotent antioxidants are molecules that have an additional pharmacological activity as well as antioxidant activity. Numerous examples of extremely potent antioxidants can be found in natural products. In addition to their antioxidant qualities, certain natural antioxidants have anti-inflammatory, anti-tumor, and platelet-

aggregation-inhibiting effects. However, the antibacterial action of many natural phenolic antioxidants is one of their most frequently mentioned characteristics. Because both effects are very desirable to keep food as fresh as possible, this is very significant to the food business [10,11].

Chemotherapeutic cytotoxic drugs are used to treat cancer. However, these drugs can cause serious side effects that continue to be a major dilemma for cancer patients [12]. In recent years, there has been an increasing interest in new medicinal plant-derived bioactive molecules for cancer drug discovery, due to their abundant and easy availability, their safety due to low toxicity, and fewer side effects [13].

Our study; It was aimed to determine the biochemical content of the extracts prepared with hexane, ethyl acetate, and ethanol of the bark, leaf, and fruit parts of the *Rhamnus cathartica* L. plant collected from the region of Kazakhstan and to examine the antioxidant, antimicrobial and antiproliferative effects.

MATERIAL AND METHOD

Plant Material

Rhamnus cathartica L. samples were collected from an altitude of 2000 m on the banks of the Talgar River in Ile Alatau, Almaty region, Republic of Kazakhstan.

Preparation of the Extract

Collected leaf, cortex and fruit samples were washed with tap water and distilled water, respectively, and dried under suitable conditions. After the dried samples were powdered in the grinder, 100 g of each sample was weighed. 250 ml of ethyl alcohol, hexane, and ethyl acetate solvents, each with a different polarity, were added to the weighed samples. The samples, which were shaken for 24 hours at 125 rpm at room temperature, were filtered through filter paper at the end of the period. Thus, plant particles are separated. The solvent was removed under vacuum with the help of an evaporator (Buchi R-100 equipped with Vacuum Pump V-300 and Control unit I-300) at a temperature not exceeding 37º C and 9 dry extracts obtained were kept at -20º C for use in analysis [14].

GC-MS Analysis

Agilent Technologies GC 7890A, equipped with a 5975 Triple Axis Detector mass spectrometer was employed to perform GC-MS analyses. DBWAXETR column (320 m x 60 m x 0.25 m), electron ionization system, and ionization energy of 70 eV were used for GC-MS detection. Helium was the carrier gas at a flow rate of 1 ml/min. The column temperate was operated under the same conditions as described above. After the oven temperature is kept at the initial temperature of 50 °C for 2 minutes, the temperature rise rate at all stages is 5 °C/minute, respectively, to 80 °C (waiting 2 minutes at this temperature), 100 °C (at this temperature 1 minute), 150 °C (holding 1 minute at this temperature), 240 °C (holding 1 minute at this temperature), and 270 °C (7 minutes standing at this temperature).

GC-MS analyses were performed by Giresun University Central Research Laboratory Application and Research Center (GRÜMLAB).

Antioxidant Assay

DPPH

2,2-Diphenyl-1-picrylhydrazyl radical scavenging activity of the resulting extract was performed as the modified method developed by Ou *et al.* [15]. Different concentrations of the extracts were prepared. Equal volumes (1000 µL) of DPPH and sample solutions were incubated with stirring for 30 min. After incubation, absorbance was measured by a spectrophotometer at 517 nm. The ascorbic acid as an antioxidant was used to compare the results. Samples and radical scavenging activity of the standard were calculated.

FRAP

The amount of FRAP antioxidant power in the analysis of the extracts of different solvents belonging to the plant *R. cathartica* L. according to the Benzie and Strain method, the amounts of the FRAP antioxidant power was calculated as the trolox equivalent. Trolox was used as a standard.

Total Flavonoid Content

Total flavonoid substance amount in the analysis of extracts of different solvents belonging to *Rhamnus cathartica* L. plant according to Chang et al method, total phenolic substance amounts were calculated as the equivalent of quercetin. Quercetin was used as a standard [16].

Antimicrobial Activity

The Broth Microdilution method was used to determine as the Minimum Inhibition Concentration (MIC) of *Rhamnus cathartica* L. plant cortex, leaf, and fruit extracts against microorganisms according to CLSI and EUCAST standards and Eloff's method [17-20]. In the study, *Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis* ATCC 29212, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922, *Bacillus cereus* ATCC 11778, *Candida albicans* ATCC 10233 and *Candida albicans* ATCC 10231 were used as test microorganisms.

The extracts were dissolved in 40% Dimethyl sulfoxide (DMSO), and concentrated solutions were prepared. Cation Adjusted Mueller Hinton Broth (CAMHB, Becton Dickenson BBL, Sparks, MD, USA) for bacteria; RPMI 1640 medium (Sigma-Aldrich R8758, Germany) was used for yeasts [18-20]. Plate wells in the 12th row were used as breeding controls. 10 µL of extract was added to the wells in the first row and serial dilutions were made. After two-fold serial dilution, bacteria and yeast were added to the wells and final concentrations of 5×10^5 cfu/ml for bacteria and $0.5\text{-}2.5\times10^3$ cfu/ml for yeast were reached in each well. Bacterial plates were incubated at 37°C and yeast plates were incubated at 25°C for 24-48 hours. Fluconazole was used as reference antifungal. After incubation, the lowest concentration at which visible growth was inhibited was accepted as the Minimum Inhibitory Concentration value (MIC) (µg/ml) [19-21]. Experiments were performed in triplicate.

Antiproliferative Activity

The antiproliferative effects of *Rhamnus cathartica* L. plant extracts were tested on Human lung cancer cell line (A549, ATCC-CCL-185), human breast cancer cell line (MCF-7, ATCC-HTB-22), mouse glioma cell line (C6, ATCC-CCL-107) and human normal lung fibroblast (WI-38, ATCC-CCL-75) cell lines using the XTT Assay method. After being seeded in growth media in a 96-well plate, the cells were treated with varying doses of test substances and incubated for 24 hours at 37°C in a humidified CO_2 atmosphere. After the incubation, each well received 100 µl of XTT solution, which was then incubated for an additional two hours. A microplate reader was used to measure the optical density values at 475 nm [22,23].

Statistical Analysis

One Way Anova test as well as the Tukey test were used for statistical analysis of the findings. For this purpose, the SPSS 16.0 (SPSS, Chicago, IL, USA) statistical program was used and a $p<0.05$ value at a 95% confidence interval was considered significant between groups.

RESULT AND DISCUSSION

Determining the Chemical Composition of Different Parts of *Rhamnus cathartica* L. Plant Extracts

The items obtained according to the results of the GC-MS analysis are shown in Table 1. According to this table, Trans-2-Decenal is the substance with the highest percentage with 2.88%. Eicosane is in the second place with a rate of 1.71%.

Table 1. The phytochemical composition of the fruit's ethyl acetate extract was analyzed by GC-MS.

Fruit's ethyl acetate	RT	Ethyl acetate (%)
Propanoic acid	3.280	1.63
3-Pentanone, 2-methyl-	3.732	0.58
Acetic acid,1-methylpropyl ester	3.830	1.06
Octane	4.448	1.49
Nonanal	13.202	0.94
Trans-2-Decenal	18.775	2.88
2-Tetradecene	22.644	0.63
1-Hexadecene	28.022	0.50
1-Heptadecene	28.022	0.50
1-Nonadecene	28.022	0.50
Pyridine-3-carbonitrile (3-cyanopyridine)	32.852	1.30
Hexadecanoic acid	45.932	0.67
9-Octadecenamide	50.281	1.54
Eicosane	56.003	1.71

Acetic acid, which is among the substances we detected as a result of GC-MS analysis (Table 1), has been used as an antiseptic agent in the disinfection of wounds for more than 6000 years in medicine [24]. Again, Pyridine-3-carbonitrile, which we determined as a result of this analysis, is a compound recently emphasized due to its extraordinary *in vitro* anticancer activity against a wide variety of cell lines. Recent studies suggest that pyridine derivatives show a range of pharmacological activities such as antibacterial, antitumor and analgesic activity [25].

It has been stated that 1-Nanodecene, which is among the substances in Table 1, shows antituberculosis, anticancer, antioxidant, and antimicrobial activity in studies [26].

DPPH Radical Sweeping Activity

According to Table 2, the fruit's ethyl acetate extract was found to have the best radical sweeping activity.

Ascorbic acid is one of the substances with the best-known antioxidant scavenging effect. Ascorbic acid was used as a positive control in the study. According to the DPPH result, the best antioxidant scavenging effect in the extracts of *R. cathartica* L. plant is observed to be 38.65±1.33 in fruit ethyl acetate when compared with ascorbic acid. While the second effect was observed in Cortex Ethyl acetate 43.87±1.74; the third effect was found to be Leaf Hexane 61.32±1.49.

Table 2. DPPH IC_{50} values

***Rhamnus cathartica* L.**	**IC_{50} (µg/ml)**
Leaf Ethyl Acetate	80.52 ± 1.23
Leaf Ethanol	135.6 ± 1.65
Leaf Hexane	61.32 ± 1.49
Fruit Ethyl Acetate	38.65 ± 1.33
Fruit Ethanol	105.4 ± 1.78
Fruit Hexane	77.35 ± 1.58
Cortex Ethyl Acetate	43.87 ± 1.74
CortexEthanol	82.74 ± 1.45
Cortex Hexane	88.96 ± 1.69
Ascorbic acid	11.07 ± 1.38

FRAP Antioxidant Power

The amount of FRAP antioxidant power in the analysis of the extracts of different solvents belonging to the plant *R. cathartica* L. according to the Benzie and Strain method, the amounts of the FRAP antioxidant power were calculated as the trolox equivalent. Trolox was used as standard (Figure 1).

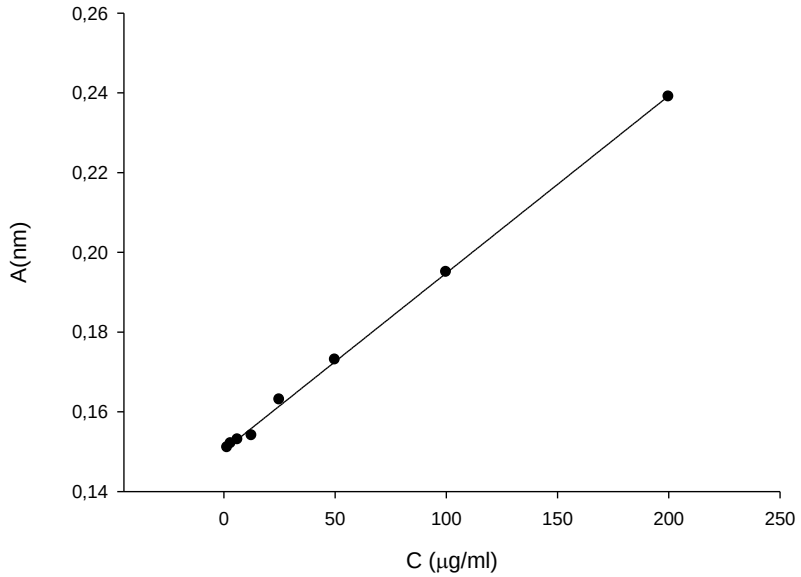


Figure 1. Trolox standard chart y = 0.1511 + 0.0005x (R^2 = 0.8772)

According to Table 3, leaf ethyl acetate, fruit ethyl acetate, and cortex ethyl acetate extracts were found to have stronger FRAP antioxidant activity than others.

In the study, fruit ethyl acetate is in first place with a value of 371.52 µg/ml, cortex ethyl acetate is in second place with a value of 349.81 µg/ml, and Leaf EA is in third place with a value of 341.58 µg/ml. Trolox was used as a positive control, and Fruit EA showed the best antioxidant results according to the FRAP results.

Table 3. FRAP Values

***Rhamnus cathartica* L.**	**(µg/g)**
Leaf Ethyl Acetate	341.58 ± 1.32
Leaf Ethanol	47.58 ± 1.12
Leaf Hexane	65.25 ± 1.18
Fruit Ethyl Acetate	371.52 ± 1.38
Fruit Ethanol	91.81 ± 1.26
Fruit Hexane	87.80 ± 1.22
Cortex Ethyl Acetate	349.81 ± 1.36
Cortex Ethanol	55.36 ± 1.08
Cortex Hexane	34.15 ± 1.04

Total Flavonoid Content

Total flavonoid substance amount in the analysis of extracts of different solvents belonging to *R. cathartica L.* plant, according to Chang et al method, total phenolic substance amounts were calculated as the equivalent of quercetin. Quercetin was used as a standard (Figure 2).

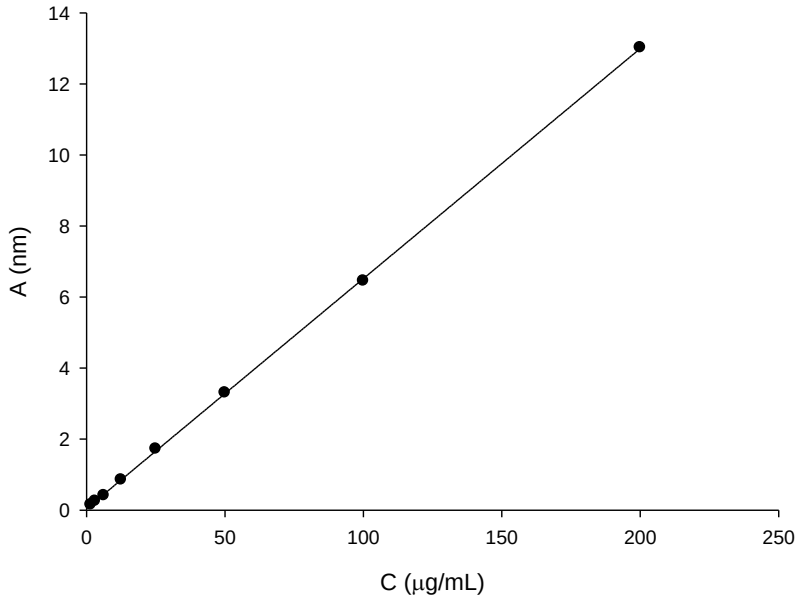


Figure 2. Quercetin standard graphic = 0.0433 + 0.0648x (R2 = 0.9999)

Table 4 shows the total flavonoid substance amount of *R. cathartica* L. ethanol, ethyl acetate, and hexane extracts.

Table 4. Flavonoid values

Rhamnus cathartica L.	**(µg/g)**
Leaf ethyl acetate	32.69 ± 0.98
Leaf ethanol	1.19 ± 0.08
Leaf hexane	0.98 ± 0.04
Fruit ethyl acetate	38.20 ± 0.93
Fruit ethanol	0.92 ± 0.03
Fruit hexane	0.87 ± 0.02
Cortex ethyl acetate	37.01 ± 0.93
Cortex ethanol	0.60 ± 0.01
Cortex hexane	0.43 ± 0.01

According to Table 4, the most flavored leaf extracts are ethyl acetate, fruit hexane, and cortex ethyl acetate extracts. The high flavonoid values indicate that they may have anticancer and antioxidant properties.

Based on the results of total flavonoid content, 38.20±0.93 flavonoid content of Fruit ethyl acetate was found to be the highest; secondly, Cortex ethyl acetate 37.01; third, Leaf ethyl acetate was found to be 32.69±0.98.

In many studies on the genus *Rhamnus*, phenolic compounds such as flavonoids, anthraquinones, and tannins, which are defined as antioxidant molecules, are high [27-30]. However, antioxidant activity studies with *Rhamnus cathartica* species are not sufficient. According to our study, it was determined that the fruit ethyl acetate extract of this species showed higher antioxidant activity than the other extracts (Table 2, Table 3 and Table 4).

Antimicrobial Activity

MIC results were evaluated according to reference sources (Highly Effective/Significant MIC

<0.1 mg/ml, Medium Effective/Moderate = 0.1 <MIC ≤ 0.625 mg/ml, Weak Effective/Weak = MIC> 0.625 µg/ml) [31].

Table 5. MIC results of *R. cathartica* L. cortex extract (mg/ml)

No	Microorganisms	Cortex (Ethyl acetate)	Cortex (Ethanol)	Cortex (Hexane)
1	*E. coli*	>5	>5	2.5
2	*P. aeruginosa*	>5	>5	>5
3	*S. aureus*	0.156	0.078	0.156
4	*B. cereus*	0.312	0.312	5
5	*C. albicans*	1.25	2.5	2.5
6	*C. tropicalis*	5	5	>5

Table 6. MIC results of *R. cathartica* L. leaf extract (mg/ml)

No	Microorganisms	Leaf (Ethyl acetate)	Leaf (Ethanol)	Leaf (Hexane)
1	*E. coli*	>5	>5	2.5
2	*P. aeruginosa*	>5	>5	>5
3	*S. aureus*	0.078	0.156	0.078
4	*B. cereus*	1.25	1.25	0.312
5	*C. albicans*	>5	5	2.5
6	*C. tropicalis*	5	2.5	2.5

Table 7. MIC results of *R. cathartica* L. fruit extract (mg/ml)

No	Microorganisms	Fruit (Ethyl acetate)	Fruit (Ethanol)	Fruit (Hexane)
1	*E. coli*	>5	2.5	>5
2	*P. aeruginosa*	>5	5	>5
3	*S. aureus*	0.156	0.312	0.312
4	*B. cereus*	>5	5	5
5	*C. albicans*	5	2.5	2.5
6	*C. tropicalis*	2.5	2.5	>5

MIC values were determined at different concentrations against microorganisms. The findings regarding the MIC values of the extracts obtained from the cortex, leaf and fruit parts of the plant *R. cathartica* L. can be seen in Table 5, Table 6 and Table 7.

The antimicrobial activity of plant extracts can be classified as (Highly Effective/Significant MIC <0.1 mg/ml, Medium Effective/Moderate = 0.1 <MIC ≤ 0.625 mg / ml, Weak Effective/Weak = MIC> 0.625 µg/ml) [31]. According to this classification, the cortex, leaf and fruit extracts of *R. cathartica* L. had MIC values of 2.5 mg/ml and 5 mg/ml for *E. coli*, *P. aeruginosa*, *C. albicans* and *C. tropicalis*. When the MIC values of the *R. cathartica* L. extracts were compared with the reference antifungal Flucanazole (MIC=0.25 µg/ml), none of them reached effective values.

It was shown to be effective against *S. aureus* even at low concentrations in different solvents and all extracts of the plant (0.078-0.156 mg/ml). The MIC value for *S. aureus* was found to be effective with a value of 0.078 mg/ml, especially in cortex and leaf ethyl acetate solvents. For *B. cereus*, cortex and leaf extracts had antimicrobial activity (0.312 mg/ml), whereas fruit showed weak MIC values (Table 5, Table 6 and Table 7) [31].

Ibrahim et al. found that *R. cathartica* L. extract had high antimicrobial activity against gram-positive bacteria *B. cereus* and *E. coli* among gram-negative strains [32]. Antimicrobial activity evaluation was performed by the disk diffusion method. It is compatible with our results.

In a study conducted by Nayyeri et al. on acne treatment, it was found that *R. cathartica* L. bark extract had antimicrobial activity against *Cutibacterium acnes, Staphylococcus epidermidis,*

and *Staphylococcus aureus* [5]. The MIC value against *S. aureus* was found to be 97.65 µg/ml. It is compatible with our results.

In another study, the antimicrobial effect of some components of *R. cathartica* L. was investigated. Three components had antibacterial activity against *E. coli* and *S. aureus* bacteria, and two components had antifungal activity against *C. albicans* [3]. Antimicrobial activity evaluation was performed by the disk diffusion method. Although our study is compatible with bacteria, it is not compatible with yeasts.

Locatelli et al. investigated the antimicrobial effects of *Rhamnus cathartica* and *Rhamnus orbiculatus* plant extracts. Both methanol extracts showed antimicrobial activity against all microbial species tested (*Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, Candida albicans, Aspergillus niger, Microsporum gypseum*) with MIC values either equal to or lower than 2.50 mg/ml [33]. Compared to our study, different MIC values were obtained on test bacteria and yeasts.

In a study performed by Kosalec et al. on 4 different *Rhamnus* species extracts, the results of antimicrobial activity can be listed as follows: *E. coli, P. aeruginosa, S. aureus* bacteria have MIC values (1.25-2.5 mg/ml), while *C. albicans* have MIC values (0.625 mg/ml) [10]. Compared to our study, although similar values were obtained on test bacteria and yeasts, mostly different MIC values were determined.

Antiproliferative Activity

In vitro cytotoxicity of *R. cathartica* L. plant extracts was evaluated in MCF-7, A549 and C6 cancer cell lines and WI-38 fibroblast cell line by XTT method. The percentage of cell viability and cell inhibition was determined by dividing the number of unstained cells by the total number of cells, and multiplying the ratio by 100.

IC_{50} values were generated from the dose-response curve and optical density. The dose-response results of the extracts are shown in Table 8.

Table 8. IC_{50} values

Rhamnus cathartica L.	***IC_{50} (µg/ml)***			
	MCF-7	**C6**	**A549**	**WI-38**
Leaf ethyl acetate	39.19 ± 0.32 a	3.55 ± 0.32 a	10.38 ± 0.18 a	44.74 ± 1.63 a
Leaf ethanol	42.85 ± 0.63 b	4.7 ± 0.18 b	13.35 ± 0.34 b	21.44 ± 1.32 b
Leaf hexane	4.79 ± 0.54 c	3.1 ± 0.21 a	10.28 ± 0.45 a	40.65 ± 1.45 c
Fruit ethyl acetate	10.28 ± 0.12 d	4.15 ± 0.10 b	15.24 ± 0.22 c	130.23 ± 1.93 d
Fruit ethanol	23.79 ± 0.28 e	9.04 ± 0.17 c	14.02 ± 0.62 d	15.24 ± 1.12 e
Fruit hexane	9.04 ± 0.16 f	6.62 ± 0.12 d	12.17 ± 0.24 e	41.67 ± 1.78 f
Cortex ethyl acetate	18.89 ± 0.41 g	8.45 ± 0.24 e	6.62 ± 0.14 f	18.89 ± 1.33 g
Cortex ethanol	13.35 ± 0.25 h	17.06 ± 0.13 f	17.06 ± 0.32 g	44.09 ± 1.65 a
Cortex hexane	25.68 ± 0.19 i	13.35 ± 0.27 g	13.35 ± 0.48 b	21.97 ± 1.48 b

Significant $p \leq 0.05$ level of analysis of variance (One Way ANOVA Tukey)

The difference between the IC_{50} values of the leaf, fruit and bark samples on the MCF-7 cell line in all solvents was found to be significant. In C6 and A549 cell lines, when the IC_{50} values are evaluated, the differences between the values obtained from all other samples and all solvents are statistically significant, except for the leaf ethylacetate and leaf hexane values.

In the WI-38 cell line, while the difference between leaf ethyl acetate and cortex ethanol was not significant, the difference between all other samples and solvents was statistically significant.

According to the SI table, F-EtAc extract was found to have the highest anticancer activity in cancer cell groups with WI-38/MCF-7= 12.67, WI-38/C6= 31.38, WI-38/A549= 8.55 values. L-EtAc extract with WI-38/C6= 12.60, WI-38/A549= 4.31 values and F-Hex extract with WI-38/MCF-7= 4.61 values take the second place (Table 9).

Table 9. SI values

Rhamnus cathartica L.	*Selectivity index (SI)*		
	WI-38 / MCF-7	**WI-38 / C6**	**WI-38 / A549**
Leaf ethyl acetate	1.14	12.60	4.31
Leaf ethanol	0.50	4.56	1.61
Leaf hexane	8.49	13.11	3.95
Fruit ethyl acetate	12.67	31.38	8.55
Fruit ethanol	0.64	1.69	1.09
Fruit hexane	4.61	6.29	3.42
Cortex ethyl acetate	1	2.24	2.86
Cortex ethanol	3.30	2.58	2.58
Cortex hexane	0.86	1.65	1.65

A significant difference was found between the IC_{50} values of the *R.cathartica* L. plant leaf, fruit and bark samples on the MCF-7 cell line in all solvents. In C6 and A549 cell lines, when the IC_{50} values are evaluated, the differences between the values obtained from all other samples and all solvents are statistically significant, except for the leaf ethyl acetate and leaf hexane values.

In the WI-38 cell line, while the difference between leaf ethyl acetate and bark ethanol was not significant, the difference between all other samples and solvents was statistically significant.

Shokoohian et al., in their study on the MCF-7 cell line, observed that different doses of *Rhamnus frangula* extracts (0.01 mg/ml, 0.1mg/ml, 1 mg/ml and 10 mg/ml) decreased cell viability as the dose increased [34].

A different study determined anticancer activity for the methanolic extract of *Rhamnus aternus* bark on U937 cells. The extract's IC_{50} value was discovered to be less than the NCI-recommended threshold for classification as an anticancer agent. In preliminary analyses, the US National Cancer Institute (NCI) established the following cytotoxicity criteria for the crude extract: $10 \leq IC_{50} < 20$ µg/ml of total/crude extract was considered cytotoxic, $20 \leq IC_{50} < 50$ µg/ml was considered moderately cytotoxic, and < 10 µg/ml was considered strongly cytotoxic. Furthermore, it was discovered that both conventional and aqueous extracts were only mildly harmful to healthy PBMCs; the methanolic extract exhibited cytotoxic activity exclusively on cancer cells (U937) [35].

Investigated the antiproliferative activity of components of *Rhamnus dysperma* roots on MCF-7 (human breast adenocarcinoma), A2780 (human ovarian adenocarcinoma), HT29 (human colon adenocarcinoma), and MRC5 (normal human fetal lung fibroblast) cells. The penduletine component exhibited the highest cytotoxic activity against the three cancer cell lines. MCF-7, A2780, and HT29 have IC_{50} values between 0.53-2.17 µM. It was also found to be 2-9 times more selective against the tested cancer cell lines than normal fibroblast cells (MRC5) [7].

The methanol extract of *R. cathartica bark* from Iran affected the Caucasian colon adenocarcinoma (HT-29) and human Caucasian gastric adenocarcinoma (AGS) cell lines. Compared to the isolated components and the whole extract, the methanol extract demonstrated a noticeably decreased cytotoxic impact at doses of 100, 200, and 400 mg/ml after 48 hours [36].

The antiproliferative effects of *R. cathartica* L. extracts in C6, A549 and MCF-7 cancer lines have not been investigated before. The extracts have antiproliferative effects in the studies discussing the genus *Rhamnus.*

Conclusion

In recent years, the rapid development of drug resistance of many infectious agents, the high side effects of synthetic chemotherapeutics, and their metabolically highly toxic and expensive nature have led researchers to search for new herbal drug active ingredients. Nowadays, drugs obtained from plants are preferred over synthetic chemotherapeutics due to their easy accessibility and broad spectrum of action. In addition, it should not be forgotten that herbal drugs can cause many harms due to their unconscious and unlimited use. In this case, a lot of work should be done by researchers about the concentration and effect spectrum of herbal drugs, and public awareness should be raised.

In our study, the biochemical content and antioxidant, antimicrobial, and antiproliferative effects of the extracts prepared with hexane, ethyl acetate, and ethanol of the cortex, leaf, and fruit parts of the *R. cathartica* L. collected from the Kazakhstan region were investigated. As a result of GC-MS analysis, trans-2-Decenal is the substance with the highest percentage, with 2.88%. According to DPPH IC_{50}, FRAP, and flavonoid values, the highest antioxidant activity was determined in the fruit ethyl acetate extract. Fruit ethyl acetate extract was found to have the highest anticancer activity in cancer cell groups, with the values in the Selectivity Index table prepared according to the IC_{50} values of WI-38/MCF-7: 12.67, WI-38/C6: 31.38, and WI-38/A549: 8.55. All plant extracts have reached high effective MIC values for *S. aureus*. Especially in cortex and leaf ethyl acetate solvents, the MIC value was found to be 0.078 mg/ml. for *B. cereus*, cortex and leaf extracts were found to have high antimicrobial efficacy (0.312 mg/ml), while the fruit reached weak effective MIC values.

The results of this study revealed that the plant extracts obtained from *Rhamnus cathartica L.* established high biological effects. Therefore, more studies are needed in this area.

ACKNOWLEDGMENTS

We would like to thank Dr. Ceylan HEPOKUR, who helped us with the antioxidant assay of our research.

AUTHOR OF CONTRIBUTIONS

Concept: A.S.T., T.T.; Design: Y.Z., T.T.; Control: A.S.T., Y.Z., T.T., H.Ö.; Sources: A.S.T., T.T.; Materials: T.T., H.Ö.; Data Collection and/or Processing: A.S.T., T.T., H.Ö.; Analysis and/or Interpretation: Y.Z., H.Ö.; Literature Review: T.T., H.Ö.; Manuscript Writing: A.S.T., Y.Z., T.T., H.Ö.; Critical Review: A.S.T., Y.Z., T.T., H.Ö.; Other: -

CONFLICT OF INTEREST

The authors declare that there are no real, potential, or perceived conflicts of interest for this article.

ETHICS COMMITTEE APPROVAL

The authors declare that ethics committee approval is not required for this study.

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J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 49(3): 756-765, 2025
Doi: 10.33483/jfpau.1646794

ORIGINAL ARTICLE / ÖZGÜN MAKALE

INVESTIGATION OF ANTIMICROBIAL, ANTIBIOFILM AND ANTI-QUORUM SENSING ACTIVITIES OF EXTRACTS FROM *SCUTELLARIA YILDIRIMLII* M. ÇİÇEK & A.E. YAPRAK

SCUTELLARİA YILDIRIMLİİ M. ÇİÇEK & A.E. YAPRAK EKSTRELERİNİN ANTİMİKROBİYAL, ANTİBİYOFİLM VE ÇOĞUNLUĞU ALGILAMA İNHİBİSYONU AKTİVİTELERİNİN ARAŞTIRILMASI

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ABSTRACT

Objective: *This study aims to investigate the antimicrobial, antibiofilm, and anti-quorum sensing (anti-QS) properties of extracts derived from the aerial parts of Scutellaria yildirimlii* M. Çiçek & A.E. Yaprak.

Material and Method: *Using a liquid-liquid extraction method, petroleum ether (PEE), ethyl acetate (EAE), and aqueous (AE) sub-extracts were obtained from the total extract (TE) prepared with 80% methanol. Broth microdilution method assessed antimicrobial activity, microplate-based biofilm model and crystal violet assay assessed antibiofilm activity, and disk diffusion method assessed anti-quorum sensing activity*

Result and Discussion: *The extracts exhibited MIC values ranging from 625 to 10000 µg/ml against the tested bacteria. Among them, the TE and PEE extracts showed the strongest antibacterial activity against both Gram-positive and Gram-negative test bacteria. Notably, only the PEE extract demonstrated antifungal activity against Candida albicans ATCC 10231, with an MIC value of 625 µg/ml, while no antifungal activity was observed for the other extracts. However, the antimicrobial effects were generally weak compared to standard antimicrobials. At a concentration of 2500 µg/ml, the biofilm inhibition percentages for AE, TE, PEE, and EAE extracts were 76.79%, 57.47%, 54.81%, and 51.09%, respectively. An anti-QS activity test, conducted based on MIC values against Chromobacterium violaceum ATCC 12472, revealed no violacein inhibition zones, indicating a lack of anti-QS activity. The test results demonstrated that S. yildirimlii possesses greater antimicrobial properties than many other Scutellaria species. This indicates that its extracts could serve as promising candidates for developing treatments against the increasing threat of antibiotic-resistant*

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Submitted / Gönderilme : 05.03.2025
Accepted / Kabul : 04.05.2025
Published / Yayınlanma : 19.09.2025

bacteria. Nonetheless, additional research is essential to thoroughly investigate and confirm the plant's potential as a medicinal resource.
Keywords: *Antibiofilm activity, antimicrobial activity, anti-quorum sensing activity, extraction, Scutellaria yildirimlii*

ÖZ

Amaç: *Bu çalışma kapsamında, Scutellaria yıldırımlii bitkisinin toprak üstü kısımlarından elde edilen ekstrelerin antimikrobiyal, antibiyofilm, ve çoğunluğu algılama mekanizması aktivite potansiyelinin araştırılması amaçlanmıştır.*
Gereç ve Yöntem: *Sıvı- sıvı ekraksiyon metodu ile, %80 metanollü total ektsreden, petrol eteri (PEE), etil asetat (EAE), ve sulu (AE) alt ekstreler elde edilmiştir. Antimikrobiyal aktivite testi, broth mikrodilüsyon metodu ile; antibiyofilm aktivite testi, mikroplaka-bazlı biyofilm modeli ve kristal viyole deneyi ile; çoğunluğu algılama mekanizması aktivite testi, disk difüzyon metodu ile yapılmıştır.*
Sonuç ve Tartışma: *Ekstreler, test edilen bakterilere karşı 625 ile 10.000 µg/ml arasında değişen MIC değerleri sergilemiştir. Bunlar arasında, TE ve PEE ekstreleri, hem Gram-pozitif hem de Gram-negatif test bakterilerine karşı en güçlü antibakteriyel aktiviteyi göstermiştir. Özellikle, PEE ekstreleri yalnızca Candida albicans ATCC 10231'e karşı antifungal aktivite göstermiş olup, MIC değeri 625 µg/ml olarak belirlenmiştir. Diğer ekstrelerde ise herhangi bir antifungal aktivite gözlemlenmemiştir. Ancak, genel olarak, antimikrobiyal etkiler standart antimikrobiyallerle kıyaslandığında zayıf kalmıştır. 2500 µg/ml konsantrasyonda, AE, TE, PEE ve EAE ekstrelerinin biyofilm inhibisyon yüzdeleri sırasıyla %76.79, %57.47, %54.81 ve %51.09 olarak ölçülmüştür. Chromobacterium violaceum ATCC 12472'ye karşı MIC değerlerine dayalı olarak gerçekleştirilen çoğunluğu algılama mekanizması aktivite testi, herhangi bir violasein inhibisyon bölgesi göstermemiştir; bu da çoğunluğu algılama mekanizması aktivitesinin bulunmadığını ortaya koymuştur. Test sonuçları, S. yıldırımlii'nin, birçok Scutellaria türüne kıyasla daha güçlü antimikrobiyal özelliklere sahip olduğunu göstermiştir. Bu durum, bitki ekstrelerinin antibiyotiklere dirençli bakterilere karşı tedavi geliştirme açısından umut verici adaylar olabileceğini düşündürmektedir. Bununla birlikte, bitkinin tıbbi potansiyelinin tam olarak anlaşılması ve doğrulanması için daha fazla araştırma yapılması gerekmektedir.*
Anahtar Kelimeler: *Antibiyofilm aktivite, antimikrobiyal aktivite, çoğunluğu algılama mekanizması aktivitesi, ekstraksiyon, Scutellaria yıldırımlii*

INTRODUCTION

The World Health Organization (WHO) recognizes medicinal plants as a fundamental component of primary healthcare resource for 80% of the global population. Herbal formulations are widely used to address various conditions, such as diabetes, arthritis, liver disorders, and coughs, as well as to enhance memory and function as adaptogens [1]. Since the 1990s, the demand for natural products and innovative applications of medicinal and aromatic plants has grown significantly, driving their increased usage and availability [2].

Scutellaria L. genus (Lamiaceae) is represented by nearly 360-469 species and is known as "skullcap" in the World [3]. Various *Scutellaria* species have been used to treat many diseases, including hepatic and gastric disorders, respiratory, cardiovascular and neurological diseases, cancer, and infectious diseases. The dried root of *Scutellaria* is among the most widely used and versatile medicinal herbs in China and several other Eastern countries. In Turkey, *Scutellaria* has also been utilized, particularly in Anatolian folk medicine, where it is known for its styptic properties, wound-healing effects, and as a general tonic to promote strength. The plant is rich in phytochemicals, including flavonoids, iridoids, neo-clerodanes, and essential oil. Their pharmacological effects, such as anticancer, antioxidative, hepatoprotective, antivirus, anti-inflammatory, and neuroprotective activities have been reported [3,4].

Scutellaria yildirimlii was first identified in the Ayaş region of Türkiye by Mehmet Çiçek and Ahmet Emre Yaprak in 2013. Although it bears botanical similarities to *Scutellaria pectinata* Montbret & Aucher ex Bentham, it differs notably in its dense, woolly hairs. Apart from a few botanical studies [5,6], there has been little to no research on its phytochemical properties or biological activities. In

Turkey, *Scutellaria yildirimlii* taxa has been reported from six different localities in the provinces of Ankara and Eskişehir [5].

The antimicrobial activity of *Scutellaria* species has generally been examined through studies on its essential oil. Studies have shown that essential oil from various *Scutellaria* species, including *S. strigillosa* Hemsley [7], *S. albida* L.subsp. *albida* [8], *S. barbata* D. Don [9], and *S. araxensis* Grosh [10] have been proven to have vigorous antimicrobial activity.

Research has demonstrated the antimicrobial potential of extracts from various *Scutellaria* species. For example, a comparative study on the antimicrobial activities of *Artemisia apiacea* and *S. baicalensis* revealed efficacy against pathogenic fungi and Gram-positive bacteria. Notably, combining the two plants in a 3:5 ratio resulted in enhanced antimicrobial effects [11]. Similarly, an investigation of three *Scutellaria* species native to Türkiye (*S. salvifolia* Benth, *S. diffusa* Benth, and *S. pontica* Karl Koch) reported robust antimicrobial activity [12].

Antibiotic resistance poses a significant global health challenge, leading to infections associated with heightened morbidity and mortality rates. This crisis underscores the urgent need for the development of novel antibacterial therapies. The persistent emergence of resistance to existing antibiotics and the inherent difficulties in discovering new compounds have necessitated the exploration of alternative therapeutic strategies [13,14].

Among these alternatives, quorum sensing (QS) has emerged as a promising target for combating pathogenicity. Quorum sensing plays a pivotal role in regulating virulence factor production and biofilm formation in bacterial pathogens. Recent research has increasingly focused on antibiofilm and anti-QS molecules as potential substitutes for traditional antibiotics. Compounds targeting QS and biofilm formation, particularly those derived from natural sources, are recognized as promising candidates for addressing antibiotic resistance while mitigating the limitations of current antibacterial treatments [15-17].

This study aimed to evaluate the antibacterial, antibiofilm, and anti-quorum sensing properties of extracts obtained from *S. yildirimlii*. The promising results from studies on various *Scutellaria* species, combined with the recent discovery of *Scutellaria yildirimlii* and the lack of research beyond its botanical characterization, have made this species the focus of the present study. This work represents the first investigation into the biological effects of this plant.

MATERIAL AND METHOD

Plant Material

The aerial parts of *S. yildirimlii* M. Çiçek & A.E. Yaprak were gathered on August 7, 2022, from Aşağıkepen village in the Sivrihisar district of Eskişehir province at an altitude of 979 meters. Prof. Dr. Hayri Duman identified the collected plant material, and a voucher specimen (AEF 30989) has been deposited in the Herbarium of the Faculty of Pharmacy at Ankara University.

Preparation of Plant Extract

When the literature is reviewed, it is seen that solvents such as methanol and ethanol, as well as their aqueous mixtures, are frequently used for the extraction of phenolic compounds from *Scutellaria* species [4,9,10]. Based on this, the dried aerial parts of *S. yildirimlii* (200.81 g) were ground and extracted with 80% methanol over a period of 12 days, with each extraction lasting 24 hours. The extract obtained was subsequently filtered and evaporated under vacuum at 40°C, resulting in 27.0977 g of crude extract.

Crude extract (20.0151 g) (TE) was dissolved with 250 ml of distilled water and transferred to a separate funnel. Based on the aqueous methanolic extract, liquid-liquid extraction was performed to obtain sub-extracts with different polarities in order to interpret the active compounds effectively [4,10]. Using petroleum ether and ethyl acetate, liquid-liquid extraction was carried out, as a result, three sub-extracts produces with various polarities: 2.90501 g of petroleum ether extract (PEE), 2.92418 g of ethyl acetate extract (EAE), and 13.0606 g of aqueous extract (AE).

Antimicrobial Activity Assay

The antibacterial and antifungal activities of the extracts were evaluated by using their MIC values with the broth microdilution method [18,19]. For the antibacterial assays, the tested microorganisms included *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 13883, *Pseudomonas aeruginosa* ATCC 27853, and methicillin-resistant *S. aureus* (MRSA) ATCC 43300. The antifungal activity was evaluated using *Candida albicans* ATCC 10231. The plant extracts were prepared in 5% dimethyl sulfoxide (DMSO), and all experiments were performed in triplicate to ensure reliability.

In the antibacterial activity assays, plant extracts were prepared through two-fold serial dilutions in Mueller-Hinton broth, with final concentrations ranging from 10.000 to 78.125 µg/ml. Bacterial inoculums were obtained from 24-hour subcultures and adjusted to a final concentration of 5×10^5 CFU/ml. After the incubation period at 35°C for 18–24 hours, the MIC was determined as the lowest extract concentration that inhibited visible bacterial growth. Negative controls included wells containing only inoculated broth with 5% DMSO, while ampicillin, ciprofloxacin, and gentamicin served as positive controls.

For the antifungal activity assay, plant extracts were prepared in RPMI 1640 broth (ICN-Flow, Aurora, OH, USA; supplemented with glutamine, lacking bicarbonate, and including a pH indicator) using serial two-fold dilutions ranging from 10.000 to 78.125 µg/ml. The fungal inoculum was standardized to a final concentration of $0.5–2.5 \times 10^3$ CFU/ml. Microplates were incubated at 35°C for 48 hours, and the MIC was determined as the lowest concentration that completely inhibited visible fungal growth. Negative controls included wells containing 5% DMSO and RPMI 1640 broth, while fluconazole served as the standard antifungal agent.

Antibiofilm Activity Assay

Before analyzing antibiofilm effects, the MIC of the plant extracts against *Pseudomonas aeruginosa* PAO1 was evaluated. For the antibiofilm evaluation, a sub-MIC concentration of 2500 µg/ml was selected. The testing was conducted using a microplate-based biofilm model and the crystal violet assay [15,20-22].

To biofilm formation, *P. aeruginosa* PAO1 was cultured in Brain Heart Infusion (BHI) Broth for 24 hours at 37°C. Final inoculum suspensions, standardized to approximately 10^6 CFU/ml, were prepared in BHI supplemented with 2% sucrose. A volume of 100 µl of the inoculums was transferred into 96-well microtiter plates in all experimental setups, with incubation at 37°C for 72 hours to achieve fully developed biofilms.

Mature biofilms were first established by removing the medium and washing away non-adherent cells with sterile PBS (pH 7.2). Subsequently, 100 µl of the plant extracts were dispensed into the wells, and the plates were incubated at 37 °C for 24 hours. After incubation, the wells were rinsed with PBS and left to air-dry at room temperature for one hour. To stain the biofilm cells, 100 µl of 0.5% crystal violet solution was added to each well and allowed to incubate for 30 minutes. The wells were then washed three times with PBS, and acetone-alcohol (30:70 v/v) was used to dissolve the bound dye within the biofilm matrix.

BHI broth supplemented with 2% sucrose was served as the control, and the optical density (OD) of the dissolved crystal violet dye was measured at 620 nm using the Microplate Spectrophotometer (Thermo Scientific Multiskan GO/Vantaa, Finland). The percentage of biofilm inhibition was evaluated using the following equation:

$$\% \text{ Biofilm inhibition} = [(OD (\text{Growth control}) - OD (\text{Sample}))/OD (\text{Growth control})] \times 100$$

Anti-Quorum Sensing Activity Assay

Prior to evaluating the anti-quorum sensing (QS) activity, the MIC of the extracts against Chromobacterium violaceum ATCC 12472 was determined. The anti-QS activity was then evaluated using the disc diffusion method, with C. violaceum ATCC 12472 as the reporter bacterium [23,24].

For the assay, the bacterial culture was standardized to a density of 1.5×10^8 CFU/ml. The bacterial suspension was evenly spread onto Luria Bertani (LB) agar plates. Sterile blank discs (6 mm

in diameter; Bioanalyse®, Ankara, Türkiye) were impregnated with 20 µl of the plant extracts and placed on the agar surface. The plates were incubated at 30°C for 24 hours. After incubation, the agar plates were examined for violacein inhibition zones around the discs. The presence of such zones indicated that the plant extracts exhibited anti-QS activity.

RESULT AND DISCUSSION

Antimicrobial Activity Assay

The MIC (minimum inhibitory concentration) values (µg/ml) of *S. yildirimlii* extracts are presented in Table 1. The MIC values against the tested strains varied between 625 and 10000 µg/ml. Among the extracts, the total extract (TE) and petroleum ether (PEE) extracts exhibited the strongest antibacterial activity, effectively inhibiting both Gram-negative and Gram-positive test bacteria. Notably, the PEE extract was the only sample to demonstrate antifungal activity, with a MIC value of 625 µg/ml against *Candida albicans* ATCC 10231, while no antifungal effects were observed for the other extracts. The antimicrobial activities of the extracts were relatively low in comparison to standard antimicrobial agents (Table 1).

Table 1. MIC values (µg/ml) of *S. yildirimlii* extracts

Plant extract	Gram-positive test bacteria		Gram-negative test bacteria			Fungus
	S. aureus ATCC 25923 (MSSA)	*S. aureus* ATCC 43300 (MRSA)	*E. coli* ATCC 25922	*K. pneumoniae* ATCC 13383	*P. aeruginosa* ATCC 27853	*C. albicans* ATCC 10231
Total extract (TE)	625	625	2500	625	5000	-
Petroleum ether extract (PEE)	625	625	625	625	2500	625
Ethyl acetate extract (EAE)	2500	2500	10000	10000	10000	-
Aqueous extract (AE)	1250	1250	10000	1250	10000	-
Ampicillin	1.6	NT	NT	NT	NT	NT
Ciprofloxacin	NT	0.5	<0.25	62.5	<0.25	NT
Gentamicin	NT	<0.25	0.5	NT	<0.25	NT
Fluconazole	NT	NT	NT	NT	NT	1.56
DMSO (%5)	-	-	-	-	-	-

NT: not tested, (-): no activity

Antibiofilm Activity Assay

The biofilm inhibition percentages of *S. yildirimlii* extracts at a concentration of 2500 µg/ml are illustrated in Figure 1. Among the extracts, the aqueous extract (AE) demonstrated the highest antibiofilm activity, achieving a biofilm inhibition rate of 76.79% (Figure 1).

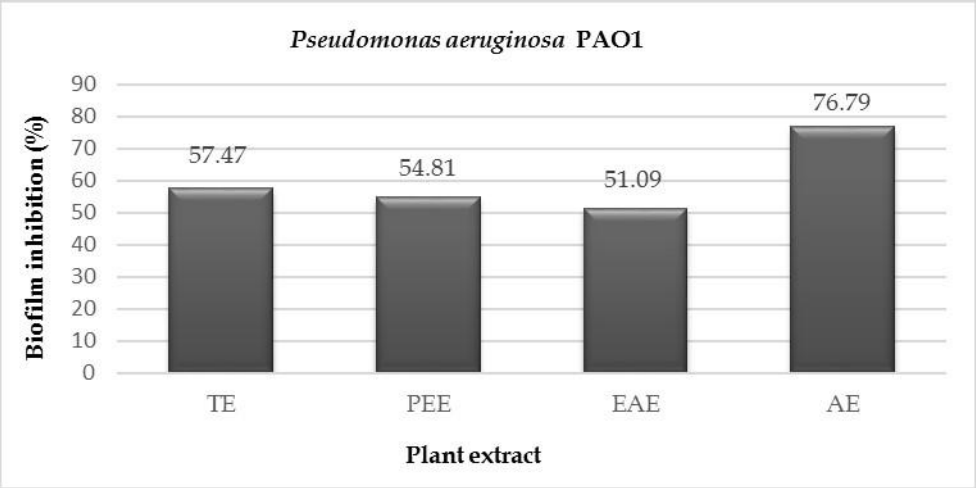


Figure 1. Biofilm inhibition (%) of the *S. yildirimlii* extracts

Anti-Quorum Sensing Activity Assay

The anti-quorum sensing (anti-QS) activity of *S. yildirimlii* extracts was evaluated based on their MIC values against *C. violaceum* ATCC 12472. None of the tested extracts demonstrated violacein inhibition, as no inhibition zones were observed (Figure 2).

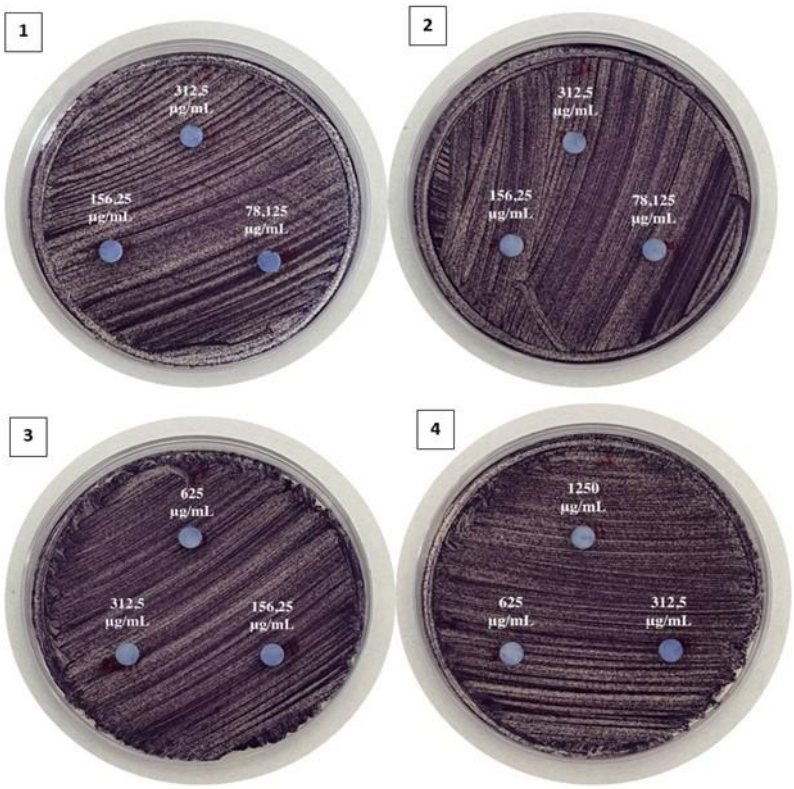


Figure 2. Anti-QS Activity of *S. yildirimlii* extracts (1: TE, 2: PEE, 3: EAE, 4: AE)

Due to the growing threat posed by antibiotic-resistant bacteria to public health, global research efforts on antibiotic resistance have significantly intensified. Studies indicate that bacteria can develop resistance through intrinsic mechanisms or by acquiring resistance to specific antibiotics. The irrational

use of antibiotics exacerbates this problem by preventing antibiotics from reaching their targets, modifying their targets, or inactivating them. These factors contribute to the development and spread of antibiotic resistance [25].

Over the years, various *Scutellaria* species have been reported to exhibit a wide range of pharmacological effects, including anticancer, antibacterial, antimicrobial, anti-inflammatory, antioxidant, antiviral, hepatoprotective, and neuroprotective activities [4]. The selected species within the scope of the study, *S. yildirimlii*, was first described by Mehmet Çiçek and Ahmet Emre Yaprak in Ayaş in 2013 [26]. In a literature study that we conducted, no biological activity and phytochemical studies were found except botanical studies [5,6]. For this reason, the study focuses on evaluating the antimicrobial, antibiofilm, and anti-quorum sensing effects of *S. yildirimlii*.

The antimicrobial activity of *Scutellaria* taxa has generally been studied on their essential oils, including *S. strigillosa* [7], *S. albida* [8], *S. barbata* [9] and *S. araxensis* [10] species, and it has been shown to have strong antimicrobial activity. Although there are few antimicrobial activity studies on extracts obtained from *Scutellaria* species, there are studies on many species, including *S. lateriflora*, *S. baicalensis*, *S. salviifolia*, *S. diffusa*, *S. pontica* and *S. lindbergii*.

S. baicalensis is one of the most studied *Scutellaria* species. A study on this plant revealed that a 70% ethanol extract completely inhibited the growth of *Staphylococcus aureus* at a 10% concentration, *Bacillus subtilis* at a 50% concentration, and *Pseudomonas syringae* at a 50% concentration [27].

In another study conducted with *S. baicalensis*, extracts were prepared using 60, 80 and 100% ethanol and their antimicrobial activity was tested. The results obtained showed a MIC value of 12.5 mg/ml against *S. aureus*, 25 mg/ml against *L. monocytogenes*, and 25 mg/ml against *S. enterica*. Table 1 shows that the least effective extract tested, ethyl acetate, had the same MIC value as the 80% ethanolic *S. baicalensis* extract. Additionally, the most effective extract, petroleum ether, demonstrated significantly stronger activity, with a MIC value of 625 µg/ml [28].

Similarly, the antimicrobial activity test performed on the 70% ethanolic extract prepared from *S. baicalensis* showed a MIC value of 4 mg/ml against *C. albicans*. This value is six times higher than the results of petroleum ether extract obtained from *S. yildirimlii* with MIC value of 625 µg/ml. In contrast, the same experiment revealed that the 70% *S. baicalensis* extract had a MIC value of 0.03125 mg/ml against *S. aureus*, which was considerably lower than the results found in our study [11].

In a study comparable to the present research, 36 extracts were derived from three *Scutellaria* species native to Türkiye (*S. salviifolia*, *S. diffusa*, and *S. pontica*) using various solvents. The antimicrobial activities of these extracts were evaluated against four bacterial strains (*E. faecalis*, *P. aeruginosa*, *E. coli*, and *S. aureus*) and three fungal species (*C. krusei*, *C. parapsilosis* and *C. albicans*). The results indicated that all tested extracts exhibited strong antimicrobial activity against both fungi and bacteria. Among them, the chloroform extract of *S. salviifolia* demonstrated the highest antifungal activity against *C. krusei* (MIC: 32 µg/ml), compared to fluconazole. For antibacterial activity, the chloroform extract from the roots of *S. salviifolia* showed the strongest effect (MIC: 128 µg/ml) against *E. faecalis*, *P. aeruginosa*, and *E. coli*. When compared to the findings of the current study, *S. salviifolia* extracts appear to exhibit higher activity against *S. aureus*, *P. aeruginosa*, and *E. coli* [12].

Another study investigated the antimicrobial activity of methanol, dichloromethane, and ethyl acetate extracts prepared from *S. lindbergii*. The methanol and ethyl acetate extracts (6.25 mg/ml) exhibited the highest activity against *S. aureus*, while the methanol extract (25 mg/ml) was most effective against *E. coli*. The ethyl acetate extract (25 mg/ml) showed the strongest activity against *P. aeruginosa*. All tested extracts had a consistent MIC value (100 mg/ml) against *C. albicans*. The findings of this study indicate that the extracts obtained from *S. yildirimlii* exhibit significantly stronger activity against *P. aeruginosa*, *S. aureus*, *E. coli* and *C. albicans*, with a MIC value of 625 µg/ml [29].

When antibiofilm studies with *Scutellaria* species were examined, a study was found with *S. lateriflora*. In this study, the hydroalkaloid extract derived from the aerial parts of the plant was found to reduce biofilm mass by 68% at a concentration of 55 mg/ml. Furthermore, when combined with 5 mg/ml of *Cistus incanus* at the same concentration, it achieved an 80% reduction in biofilm mass which was slightly higher than the aqueous extract obtained from *S. yildirimlii* (76.79%) [30]. Compared with our study's results, the aqueous extract obtained from *S. yildirimlii* has higher antibiofilm activity than

S. lateriflora alone. No anti-quorum sensing activity test studies conducted with *Scutellaria* species were found.

This study examined the antimicrobial, antibiofilm and anti-QS activities of total (80% methanol), ethyl acetate, petroleum ether and aqueous extracts obtained from the aerial parts of the *S. yildirimlii*. The antimicrobial activity test results revealed that the total and petroleum ether extracts exhibited the most potent antibacterial effects against Gram-positive test bacteria. Additionally, the petroleum ether extract showed the highest antimicrobial activity against both Gram-negative test bacteria and the tested fungus. The antibiofilm activity results indicated that the aqueous extract exhibited the strongest activity, with an inhibition effect of 76.79%. Lastly, no extract tested showed any inhibition of violacein in anti-QS activity results. When these results are compared with literature data, extracts obtained from *S. yildirimlii* have a higher antimicrobial effect than most *Scutellaria* species. This shows that these extracts obtained from *S. yildirimlii* are promising drug candidates against increasingly widespread antibiotic-resistant bacteria. However, more studies certainly needed, to be done with *S. yildirimlii*. The drug potential of the plant should be evaluated further.

ACKNOWLEDGEMENTS

This work was financially supported by the Ankara University Scientific Research Projects Coordination Unit [Project Number: TYL-2023-2946].

AUTHOR CONTRIBUTIONS

Concept: D.H.A., H.G.S.I., M.E.; Design: D.H.A., H.G.S.I., H.K.I.O., M.E; Control: D.H.A., H.G.S.I., M.E.; Sources: H.G.S.I., M.E.; Materials: H.G.S.I., M.E.; Data Collection and/or Processing: D.H.A., S.S.R., H.K.I.O., M.K., M.E.; Analysis and/or Interpretation: D.H.A., S.S.R., H.K.I.O., M.K., M.E.; Literature Review: D.H.A., S.S.R., H.K.I.O., M.K.; Manuscript Writing: D.H.A., H.G.S.I., S.S.R., H.K.I.O., M.E.; Critical Review: D.H.A., H.G.S.I., S.S.R., H.K.I.O., M.K., M.E.; Other: -

CONFLICT OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest for this study.

ETHICS COMMITTEE APPROVAL

The authors declare that the ethics committee approval is not required for this study.

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J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 49(3): 766-772, 2025
Doi: 10.33483/jfpau.1663313

ORIGINAL ARTICLE / ÖZGÜN MAKALE

INTEGRATED TRAINING PROGRAMS FOR ANTIMICROBIAL STEWARDSHIP AND INFECTION PREVENTION AND CONTROL FOR PRIMARY CARE PHYSICIANS: A REVIEW OF THE CURRENT LITERATURE

BİRİNCİ BASAMAK HEKİMLERİ İÇİN ANTİMİKROBİYAL YÖNETİM İLE ENFEKSİYON ÖNLEME VE KONTROLÜNE YÖNELİK ENTEGRE EĞİTİM PROGRAMLARI: MEVCUT LİTERATÜRÜN BİR İNCELEMESİ

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ABSTRACT

Objective: *Antimicrobial resistance (AMR) is a growing global threat, recognized by the World Health Organization (WHO) as a silent pandemic. Its impact endangers health systems worldwide, compromising progress toward Universal Health Coverage (UHC) and the Sustainable Development Goals (SDGs). Primary health care (PHC) plays a crucial role in ensuring access to high-quality, affordable healthcare, disease prevention, and community health improvement. However, the risk of infection transmission in PHC settings highlights the need for robust infection prevention and control (IPC) measures alongside antimicrobial stewardship (AMS) to ensure responsible antibiotic use and optimal patient outcomes.*

Material and Method: *This research examines the current literature on AMS and IPC training programs for primary care physicians. A systematic search using PubMed, Google Scholar, and Scopus identified 95 relevant articles, excluding settings outside PHC and specific AMS or IPC interventions beyond training.*

Result and Discussion: *Despite the recognized importance of AMS and IPC, no integrated training programs addressing both strategies in PHC settings were identified. The findings suggest an urgent need for structured, evidence-based training programs to enhance AMS and IPC implementation in primary care. Developing and evaluating such programs could be a key step in combating AMR at the frontline of healthcare.*

Keywords: *Antibiotic resistance, antimicrobial stewardship, infection prevention and control, integrated training program, primary health care*

ÖZ

Amaç: *Antimikrobiyal direnç (AMD), Dünya Sağlık Örgütü (DSÖ) tarafından sessiz bir pandemi olarak tanımlanan ve giderek büyüyen küresel bir tehdittir. Bu durum, sağlık sistemlerini dünya*

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Submitted / Gönderilme : 22.03.2025
Accepted / Kabul : 04.05.2025
Published / Yayınlanma : 19.09.2025

genelinde tehlikeye atarak Evrensel Sağlık Kapsamı (UHC) ve Sürdürülebilir Kalkınma Amaçalarına (SKA'lar) yönelik ilerlemeyi sekteye uğratmaktadır. Birinci basamak sağlık hizmetleri, yüksek kaliteli, erişilebilir ve uygun maliyetli sağlık hizmetleri sunarak hastalıkların önlenmesi ve toplum sağlığının iyileştirilmesinde kritik bir rol oynamaktadır. Ancak, birinci basamak sağlık hizmeti sunulan ortamlarda enfeksiyon yayılma riski, sorumlu antibiyotik kullanımı ve en iyi hasta sonuçlarını sağlamak için güçlü enfeksiyon önleme ve kontrol (EÖK) önlemlerinin yanı sıra AMD ile mücadele uygulamalarına duyulan ihtiyacı ortaya koymaktadır.

Gereç ve Yöntem: *Bu araştıma, birinci basamak hekimleri için AMY ve EÖK eğitim programlarına ilişkin mevcut literatürü incelemektedir. PubMed, Google Scholar ve Scopus veritabanlarında yapılan sistematik bir arama sonucunda birinci basamak sağlık hizmeti dışındaki ortamlar ve eğitim dışındaki spesifik AMY veya EÖK müdahaleleri hariç tutularak 95 ilgili makale belirlenmiştir.*

Sonuç ve Tartışma: *AMY ve EÖK'nın önemine dair güçlü kanıtlar bulunmasına rağmen, birinci basamak sağlık hizmeti sunulan ortamlarda her iki stratejiyi bir arada ele alan entegre eğitim programlarına rastlanmamıştır. Bulgular, birinci basamak sağlık hizmetlerinde AMY ve EÖK uygulamalarını güçlendirmek için yapılandırılmış, kanıta dayalı eğitim programlarına acil bir ihtiyaç olduğunu göstermektedir. Bu tür programların geliştirilmesi ve değerlendirilmesi, sağlık hizmetlerinde AMR ile mücadelede önemli bir adım olabilir.*

Anahtar Kelimeler: *Antibiyotik direnci, antibiyotik yönetimi, birinci basamak sağlık hizmetleri, enfeksiyon önleme ve kontrolü, entegre eğitim programı*

INTRODUCTION

Antimicrobial Resistance (AMR) has been declared as a silent pandemic by World Health Organization (WHO). It's devastating impact puts immense pressure on health systems worldwide, threatening progress toward Universal Health Coverage (UHC) and the Sustainable Development Goals (SDGs).

In response, Member States have adopted the 2024 UNGA High-Level Political Declaration on AMR, building on the 2023 UNGA declaration on pandemic preparedness. This declaration makes it clear: preventing future pandemics and tackling AMR go hand in hand. Now more than ever, global action is urgently needed [1,2]. It reinforces global commitment through increased investment, stronger regulations, better surveillance, and equitable access to treatments—key elements for preventing future health crises and building resilient health system [2]. The strong association between the AMR and misusage of antimicrobials is widely demonstrated with the scientific evidence [3].

Multisectoral approach is needed for tackling AMR [1,2,3]. An OECD study highlights that simple infection prevention measures, such as vaccinations and improved hand hygiene in healthcare settings, can more than halve the risk of death and reduce AMR burden. Additionally, integrated policies promoting hospital hygiene, AMS, diagnostic tests, and public awareness campaigns could save 1.6 million lives by 2050 across 33 countries. The study also found these interventions to be cost-effective, with investments paying off within a year and generating annual savings of up to US$ 4.8 billion [4].

Primary health care (PHC) plays a key role in making sure everyone gets the health services they need-safe, affordable, and high quality. It's the foundation for reaching UHC and the SDGs. Countries around the world have promised to take bold action: putting health at the center of all policies, building stronger PHC systems, giving people and communities more control over their health, and making sure efforts from all partners align with national plans. It's all part of the shared vision laid out in the Declaration of Astana [5].

Primary care plays a vital role in delivering healthcare, preventing diseases, and improving community health outcomes and safety. However, the potential for infections to spread in these settings, where diverse patient populations seek care, highlights the urgent need for evidence-based infection prevention and control (IPC) measures. Infection transmission can occur in health facilities that offer a variety of health services, such as those found in PHC Centers [6]. Therefore, it is crucial to assess the level of IPC knowledge among health care workers. Understanding the determinants and availability of administrative control measures is essential to interrupt the chain of infection transmission [7]. WHO issued guidelines on core IPC components in 2016, these primarily focused on acute healthcare settings. In response, the WHO has recently developed minimum IPC requirements and resources tailored to

primary care facilities, drawing on existing IPC guidance. Despite these efforts, there remains a significant need for tailored IPC guidelines and implementation strategies specifically designed for primary care settings to effectively address healthcare-associated infections (HAI) and AMR [8,9]. It has been demonstrated that effective IPC programs have the potential to reduce HAI rates by over 30%, while improved hand hygiene practices can decrease pathogen transmission in healthcare facilities by 50% [10].

Antimicrobial stewardship (AMS) is one of the important tools for tackling misuse of antibiotics while achieving the optimal patient outcomes during the treatment [11].

Stewardship's meaning in dictionary refers to the careful and responsible management of resources entrusted to one's care which meets the whole meaning for the responsible use of antibiotics. Strengthening the health care system requires an integrated approach which builts on three key pillars: AMS, IPC, and patient and medicine safety. Education and training serve as foundational components of effective AMS programs [12]. Although substantial advancements have been achieved in hospital settings, progress in primary care-where most antimicrobial prescriptions are written-remains comparatively limited [13]. It has been demonstrated that with 80-90% of antibiotic consumption occurring in outpatient settings, there is a significant and unmet need to promote AMS initiatives in the ambulatory care sector [14,15,16].

IPC, together with AMS, are essential strategies for combating AMR, the literature underscores the importance of ongoing training in AMS practices, focusing on the use of data-driven strategies combined with innovative clinical approaches to optimize antimicrobial use and tackle AMR challenges. Utilizing real-time data from routine healthcare facilitates continuous knowledge development, improves precision care, and reinforces public health protection [17].

It has been clearly demonstrated from both high- and low-income settings that AMS programs are significantly more effective in hospitals when implemented alongside IPC measures, particularly those aimed at improving hand hygiene (HH) compliance, compared to implementing AMS alone [18].

According to the WHO Guidance on AMS Activities, a systematic approach grounded in public health principles is necessary for implementing integrated AMS interventions. This involves crucially integrating core IPC practices within healthcare facilities. Furthermore, the WHO recommends that training for health workers should include basic principles of antimicrobial use and IPC, along with communication skills [19].

Effective collaboration, knowledge sharing, and capacity building are suggested as means to tackle disparities and enhance the implementation of IPC and AMS worldwide across various settings [20]. However, all the findings in the literature are derived from secondary and tertiary care facilities. Our review did not find any publications from primary health care settings that explore the synergistic effects between AMS and IPC implementation.

Purpose of our study is to analyze current training programs for AMS and IPC aimed specifically for physicians who are serving on primary care level and identify gaps or best practices in this regard.

MATERIAL AND METHOD

First the initial search made for identifying the relevant key words. Search strategy was developed for the PubMed, Google scholar and scopus. During the literature search "Antimicrobial Stewardship & Primary Health Care & Training (243)" "Antimicrobial Stewardship & Infection Prevention and Control & Training (400)" Antimicrobial Stewardship & Infection Prevention and Control & Primary Health Care (271)", "Infection Prevention and Control & Primary Health Care & Training (1458)", keywords were used in the databases. The reference lists of relevant papers were screened to identify any additional studies. Mendeley was used as reference management system. Target settings other than the PHC, patient trainings, residential home care/long term care settings and specific AMS and IPC interventions other than training were excluded from the review. In total 95 articles were reviewed, and all the resources of this article have been analyzed. This review did not involve any human subjects, so institutional ethics approval was not needed.

RESULT AND DISCUSSION

A systematic review of global studies conducted before 2019 identified 17 categories of policy interventions, with behavioral approaches, such as guidelines and professional engagement strategies, being the most commonly implemented internationally [21]. A recent systematic review, explores national interventions and policies implemented in England from 2013 to 2022 to optimize antibiotic use in healthcare settings, highlights the broad effort to reduce antibiotic prescribing, structural interventions like financial incentives showed the most significant impact on prescribing, albeit with a limited evidence base. Behavioral approaches, such as guidelines and training, were easier to implement but often lacked robust evaluations [22].

In the literature, some studies integrated AMS practices within IPC programs, particularly in hospital settings. Others combined IPC practices with measures like vaccination and social distancing under AMS activities to prevent infections and, consequently, reduce antibiotic consumption. However, none of these articles specifically addressed the unique challenges of primary healthcare or the training requirements of healthcare staff [23].

Lee et al. (2020) conducted a mixed-methods study in Canada to assess the need for, and effectiveness of, an educational tool designed to support AMS in primary care settings. The study explored healthcare providers' views on current AMS education and the accessibility of related resources. Results showed that more than 92% of respondents, across various professional roles, believed there is a significant need for increased public education on appropriate antimicrobial use. However, fewer than 58% felt they had adequate access to the tools or resources necessary to educate patients effectively. Despite this shortfall, over 80% of participants expressed strong interest in further educational opportunities, including seminars, workshops, and online learning, to strengthen their knowledge and capacity in AMS [24]. This study also highlights the need for primary health care providers to have access to and training in using educational tools that promote appropriate antimicrobial use.

March-Lopez et al. (2020) introduced a set of AMR interventions, including the development of local treatment guidelines and hands-on training workshops. These efforts paid off, leading to a 16.8% drop in antibiotic use between 2016 and 2018. The interventions focused on common conditions like pharyngotonsillitis, acute otitis media, sinus infections, bronchitis, and urinary tract infections-illnesses frequently diagnosed in their local setting and often linked to antibiotic overprescribing [25].

Gonen et al (2021) have presented comprehensive analysis of a quarter billion primary care prescriptions in Turkey as part of a nationwide AMS program in Türkiye. They have reported an average decrease of 8.33% in antibiotic prescriptions for acute upper respiratory infections (AURI) across the country with AMS interventions including feedback reports to physicians on their antibiotic prescribing patterns, educational programs, and public awareness campaigns. Authors suggested ongoing monitoring and adaptation of AMS strategies, reinforced by continuous education for healthcare providers and public engagement to sustain reduced levels of antibiotic prescriptions [26].

Muller et al. documented significant improvements in hand hygiene compliance and knowledge among healthcare workers in primary healthcare centers in Guinea, following targeted training and education. These improvements occurred after implementing the WHO's hand hygiene strategy [27].

Qureshi et al. reviewed the guidelines for occupational infection prevention and control (OIPC) training, which is meant to protect healthcare workers from infectious diseases. They found that while most guidelines push for mandatory trainings, there's not any standards on in how it's delivered, how often it happens, and what exactly gets covered-especially across different countries. Their study stressed the importance of making these programs more standardized and effective by incorporating adult learning principles and regularly evaluating their impact [28].

Truppa et al. (2023) emphasized a similar point on the need for tailor made AMS strategies in conflict-affected areas. They reflected that healthcare workers in these challenging settings need strong training and capacity-building programs to fight the growing threat of AMR. Without these measures, the fight against resistant infections becomes even tougher [29].

Baur et al. reported that studies implementing a hand hygiene (HH) intervention alongside an AMS programme achieved a 66% reduction in AMR bacteria, compared to only 17% in those without

such an intervention [30]. A systematic review and meta-analysis examining the impact of AMS programs in Asia found a significant reduction in healthcare-associated infections (HAIs) in studies that incorporated a hand hygiene (HH) program. Customizing educational strategies to address the needs of all disciplines simultaneously is a key enabler for fostering synergy between IPC and AMS [31]. Medical education faces significant gaps in infectious diseases and AMS training, with poor infection prevention practices, limited exposure to clinical ID rotations, and no consensus on optimal curriculum strategies, underscoring the need for improved and innovative educational approaches [32]. The findings of Mbamalu et al highlight the critical need for targeted training and customized IPC and AMS interventions to effectively address the gaps identified during pandemic responses [33].

Poje et al. have reported on the perspectives of primary care physicians on academic detailing (AD) for AMS regarding its feasibility and impact. According to their findings, primary healthcare physicians in Croatia recognized AD as a valuable tool for AMS. Participants highlighted the importance of continuous education, advocating for workshops, educational materials, and live lectures to reinforce AMS principles effectively [34].

Harrigan et al. (2024) took a similar approach, looking at how antibiotic prescribing for respiratory tract illnesses changed after an education and feedback intervention in primary care. Their study found that educational programs, combined with peer-comparison feedback, significantly reduced inappropriate antibiotic use. However, they also pointed out the challenge of keeping these improvements going over time [35]. Likewise, Dutcher et al. (2022) highlighted how these strategies can help cut down on unnecessary antibiotic prescriptions in primary care, presenting a scalable model for AMS efforts [36].

Motta et al. have highlighted the need of for local improvement in AMS across primary care: to work with prescribers to prescribe in line with guidance on recommended duration of treatment for common infections; to assist prescribers in understanding when macrolides are an appropriate choice; and to ensure relevant healthcare professionals are aware of the evolving focus on how primary care could manage potentially incorrect penicillin allergy labels [37].

Our literature search revealed a significant emphasis on the importance of AMS and IPC practices in PHC settings. However, these two approaches have not been addressed as a comprehensive, integrated strategy. We found no evidence of integrated training programs specifically designed for AMS and IPC practices in PHC settings. Implementing such training programs and evaluating their short- and long-term impacts could prove valuable in addressing the challenge of AMR in PHC settings.

This study had certain limitations. Our analysis included only English-language articles with accessible full texts, and we did not utilize paid databases for screening. Due to the absence of specific literature on Integrated Training Programs for AMS and IPC tailored to Primary Care Physicians, we broadened our scope. We analyzed AMS training for Primary Care Physicians, AMS and IPC training across various settings, and IPC training specific to Primary Care Physicians.

AUTHOR CONTRIBUTIONS

Concept: M.K., M.Ç.; Design: M.K., M.Ç., C.H.H.; Control: C.H.H., F.N.B.A.; Sources: M.K., M.Ç.; Materials: M.K., M.Ç.; Data Collection and/or Processing: M.K., M.Ç.; Analysis and/or Interpretation: C.H.H., F.N.B.A.; Literature Review: M.K., M.Ç.; Manuscript Writing: M.K., Critical Review: C.H.H., F.N.B.A.; Other: -

CONFLICT OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

The authors declare that the ethics committee approval is not required for this study.

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J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 49(3): 773-784, 2025
Doi: 10.33483/jfpau.1648214

ORIGINAL ARTICLE / ÖZGÜN MAKALE

SYNTHESIS AND CHARACTERIZATION OF NOVEL 5-FLOUROINDOLYLMETHYLEN HYDRAZON DERIVATIVES AS SCHIFF BASE COMPONDS: *IN VITRO* AND *IN SILICO* EVALUATION AS POTENTIAL CARBONIC ANHYDRASES (CAI-CAII) INHIBITORS

YENİ 5-FLOROİNDOLİLMETİLEN HİDRAZON TÜREVLERİNİN SCHİFF BAZ BİLEŞİKLERİ OLARAK SENTEZİ VE KARAKTERİZASYONU: POTANSİYEL KARBONİK ANHİDRAZ (CAI-CAII) İNHİBİTÖRLERİ OLARAK İN VİTRO VE İN SİLİCO DEĞERLENDİRMESİ

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ABSTRACT

Objective: *In this study, novel 5-fluoroindolylmethylene hydrazone derivatives were synthesized and assessed for their potential inhibitory effects on human carbonic anhydrase I and II (hCA I and II) enzymes through both in vitro and in silico approaches.*
Material and Method: *Accordingly, starting from 5-fluoroindole, 5-fluoroindole-3-carboxaldehyde was synthesized. The target final compounds were then obtained by condensing substituted phenylhydrazine with 5-fluoroindole-3-carboxaldehyde, resulting in three Schiff base derivatives (2a, 2b, and 2c).*
Result and Discussion: *The synthesized compounds demonstrated effective inhibition of cytosolic carbonic anhydrase isoforms hCA I and II, with Ki values ranging from 32.28±7.09 to 74.86±10.90 nM for hCA I and 8.79±1.92 to 60.40±14.64 nM for hCA II. Among them, compound 2a exhibited the most potent inhibitory effect on both isoenzymes. In vitro results were verified with the results obtained by docking studies and interactions with enzymes were demonstrated. These novel 5-fluoroindolylmethylene hydrazone derivatives show promise as potent inhibitors of cytosolic CA isoenzymes.*
Keywords: *5-Flouroindole, carbonic anhydrase, glaucoma, inhibition*

ÖZ

Amaç: *Bu çalışmada, 5-floroindolilmetilen hidrazonun yeni türevleri sentezlendi ve bunların insan karbonik anhidraz I ve II (hCA I ve II) enzimleri üzerindeki potansiyel inhibitör etkileri in vitro ve in silico olarak değerlendirilmesi amaçlandı.*
Gereç ve Yöntem: *Buna göre, 5-floroindolden başlayarak, 5-floroindol-3-karboksaldehit sentezlendi. Hedef nihai bileşikler daha sonra ikame edilmiş fenilhidrazinin 5-floroindol-3-karboksaldehit ile yoğunlaştırılmasıyla elde edildi ve üç Schiff baz türevi (2a, 2b ve 2c) elde edildi.*

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Submitted / Gönderilme : 27.02.2025
Accepted / Kabul : 05.05.2025
Published / Yayınlanma : 19.09.2025

Sonuç ve Tartışma: *Sentezlenen bileşikler, hCA I için 32.28±7.09 ila 74.86±10.90 nM ve hCA II için 8.79±1.92 ila 60.40±14.64 nM aralığında değişen Ki değerleri ile sitozolik karbonik anhidraz izoformları hCA I ve II'nin etkili inhibisyonunu göstermiştir. Bunlar arasında, bileşik 2a her iki izoenzim üzerinde en güçlü inhibitör etkiyi göstermiştir. İn vitro sonuçlar, yerleştirme çalışmalarıyla elde edilen sonuçlarla doğrulanmış ve enzimlerle etkileşimler gösterilmiştir. Bu yeni 5-floroindolilmetilen hidrazon türevleri, sitozolik CA izoenzimlerinin güçlü inhibitörleri olarak ümit verici görünmektedir.*
Anahtar Kelimeler: *5-Floroindol, glokom, inhibisyon, karbonik anhidraz*

INTRODUCTION

Metalloenzymes consist of one or more metal ions that are tightly bound within a protein. Using the enzyme as a structural framework for transition metal complexes, these systems enable selective chemical transformations. It is well known which metal ions strongly bind to proteins. Identifying these bound metal ions is crucial for advancing enzymatic analysis [1,2]. These enzymes play crucial roles in protein degradation, detoxification, nucleic acid modification and other vital processes [2,3]. The participation of metalloenzymes in these functions also connects them to the development of various diseases, highlighting their potential as therapeutic targets [3]. Human carbonic anhydrase (hCA; carbonate hydrolases, EC 4.2.1.1) belongs to the metalloenzyme family, which includes sixteen isoenzymes (CA I–XVI) in mammals. Because they contain zinc ions, most carbonic anhydrases are categorized as metalloenzymes. Human carbonic anhydrases facilitate the reversible reaction of carbon dioxide (CO_2) with water, yielding a proton (H^+) and a bicarbonate ion (HCO_3^-). This reaction plays a crucial role in pH regulation across various tissues, particularly in erythrocytes [4-9]. Several CA isozymes play essential roles in physiological functions, making them important therapeutic targets. Modulating their activity, either through inhibition or activation, holds therapeutic potential for conditions like edema, cancer, epilepsy, osteoporosis, glaucoma and obesity [5-11]. Carbonic anhydrase inhibitors (CAIs) such as zonisamide (ZNS), acetazolamide (AZA) and methazolamide (MZA) are commonly used in clinical settings as diuretics and to manage intraocular pressure in glaucoma patients [12,13]. The inhibitory effects of several compounds, including anions, metal ions, phenols [14-16], Schiff bases, sulfonamides and their amines, amides and 1H-indazole derivatives [17-20], chalcones [21], pyrimidine-thiones [22], and certain amino methyl derivatives [23], have been investigated against numerous CA isozymes to date. CA II inhibitors have a number of uses, including the treatment of epilepsy and glaucoma, as well as their use as antitumor agents, diagnostic tools, and diuretics [6,8,24,25]. Currently, there is significant scientific interest in the discovery of novel CA inhibitors [6,8,14,24-27]. Recent studies have shown that various phenol derivatives [8,14], salicylic acid derivatives [28], and different benzene and bisphenol compounds with antioxidant properties, along with their derivatives [29,30], are potential inhibitors of hCA I-II isozymes. Recently, researchers in this field have focused on evaluating the pharmacological activity of indole and its derivatives. Numerous studies have demonstrated the diverse therapeutic potential of indole derivatives, including antioxidant [31], antimicrobial [32], and anticancer [33], properties, as well as their potential in Alzheimer's disease [34,35].

This study is primarily focused on the synthesis of three novel indole-derived Schiff base compounds and the evaluation of their inhibitory potential against the hCA I-II enzymes. In this context, firstly, the compound 5-fluoroindole-3-carboxaldehyde it resulted from the reaction of 5-fluoroindole with DMF and $POCl_3$ [36]. Then, 5-fluoroindole-3-carboxaldehyde was reacted with substituted phenylhydrazine derivatives or hydrazine to synthesis the three Schiff bases compounds. The synthesized compounds were evaluated using a variety of characterization techniques like ^1H and ^{13}C NMR and mass spectra analyses. The CA inhibitory effects of the newly synthesized compounds targeting the CA I and II isoenzymes, isolated from human erythrocytes, were assessed based on two parameters: IC_{50} and Ki. IC_{50} values were determined by measuring the percentage of enzyme activity at various inhibitor concentrations, while keeping the substrate concentration fixed, followed by graphical determination of the inhibitor concentration required for 50% inhibition. The results were subsequently compared to acetazolamide.

MATERIAL AND METHOD

General

A melting point for Stuart The SMP30 instrument was used to determine uncorrected melting points. A Varian 500 MHz and 125 MHz spectrometer (Palo Alto, CA) The ^{1}H and ^{13}C NMR spectra were recorded using TMS for internal standard, with DMSO-d6 and $CDCl_3$ serving as the solvents. The ESI mass spectra were obtained using a Waters Micromass ZQ apparatus. Every spectral analysis was carried out at Ankara University's Instrumental Laboratory of the Faculty of Pharmacy. Using Merck silica gel 60 (230–400 mesh ASTM), chromatography was performed. Sepharose-4B, protein assay reagents, 4-nitrophenylacetate and other chemicals used for purification were purchased from Sigma–Aldrich Co. All other chemicals used for kinetic analysis were analytical grade and obtained from Merck. The chemical reagents and other compounds that were utilized in the synthesis.

General Procedure for Synthesis of Compounds

Compound 5-fluoroindole-3-carboxaldehyde it resulted from the reaction of 5-fluoroindole with DMF and $POCl_3$ [36]. The final three Schiff bases compounds were synthesized by condensation of substituted phenylhydrazine hydrochloride or hydrazine hydrate with 5-fluoroindole-3-carbaldehyde (Figure 1). The novel compounds were synthesized using a Kidwai-adapted technique [37].

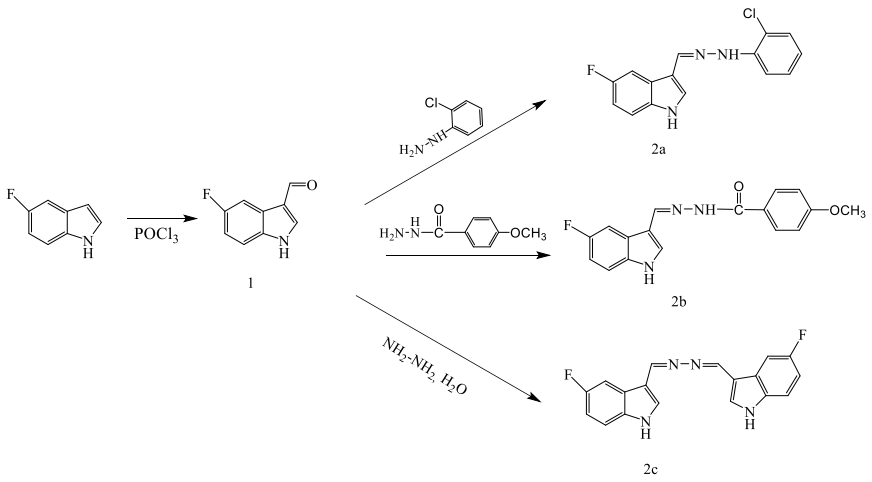


Figure 1. Synthesis of indole hydrazine derivatives

Synthesis of 5-fluoroindole-3-carbaldehyde (1)

5-Fluoroindole (1 mmol) was reacted with $POCl_3$ in DMF. $POCl_3$ was dissolved in DMF at temperatures below 10 ºC, and then 5-fluoroindole, Dissolved in DMF, it was introduced into the mixture, which was then stirred at 35 ºC for 1.5 hours. Afterward, it cooled to 10 ºC, and 1N NaOH was added until the solution became alkaline. The mixture was subsequently heated to 60 ºC and then allowed to cool. Finally, it was filtered, rinsed with water, and left to dry.

Yield: 77% (0.126 g), mp: 163-165°C. ^{1}H-NMR (500 MHz, $CDCl_3$, δ): 8.99 (s, 1H), 8.02 (dd, 1H, J=9.2, J'=2.3), 7.91 (d, 1H, J=3.1), .7.40 (dd, 1H, J=8.8, J'=4.3), 7.28 (s, 1H),7.12-7.08 (m, 1H). ^{13}C-NMR (125 MHz, DMSO, δ): 184.99, 160.74, 158.85, 136.41, 133.03, 119.76, 113.08, 112.87, 112.43, 112.35, 107.58, 107.39. MS (ESI+) m/z: 164.03 (M+H, 55%). 191.29 (M+28, 100%).

Synthesis of Compounds 2a and 2b

A mixture of 5-fluoroindole-3-carbaldehyde (1 mmol), 2-chlorophenyl hydrazine hydrochloride or 4-methoxybenzohydrazide (1.2 mmol) was dissolved in 20 ml of absolute ethanol. Sodium acetate (0.4 g) was added as a catalyst, and the reaction was heated at 80ºC for 2 hours. Upon completion, the

reaction mixture was allowed to cool to room temperature. The resulting precipitate was filtered, washed with cold water, and recrystallized from ethanol to afford the desired compounds.

5-fluoroindole-3-carboxaldehyde (2-chlorophenyl) hydrazone (2a): Yield:51% (0.145 g), mp: 147-148°C. ^{1}H-NMR (500 MHz, DMSO, δ): 11.55 (s, 1H, hydrazon-NH), 9.44 (s, 1H), 8.49 (s, 1H, azomethine-CH), 7.90 (dd, 2H, J = 10, J'=2.6 Hz), 7.76 (d, 1H, J = 2.7 Hz), 7.52 (dd, 1H, J = 8.6, J'=1.4 Hz), 7.47-7.44 (m,1H), 7.34-7.31 (m, 2H), 7.09-7.05 (m, 1H), 6.76-6.73 (m, 1H). ^{13}C-NMR (125 MHz, DMSO, δ): 158.97, 157.12, 142.46, 138.93, 134.15, 130.45, 129.74, 128.63, 124.84, 124.76, 118.93, 116.13, 113.52, 113.41, 113.33. 113.03, 112.99, 111.04, 110.84, 106.64, 106.46. MS (ESI+) m/z: 288.38 (M+H, 100%).

N'-((5-fluoro-1H-indol-3-yl)methylene)-4-methoxybenzohydrazide (2b): Yield: 37%(0.114 g), mp: 220-222°C. ^{1}H-NMR (500 MHz, DMSO, δ): ^{1}H-NMR (500 MHz, DMSO, δ): 11.68 (s, 1H, hydrazon-NH), 11.47 (s, 1H), 8.60 (s, 1H, azomethine-CH), 8.03 (dd, 1H, J=10, J'=2.5 Hz), 7.94-7.90 (m, 3H), 7.47-7.44 (m, 1H), 7.09-7.05 (m, 3H), 3.84 (s, 3H). ^{13}C-NMR (125 MHz, DMSO, δ): 162.49, 162.20, 159.12, 157.27, 144.36, 134.10, 132.20, 129.78, 126.46, 125.18, 125.10, 114.1, 113.38, 113.31, 112.47, 112.43, 111.27, 111.06, 107.33, 107.14, 55.86. MS (ESI+) m/z: 312.46 (M+H, 100%).

Synthesis of 1,2-bis((5-fluoro-1*H*-indol-3-yl)methylene)hydrazine (2c)

5-Fluoroindole-3-carbaldehyde (2 mmol) was combined with hydrazine hydrate (1 mmol) in 20 mL of absolute ethanol and heated on a hot water bath for 2 hours. After completion, the reaction mixture was cooled to room temperature. The resulting precipitate was filtered, washed with cold water, recrystallized from ethanol to obtain the desired compounds.

Yield: 77% (0.248 g), mp: 324-325°C. ^{1}H-NMR (500 MHz, DMSO, δ): 11.84 (s, 2H, hydrazon-NH), 8.92 (s, 2H), 8.05 (dd, 2H, J=10, J'=2.6 Hz), 7.99 (s, 2H, azomethine-CH), 7.51-7.48 (m, 2H), 7.11-7.06 (m, 2H). ^{13}C-NMR (125 MHz, DMSO, δ): 185.44, 159.23, 157.37, 155.60, 134.26, 133.96, 125.60, 125.52, 113.59, 113.51, 112.59, 112.55, 111.30, 111.09, 107.36, 107.17. MS (ESI+) m/z: 323.47 (M+H, 100%).

Molecular Docking Studies

The protein data bank provided hCA I (PDB: 2FOY) [38] and hCA II (PDB: 1IF7) [38], which were downloaded in pdb format for the molecular docking investigation. AutoDockTools 1.5.6 [39,40] was used to remove water, ions and ligands from the downloaded proteins, add polar hydrogens, and add a gaister charge before saving the protein in pdbqt format. Ligands were prepared in the ChemDraw3D 19.0 application. Molecular docking analysis was performed using the most recent AutoDock Vina software [40] following conversion to pdbqt format using AutoDockTools 1.5.6. The results are shown in 2D and 3D the Discovery Studio Client program [41].

Biological Activity Studies

Carbonic Anhydrase Isoenzymes Inhibition Analysis

The enzyme purification process was conducted in accordance with established protocols from previous research [42-46]. The protein concentration in active fractions was measured spectrophotometrically at 595 nm using the Bradford assay [47]. Carbonic anhydrase (CA) isoenzyme activity was determined based on the method outlined by Verpoorte et al., with spectrophotometric absorbance monitored at 348 nm to track changes in the reaction medium [48].

To assess the inhibitory potential of thiophene derivative Schiff base compounds (2a-c) on human CA isoenzymes, a graph representing enzyme activity percentage against inhibitor concentration was generated. The IC_{50} values were extracted from these plots. For Ki determination, three different inhibitor concentrations were tested alongside five varying substrate concentrations. Additionally, inhibition graphs for the most effective compounds were plotted and analyzed. The same approach used for AZA, the reference inhibitor for CA isoenzymes, was used to calculate Ki and IC_{50} values.

RESULT AND DISCUSSION

Studies on the inhibition of carbonic anhydrase (CA) enzyme activity have demonstrated its

potential as a therapeutic approach for treating glaucoma. These studies have also aimed to clarify the enzyme's catalytic mechanisms, while revealing its distribution in tissues and its vital roles within them. These findings have led to a notable rise in the development of enzyme inhibitors and activators. Consequently, various enzyme inhibitors have been developed. Beyond its use in glaucoma treatment, CA inhibitors are also widely utilized in clinical settings for their antitumor, analgesic, anticonvulsant, antiulcer, diuretic, antibiotic, and neurological properties [49]. Our previous research has demonstrated that indole derivatives containing Schiff bases inhibit the cytosolic CA isozymes hCA I and hCA II. As a continuation of our studies, the synthesis of three new indole-derived Schiff base compounds and the investigation of their inhibitory effects on hCA I and II enzymes were evaluated [50]. This work synthesized new compounds and investigated their in vitro effects on the hCAI and hCAII isoenzymes after first doing in silico assessments of novel Schiff bases generated from indole. After purifying the CAI and CAII isoenzymes using the CNBr-activated Sepharose-4B-L-tyrosine sulfanilamide affinity chromatography method, the effects of the new indole derivative Schiff base compounds on these isoenzymes were investigated (Table 1, Figure 2-5). Compounds 2a and 2c exhibited strong inhibition effects on hCAI and hCAII isoenzymes at very low concentrations. The inhibitor concentration and % activity for the compounds 2c and 2a, which showed inhibitory effects on the hCAI and hCAII isoenzymes, respectively, were shown in graphs (Figure 2 and Figure 4). Ki values were determined for each compound at three different concentrations. The compounds were found to exhibit inhibitory effects across all three concentrations tested. Lineweaver-Burk plots were drawn to determine the Ki values and the type of inhibition. The Ki and IC_{50} values obtained for hCAI and hCAII are provided in Table 1. It has been established that the Ki values for hCAI range from 32.87 ± 10.64 to 74.86 ± 10.90 nM and for hCAII from 8.79 ± 1.92 to 60.40 ± 14.67 nM. In comparison to the control compound AZA, compound 2a displayed the strongest inhibitory activity against the hCAI isoenzyme, while compound 2c exhibited the most potent inhibition of the hCAII enzyme (Figure 2 and Figure 4) (Table 1).

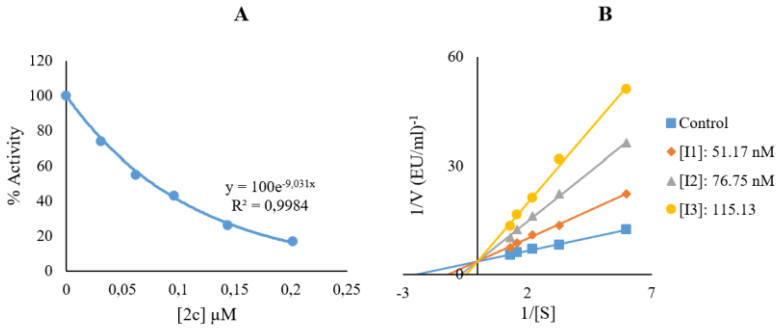


Figure 2. IC_{50} graph (A) and Lineweaver-Burk graph (B) of excellent inhibitor (**2c**) for hCAI

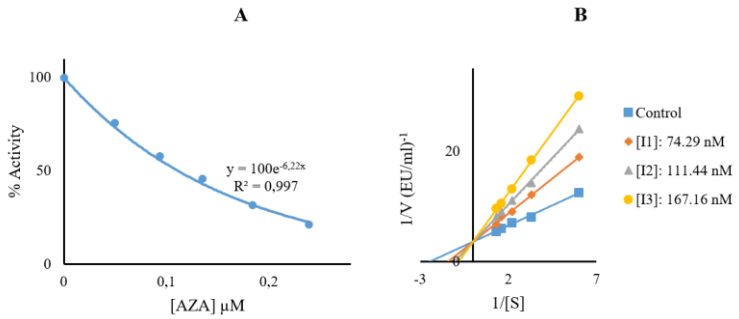


Figure 3. IC_{50} graph (A) and Lineweaver-Burk graph (B) of Asetazolamide (AZA) for hCAI

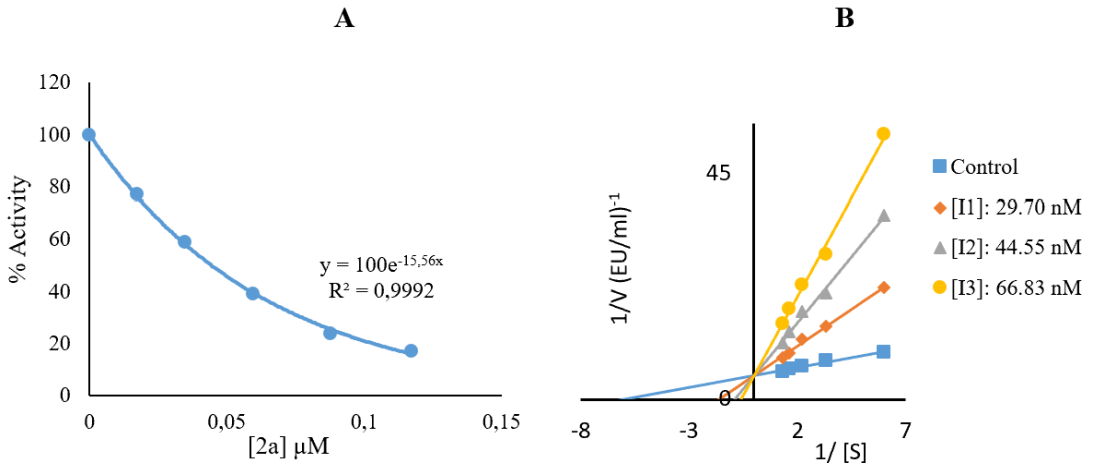


Figure 4. IC_{50} graph (A) and Lineweaver-Burk graph (B) of excellent inhibitor (**2a**) for hCAII

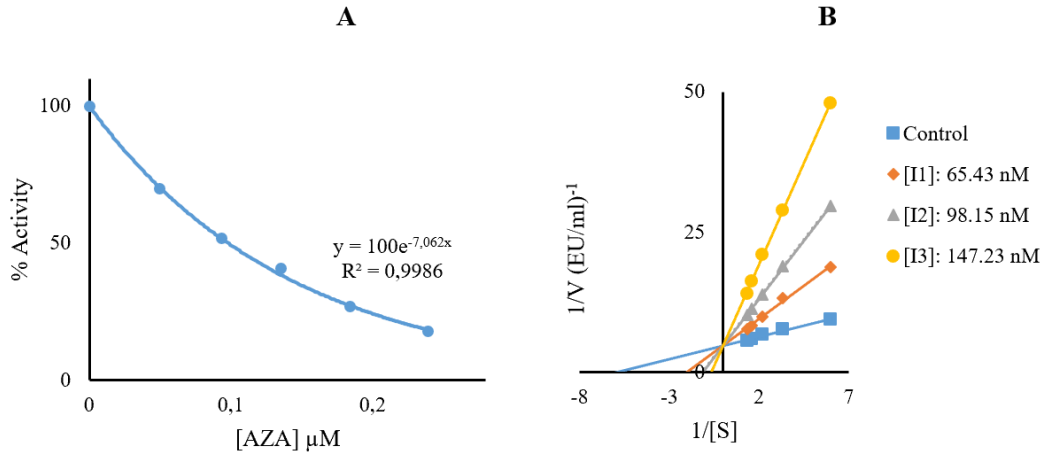


Figure 5. IC_{50} graph (A) and Lineweaver-Burk graph (B) of Asetazolamide (AZA) for hCAII

Table 1. The Ki constants and IC_{50} values determined for compounds (2a-c) and acetazolamide (AZA) drugs having inhibitory effects on hCAI and hCAII.

Compounds	IC_{50} (nM)				Ki (nM) ± STD	
	hCAI	R^2	hCAII	R^2	hCAI	hCAII
2a	59.09	0.9991	44.55	0.9971	34.20 ± 7.09	8.79 ± 1.92
2b	196.70	0.9947	165.38	0.9889	74.86 ± 10.90	60.40 ± 14.67
2c	76.75	0.9975	64.48	0.9971	32.87 ± 10.64	14.45 ± 4.28
AZA	111.44	0.9939	98.15	0.9976	89.70 ± 9.43	24.98 ± 7.71

Molecular docking studies revealed that the binding energies of compounds 2a-c to the hCA-I and hCA-II enzymes ranged from from -6.8 to -8.8 kcal/mol. The binding energy of the reference drug AZA for the hCAI enzyme is -5.7 kcal/mol, and for the hCAII enzyme, it is -6.1 kcal/mol (Table 2). All compounds were observed to bind with lower binding energies than the reference drug AZA.

When the interactions of the compounds with the hCAI enzyme were evaluated, hydrogen bonds and pi interactions were observed in the interactions of all compounds and acetazolamide with the active site of the hCAI enzyme. In compound 2b, hydrogen bonds are predominant and there are fewer hydrophobic interactions. Considering the in vitro hCAI activity of 2b, it is thought that hydrophobic

interactions may play an important role in enzyme inhibition activity. In compounds 2a and 2c, pi interactions are also seen in addition to hydrogen bonds. There are hydrogen bonds and pi interactions in the interaction of the reference drug acetazolamide with the hCAI enzyme's active site (Figure 6).

Table 2. The binding energy and protein-ligand interaction for compounds (2a-c) and acetazolamide (AZA) drugs having inhibitory effects on hCAI and hCAII

Compunds	Binding energy for hCA-I (kcal/mol)	Protein-ligand interaction (for hCA-I)	Binding energy for hCA-II (kcal/mol)	Protein-ligand interaction (for hCA-II)
2a	-6.9	VAL A:239, LYS A:170, PRO A:240, TYR A:7	-7.2	PHE A:231, PRO A:237, GLU A:236, GLY A:63, GLU A:239
2b	-6.8	GLY A:63, GLN A:242, HIS A:243, LYS A:170, SER A:231, PRO A:241	-7.8	PHE A:231
2c	-7.8	LEU A:231, HIS A:94, ALA A:121, PHE A:91, ASN A:69, HIS A:200	-8.8	TYR A:7, PHE A:231, LYS A:170, ASN A:32, VAL A:242
AZA	-5.7	LEU A:131, ALA A:121, HIS A:100, GLU A:92, HIS A:96, THR A:199, HIS A:94, HIS A:119	-6.1	PHE A:231, HIS A:4, LYS A:140, GLY A:63, TYR A:7, GLU A:239, ASN A:11

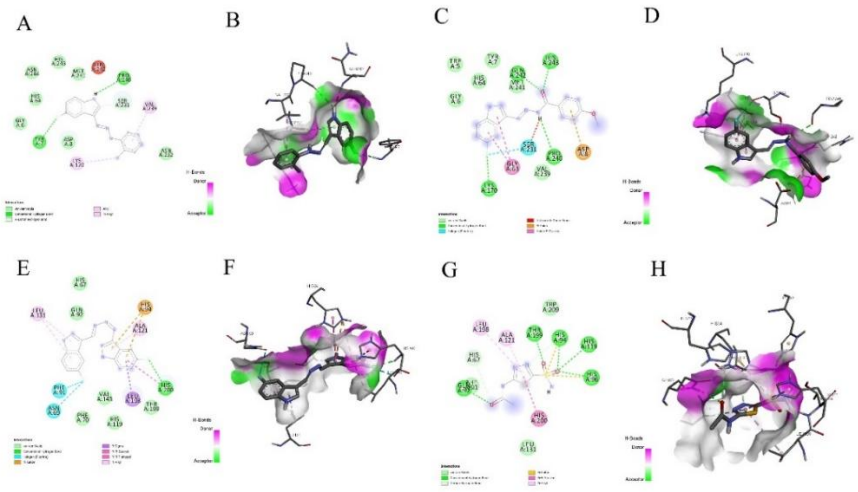


Figure 6. The active site of the hCA I enzyme contains 2D molecular docking images of compounds 2a (A), 2b (C), 2c (E), and the reference drug acetazolamide (G), as well as 3D molecular docking images of compounds 2a (B), 2b (D), 2c (F), and the reference drug acetazolamide (H). Interactions of compound 2a:VAL A:239(π interactions), LYS A:170(alkyl interactions), PRO A:240(H-bonds), TYR A:7(H-bonds), interactions of compound 2b: GLY A:63 (π-alkyl interactions), GLN A:242(H-bonds), HIS A:243(H-bonds), LYS A:170(H-bonds), SER A:231(Halogen interactions), PRO A:241 (H-bonds), Interactions of compound 2c: LEU A:231 (π interactions), HIS A:94 (π interactions), ALA A:121 (π interactions), PHE A:91 (Halogen interactions), ASN A:69(Halogen interactions), HIS A:200 (π interactions and H-bonds), interactions of reference drug acetazolamide; LEU A:131(π interactions), ALA A:121(π interactions), HIS A:100(π interactions), GLU A:92(H-bonds), HIS A:96, (H-bonds), THR A:199(H-bonds), HIS A:94(H-bonds), HIS A:119 (H-bonds)

When the interactions of compounds with hCAII enzyme are evaluated, hydrogen bonds and pi interactions are observed in the interactions of compounds 2a and 2b with the active site of hCAII enzyme. Hydrophobic interaction with Phe231 is seen as common in all compounds and acetazolamide. Hydrophobic interaction with Gly63, Glu239 is similar in compounds 2a and acetazolamide. Moreover, the low binding energy of compound 2a is low compared to acetazolamide. The fact that this compound has a more potent activity compared to acetazolamide is correlated with molecular docking study, and it can be thought that the hydrophobic interactions observed in the interaction of the compound with the enzyme contribute to the activity. It was observed that compound 2b has both hydrophobic and hydrogen bond interactions with Phe231 in the active site of the enzyme. Although its binding energy to the enzyme is high, it is thought that its lower activity compared to acetazolamide and compound 2a contributes to the activity due to hydrophobic interactions and hydrogen bonds. In the interactions of compound 2c with protein, more hydrophobic interactions are observed compared to acetazolamide. Compound 2c binds to the target protein with lower binding energy and correlates with in vitro activity. (Figure 7).

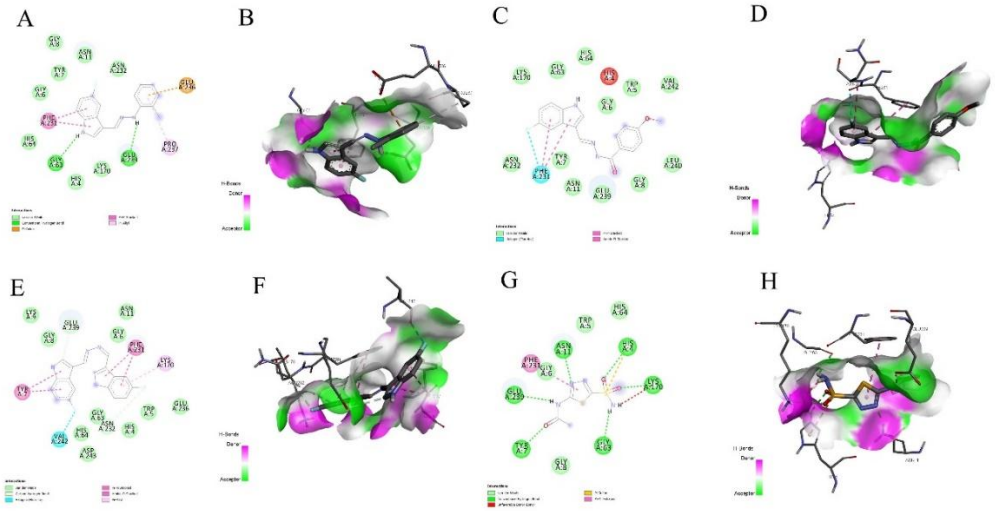


Figure 7. The hCA II enzyme's active site contains 2D molecular docking images of compounds 2a (A), 2b (C), 2c (E), and the reference drug acetazolamide (G). The hCA II enzyme's active site contains 3D molecular docking images of compounds 2a (B), 2b (D), 2c (F), and the reference drug acetazolamide (H). Interactions of compound 2a: PHE A:231(π interactions), PRO A:237, GLU A:236 (π interactions), GLY A:63(H-bonds), GLU A:239(H-bonds), interactions of compound 2b: PHE A:231(Halogen interactions and H-bonds), interactions of compound 2c: TYR A:7 (π interactions), PHE A:231 (π interactions), LYS A:170 (π interactions), ASN A:32 (H-bonds), VAL A:242 (Halogen interactions), interactions of reference drug acetazolamide; PHE A:231(π interactions), HIS A:4(H-bonds), LYS A:140(H-bonds),, GLY A:63(H-bonds), TYR A:7(H-bonds), GLU A:239(H-bonds), ASN A:11(H-bonds)

Conclusion

In this study, three different indole-derived Schiff base compounds were synthesized, and the inhibitory profiles of the synthesized compounds against the hCA I and hCA II enzymes were investigated. The investigated compounds exhibited more effective inhibitory profiles compared to AZA which is utilized as a reference drug. Consequently, the nnovel compounds inhibited hCAI and hCAII activities at lower concentrations. The synthesized compounds demonstrated effective inhibition of cytosolic carbonic anhydrase isoforms hCA I and II, with Ki values ranging from 32.28±7.09 to

74.86±10.90 nM for hCA I and 8.79±1.92 to 60.40±14.64 nM for hCA II. Compound 2a exhibited the most potent inhibitory effect on both isoenzymes. Docking studies were used to confirm the in vitro results, and interactions with enzymes were shown. The lower activity of compound 2b compared to 2a and 2c is believed to be due to the intramolecular hydrogen bond formed between the carbonyl oxygen and the hydrogen on the hydrazine nitrogen in 2b. This bond reduces the molecule's ability to form hydrogen bonds with the enzyme, leading to decreased activity. Even if our findings are encouraging, we intend to carry out more research to examine the cytotoxicity and mode of action of these substances in order to create an ideal and trustworthy hCA I and II enzyme inhibitor. Therefore, we believe that these novel 5-fluoroindolylmethylene hydrazone derivatives hold great potential as lead compounds for the development of improved CA inhibitors.

ACKNOWLEDGEMENTS

The authors appreciate the Scientific Research Council of Selcuk University for their support of the Project (Project No: 24202055).

AUTHOR CONTRIBUTIONS

Concept: C.K., N.F., E.D., H.Ş.; Design: C.K., N.F., E.D., H.Ş.; Control: C.K., N.F., E.D., H.Ş.; Sources: C.K., N.F., E.D., H.Ş.; Materials: C.K., N.F., E.D., H.Ş.; Data Collection and/or Processing: C.K., N.F., E.D., H.Ş.; Analysis and/or Interpretation: C.K., N.F., E.D., H.Ş.; Literature Review: C.K., N.F., E.D., H.Ş.; Manuscript Writing: C.K., N.F., E.D., H.Ş.; Critical Review: C.K., N.F., E.D., H.Ş.; Other: -

CONFLICT OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

The authors declare that the ethics committee approval is not required for this study.

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J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 49(3): 785-789, 2025
Doi: 10.33483/jfpau.1601950

ORIGINAL ARTICLE / ÖZGÜN MAKALE

COMPARISON OF THE ANTIBACTERIAL EFFICIENCY OF SUPER ANIONIC OXYGEN SOLUTION CONTAINING SILVER NANOPARTICLES (NPAG) AND SUPER ANIONIC OXYGEN SOLUTION (H_2O_8) AGAINST *E. FAECALIS* AND *S. MUTANS* – AN *IN VITRO* STUDY

GÜMÜŞ NANOPARTİKÜLLERİ İÇEREN SÜPER ANYONİK OKSİJEN SOLÜSYONU (NPAG) İLE SÜPER ANYONİK OKSİJEN SOLÜSYONU'NUN (H_2O_8) E. FAECALİS VE S. MUTANS'A KARŞI ANTİBAKTERİYEL ETKİNLİĞİNİN KARŞILAŞTIRILMASI – İN VİTRO ÇALIŞMA

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ABSTRACT

Objective: *The purpose of this study is to compare the antimicrobial effect of super anionic oxygen solution containing silver nanoparticles (NpAg) and super anionic oxygen solution (H_2O_8) in vitro*
Material and Method: *"The antimicrobial activity of the solutions was tested against Enterococcus faecalis ATCC 29212 (E. faecalis) and Streptococcus mutans ATCC 25175 (S. mutans) standart bacteria. The effects of them applied on the bacterial suspension after 10 minutes were examined in terms of colony reduction.*
Result and Discussion: *As a result of the efficacy tests, NpAg solution showed a 46.67% (0.27 log) decrease in E. faecalis concentration and a 58.8% (0.38 log) decrease in S. mutans concentration. Similarly, H_2O_8 solution showed an 84.67% (0.81 log) decrease in E. faecalis concentration and a 62.94% (0.43 log) decrease in S. mutans concentration. Under the limitations of this study, it was concluded that super anionic oxygen solutions (NpAg) and super anionic oxygen solutions (H_2O_8) have a significant antimicrobial flora against E. faecalis and S. mutans, but are attenuated when compared to irrigants routinely used clinically. Since this study did not simulate the oral environment, future studies may provide more effective results.*
Keywords: *Biofilms, dentin, endodontics, nanoparticles, root canal irrigants*

ÖZ

Amaç: *Bu çalışmanın amacı gümüş nanopartikülleri içeren süper anyonik oksijen solüsyonu (NpAg) ile süper anyonik oksijen solüsyonu (H_2O_8) antimikrobiyal etkisini karşılaştırmaktır.*
Gereç ve Yöntem: *Solüsyonların antimikrobiyal aktivitesi Enterococcus faecalis ATCC 29212 (E. faecalis) ve Streptococcus mutans ATCC 25175 (S. mutans) standart bakterilere karşı test edilmiştir.*

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Submitted / Gönderilme : 20.12.2024
Accepted / Kabul : 06.05.2025
Published / Yayınlanma : 19.09.2025

Bakteri süspansiyonuna 10 dakika sonra uygulananların koloni azalması açısından etkileri incelenmiştir.
Sonuç ve Tartışma: *Yapılan etkinlik testleri sonucunda NpAg solüsyonu, E. faecalis konsantrasyonunda %46.67 (0.27 log), S. mutans konsantrasyonunda ise %58.8 (0.38 log) azalma gösterdi. Benzer şekilde H_2O_8 solüsyonu, E. faecalis konsantrasyonunda %84.67 (0.81 log) azalma ve S. mutans konsantrasyonunda %62.94 (0.43 log) azalma gösterdi. Bu çalışmanın sınırlamaları altında, süper anyonik oksijen çözeltilerinin (NpAg) ve süper anyonik oksijen çökeltilerinin (H_2O_8) E. faecalis ve S. mutans'a karşı önemli bir antimikrobiyal etkiye sahip olduğu ancak klinikte rutin olarak kullanılan irrigasyonlarla karşılaştırıldığında zayıf kaldığı sonucuna varılmıştır. Bu çalışma ağız ortamını simüle etmediği için gelecekteki çalışmalar daha etkili sonuçlar verebilir.*
Anahtar Kelimeler: *Biofilm, dentin, endodonti, kök kanal irrigasyonu, nanopartikül*

INTRODUCTION

The goal of root canal therapy is to fully clean and debride the root canal system, allowing it to be sealed without microleakage. This procedure is also known as the chemico-mechanical process, in which the canal is dilated using chemical irrigants and mechanical devices [1]. NaOCl has several drawbacks, including a disagreeable taste, cytotoxicity, and the difficulty to remove the smear layer. Furthermore, various studies have shown that NaOCl is unable to penetrate deeper into dentin, and that long-term exposure to high concentrations of sodium hypochlorite can have a negative effect on dentin elasticity and flexural strength, predisposing the tooth to vertical fractures [2,3]. Clinical endodontics does not yet have a non-toxic irrigation solution capable of eliminating biofilm. As a result, research on the development of novel antimicrobial methods is extremely significant.

Because of its strong antibacterial action against a wide range of microbes, silver nanoparticles (AgNPs) have been used successfully in a variety of applications. AgNP has use in dentistry for disinfection, prophylaxis, and infection prevention. Metallic nanoparticles have stood out in scientific studies due to their antibacterial capabilities and biological activity against bacteria, fungus, and enveloped viruses. Since some researchers have chosen to include antimicrobial agents into dental biomaterials, AgNPs have emerged as a viable molecule for usage in dentistry [4].

Super anionic oxygen solution (H_2O_8) has antiviral and antibacterial effects. In this study, the antimicrobial evaluation of super anionic oxygen solution with nanoparticle silver (NpAg) added will be made and it is aimed to compare it with NaOCl and CHX, which are routinely used in endodontic treatment. The results of the study will shed light on clinicians.

MATERIAL AND METHODS

The antimicrobial activity of samples was evaluated with *Enterococcus faecalis* (*E. faecalis*) ATCC 29212 and *Streptococcus mutans* (*S. mutans*) ATCC 25175 strains [5]. Briefly, bacterial suspensions of fresh bacterial cultures were adjusted to McFarland 0.5 turbidity and diluted at a ratio 1:100 in Brain-Heart Infusion (BHI) broth (Merck, Germany) medium. One milliliter of samples was added the tubes and combined with an equal amount of bacteria suspension and allowed to stand for 10 min. Tubes containing only the bacterial suspension were used as positive controls. At the end of the period, samples were taken from each tube, diluted and 20 µl inoculated on BHI agar (Merck, Germany). All petri dishes were incubated overnight at 37 °C. The following day, the number of viable bacteria in each petri dish was calculated. The log and percent reduction values of the samples were also determined.

Statistical Analysis

Each experiment was repeated three times. The results were presented as mean values and standard deviation values of the mean. One-way Anova with Tukey's post-hoc tests ($p < 0.05$; GraphPad Prism version 5) was used to determine statistically significant differences between the treatment and control groups.

RESULT AND DISCUSSION

The antimicrobial effect of NpAg and H_2O_8 (4000 ppm) was investigated on *E. faecalis* (Table 1) and *S. mutans* (Table 2) standard strains in BHI broth. An initial concentration of *E. faecalis* was $1.5x10^6$ colony forming units/mL (CFU/mL) and an initial concentration of *S. mutans* was $1.7x10^6$ CFU/mL. The results are shown in Figure 1. While the concentration of *E. faecalis* was decreased to 0.27 log for NpAg and 0.81 log for H_2O_8, the concentration of *S. mutans* was decreased to 0.38 log for NpAg and 0.43 log for H_2O_8. A statistically significant decrease was observed in bacterial concentration in both solutions compared to the control ($p<0.05$). No significant difference was observed between the effectiveness of the solutions ($p>0.05$). The percentage reduction in *E. faecalis* was 46.67% for NpAg and 84.67% for H_2O_8. It is seen that the efficacy of the samples in *S. mutans* decreased slightly. The percentage reduction on *S. mutans* was found to be 58.8% for NpAg and 62.94% for H_2O_8. For both bacterial samples, there was a decrease in the activity of the Np-rendered sample. On the other hand, chlorhexidine solution (2%), NaOCl solution at concentrations of 5%, 2.5%, and 1% which we used as standards, completely inhibited the all bacteria in the tubes. No bacterial growth was detected and therefore was not included in the Figure 1.

Table 1. Antimicrobial effect of NpAg, H_2O_8, chlorhexidine solution (2%), and NaOCl solution (5%, 2.5%, and 1%) against *E. faecalis* ATCC 29212

Sample	*E. faecalis* ATCC 29212 (CFU/mL)	Log reduction	Percentage reduction
Control	$1.5x10^6$		
NpAg	$0.8\ x10^6$	0.273	46.67
H_2O_8	$2.3x10^5$	0.81	84.67
Chlorhexidine solution, 2%	0	-	100
NaOCl solution, 5%	0	-	100
NaOCl solution, 2.5%	0	-	100
NaOCl solution, 1%	0	-	100

Table 2. Antimicrobial effect of NpAg, H_2O_8, chlorhexidine solution (2%), and NaOCl solution (5%, 2.5%, and 1%) against *S. mutans* ATCC 25175

Sample	*S. mutans* ATCC 25175 (CFU/mL)	Log reduction	Percentage reduction
Control	$1.7x10^6$		
NpAg	$0.7x10^6$	0.38	58.82
H_2O_8	$6.3x10^5$	0.43	62.94
Chlorhexidine solution, 2%	0	-	100
NaOCl solution, 5%	0	-	100
NaOCl solution, 2.5%	0	-	100
NaOCl solution, 1%	0	-	100

E. faecalis is commonly utilized in in vitro testing due to its role in endodontic infections and its ability to persist in an environment with limited nutrition and minimal contact with other bacteria [6]. The functionalization and targeting of antimicrobials in the form of nanoparticles provides an opportunity to effectively combat these pathogens [7]. Interest in the use of metal nanoparticles has increased in recent years. The most commonly used metal nanoparticles are silver, gold and silver-gold, as they easily penetrate cells and do not have toxic effects. However, there are inconsistencies regarding their antimicrobial efficacy, which may be influenced by parameters such as synthesis method, nanoparticle size, bacterial cell structure, and external surface modification [8].

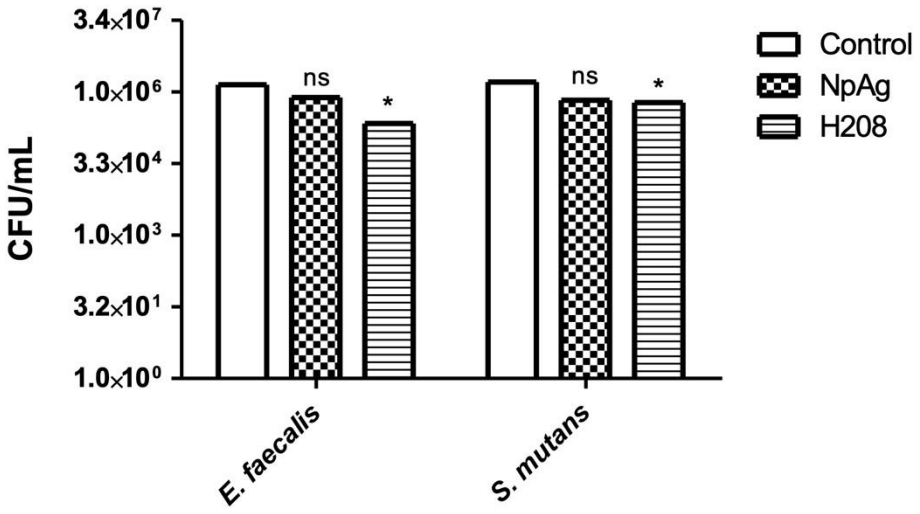


Figure 1. Antimicrobial effect of NpAg and H_2O_8 against test bacteria. *p < 0.05, ns p>0.05 significant difference

In a study conducted using computerized micro tomography, it was determined that 35% or more of the canal surfaces were not touched in the applied canal shaping techniques [9]. This highlights the role of irrigation in endodontics. An ideal endodontic solution should have a broad antimicrobial spectrum, be biocompatible, and not irritate periapical tissues. While this is striking, various solutions are tried in the search for a solution that combines all the features.

Higher concentrations of NaOCl and chlorhexidine solution have been shown to have superior antibacterial properties [10]; but their cytotoxic effects may also rise. On the one hand, medications in larger concentrations kill bacteria more efficiently; on the other, such treatments may cause harm to periradicular tissues and cells [11].

NaOCl has a wide spectrum and the ability to dissolve necrotic tissues. However, when NaOCl overflows from periapical tissues, ecchymosis, edema, sudden pain and bleeding may occur.

It was found that 2% chlorhexidine gluconate had a greater antibacterial effect than 5.25% NaOCl in infected root canals with necrotic pulp and periapical pathology [12]. Since it does not have tissue-dissolving properties, it cannot replace NaOCl.

Therefore, as an alternative to these irrigation solutions, H_2O_8, which has no toxic effects and is reported to have antiviral, antiseptic, antihistaminic, antipuritic and anti-inflammatory effects, and its functionalization in the recently popular nanoparticle form has been considered [13]. However, the results collected in this in vitro study showed that no significant difference was observed between the effectiveness of the solutions. Solutions we routinely use in the clinic, NaOCl solution and chlorhexidine completely inhibited all bacteria in the tubes. Although NpAg and H_2O_8 showed comparatively less antimicrobial effect than NaOCl and chlorhexidine against *E. faecalis* and even less effect against *S. mutans*. There is no study in the literature comparing the antibacterial efficacy of NpAg and H_2O_8 as irrigation solutions.

Based on the limitations of this investigation, it was determined that NpAg and H_2O_8 have a significant antimicrobial flora against *E. faecalis* and *S. mutans*, but are attenuated when compared to irrigants routinely used clinically. Since this study did not simulate the oral environment, future studies may provide more effective results.

ACKNOWLEDGEMENTS

We would like to thank all team members for their dedication, collaboration, and valuable contributions throughout this project. Their efforts were essential to the successful completion of this work.

AUTHOR CONTRIBUTION

Concept: D.G., F.G.Z.; Design: D.G., H.B.E., F.G.Z., B.K.; Control: D.G., H.B.E., F.G.Z.; Sources: D.G., H.B.E; Materials: D.G., H.B.E.; Data Collection and/or Processing: H.B.E., B.K.; Analysis and/or Interpretation: D.G., H.B.E., B.K.; Literature Review: D.G., H.B.E.; Manuscript Writing: D.G., H.B.E.; Critical Review: D.G., H.B.E.; Other: D.G., H.B.E., F.G.Z., B.K.

CONFLICT OF INTEREST

The authors declare that there is no real, potential, or perceived conflicts of interest for this article.

ETHICS COMMITTEE APPROVAL

The authors declare that ethics committee approval is not required for this study.

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J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 49(3): 790-808, 2025
Doi: 10.33483/jfpau.1588979

ORIGINAL ARTICLE / ÖZGÜN MAKALE

IDENTIFICATION OF SIRTUIN-ACTIVATING BIOACTIVE FROM *TARAXACUM OFFICINALE* THROUGH VIRTUAL DISCOVERIES FOR ANTI-AGING AND STRESS RESISTANCE APPLICATIONS

TARAXACUM OFFICINALE'DEN SİRTUİN AKTİVE EDEN BİYOAKTİFİN ANTİ-YAŞLANMA VE STRES DİRENCİ UYGULAMALARI İÇİN SANAL KEŞİFLER YOLUYLA TANIMLANMASI

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ABSTRACT

Objective: *This study aims to investigate the interactions between sirtuins, a class of NAD+-dependent deacetylases, and bioactive ligands derived from Taraxacum officinale, focusing on their potential to modulate pathways associated with aging and stress resistance.*
Material and Method: *A comprehensive dataset of ligands was compiled from Dr. Duke's Phytochemical and Ethnobotanical Databases, and assessed for ADMETox (absorption, distribution, metabolism, excretion, and toxicity) properties using SwissADME. For virtual screening, AutoDock Vina was employed to perform molecular docking between the active sites of Sirt1-7 enzymes and a library of 51 bioactive compounds from Taraxacum officinale. Finally, BIOVIA Discovery Studio 2024 was utilized for the visualization of protein-ligand interactions.*
Result and Discussion: *The observed protein-ligand interactions highlight the potential of Taraxacum officinale bioactive compounds to modulate sirtuins, which may lead to beneficial effects on metabolic health and cellular resilience. In particular, the compounds such as taraxerol, taraxasterol, and beta-amyrin, appear in top 10 highest having strong interaction to sirtuin protein (Sirt1-7), underscore the significance of Taraxacum officinale bioactive in therapeutic strategies aimed at targeting aging and stress-related conditions. This study serves as a valuable foundation for discovering novel therapeutic agents that target sirtuins to promote healthy aging and enhance stress resilience.*
Keywords: *Anti-aging, sirtuin, stress resistance, Taraxacum officinale, virtual screening*

ÖZ

Amaç: *Bu çalışma, NAD+-bağımlı deasetilazlar sınıfı olan sirtuinler ile Taraxacum officinale'den türetilen biyolojik aktif liganlar arasındaki etkileşimleri incelemeyi amaçlamaktadır. Çalışma, bu etkileşimlerin yaşlanma ve stres direnci ile ilişkili yolları modüle etme potansiyeline odaklanmaktadır.*

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Submitted / Gönderilme : 29.11.2024
Accepted / Kabul : 12.05.2025
Published / Yayınlanma : *early access*

Gereç ve Yöntem: *Dr. Duke'un Fitokimyasal ve Etnobotanik Veritabanlarından bir ligan veri seti derlenmiş ve SwissADME kullanılarak ADMETox (emilim, dağılım, metabolizma, atılım ve toksisite) özellikleri açısından değerlendirilmiştir. Sanal tarama için, Sirt1-7 enzimlerinin aktif bölgeleri ile Taraxacum officinale'den elde edilen 51 biyolojik aktif bileşen kütüphanesi arasında moleküler yerleştirme yapmak üzere AutoDock Vina kullanılmıştır. Son olarak, protein-ligan etkileşimlerinin görselleştirilmesi için BIOVIA Discovery Studio 2024 kullanılmıştır.*

Sonuç ve Tartışma: *Gözlemlenen protein-ligand etkileşimleri, Taraxacum officinale biyoaktif bileşiklerinin sirtuinleri düzenleme potansiyelini vurgular ve bu da metabolik sağlık ve hücresel dayanıklılık üzerinde faydalı etkilere yol açabilir. Özellikle, taraxerol, taraxasterol ve beta-amirin gibi bileşikler, sirtuin proteini (Sirt1-7) ile güçlü etkileşime sahip en yüksek 10'da yer alır ve yaşlanma ve stresle ilişkili durumları hedeflemeyi amaçlayan terapötik stratejilerde Taraxacum officinale biyoaktifinin önemini vurgular. Bu çalışma, sağlıklı yaşlanmayı desteklemek ve stres dayanıklılığını artırmak için sirtuinleri hedefleyen yeni terapötik ajanları keşfetmek için değerli bir temel görevi görür.*

Anahtar Kelimeler: *Anti-aging, sanal tarama, sirtuin, stres direnci, Taraxacum officinale*

INTRODUCTION

Aging in humans involves complex biological changes that lead to decreased function and increased disease susceptibility due to a combination of genetic, environmental, and lifestyle factors [1]. Key mechanisms driving aging that leads to the deterioration of an organism include [2]: 1) Cellular senescence, where damaged cells become inactive and promote inflammation [3], 2) Oxidative stress, which results from an imbalance between reactive oxygen species and antioxidant defenses [4], 3) Telomere shortening, which limits the number of cell divisions [5], and 4) Mitochondrial dysfunction, which reduces energy production [6]. Understanding these complicated processes, particularly their interactions with hormones, genetics, and the immune system, is vital [7].

Chronic stress can accelerate aging by increasing inflammation, oxidative stress, and cellular damage, leading to age-related diseases such as cardiovascular issues and neurodegeneration [8]. In contrast, strong stress resistance, which characterized by effective coping mechanisms and adaptive responses, can mitigate these harmful effects, promoting cellular repair and longevity [9], thus, effective stress management and resilience critical for promoting longevity [10]. Given the critical role of these mechanisms in aging, the sirtuin family emerges as a key player in modulating these processes and enhancing cellular resilience.

The sirtuin family consists of seven mammalian proteins (SIRT1-7) that are NAD+-dependent deacetylases, playing crucial roles in various cellular processes such as histone deacetylation, metabolism regulation, oxidative stress response, and potential links to longevity [11]. SIRT1, primarily found in the nucleus, influences gene expression, cell survival, and stress response. It particularly well-known for its regulatory functions in metabolism and gene expression. SIRT1 achieves this by deacetylating lysine residues on histones and transcription factors such as p53 and NF-κB, thereby altering chromatin structure and influencing gene activity. This process is closely linked to cellular energy status, as SIRT1 activity is dependent on NAD+ availability. By enhancing fat oxidation and reducing lipogenesis through deacetylation of metabolic regulators such as PGC-1α, SIRT1 has a significant impact on metabolic pathways [12]. SIRT2 and SIRT3 also contribute to important cellular functions. SIRT2 primarily operates in the cytoplasm, where it regulates the cell cycle by deacetylating tubulin, thereby influencing microtubule dynamics and mitotic progression. In addition, it interacts with proteins associated with neurodegenerative diseases, promoting cell survival [13]. In contrast, SIRT3 is located in mitochondria and focuses on regulating mitochondrial metabolism and oxidative stress responses. SIRT3 deacetylates key mitochondrial enzymes to increase their activity, activates antioxidant enzymes such as superoxide dismutase, and promotes fatty acid oxidation, thus playing a critical role in energy metabolism and cellular health [14].

The remaining sirtuins, SIRT4 through SIRT7, also have unique functions. SIRT4, located in mitochondria, combines deacetylase and ADP-ribosyltransferase activities to regulate insulin secretion and modify protein function [15]. SIRT5 is involved in regulating metabolic pathways, increasing

enzyme activity through deacetylation and demalonylation [16]. SIRT6 and SIRT7 are important for DNA repair and ribosome biogenesis, respectively. SIRT6 maintains genomic stability through its role in the base excision repair pathway and influences glucose metabolism [17], while SIRT7 enhances protein synthesis by deacetylating ribosome assembly proteins and regulating ribosomal RNA transcription [18]. Together, these sirtuins form a complex network that modulates vital physiological processes and cellular responses to stress. Sirtuins are integral to maintaining cellular homeostasis and responding to stressors, making them potential therapeutic targets for age-related diseases and metabolic disorders [19].

The selection of sirtuins (1 to 7) as therapeutic targets, compared to other potential receptors, was based on several important criteria: their critical role in regulating important biological processes such as metabolism, aging, and cellular stress responses; their association with a variety of diseases, including neurodegenerative disorders and cancer [20]; their unique functions and substrate specificity, which allow for tailored therapeutic strategies; their proven therapeutic potential in preclinical studies; and the existence of specific inhibitors or activators that support drug research and development [21]. These considerations underscore the importance of sirtuins in health and disease, positioning them as promising candidates for targeted therapies.

Taraxacum officinale, commonly known as dandelion, has a long history of use as a traditional herbal remedy, with its diverse medicinal properties in various cultures around the world. Dandelion is recognized for its potential health benefits, including anti-inflammatory, antioxidant, and diuretic effects [22]. The roots, leaves, and flowers of dandelion are rich in bioactive compounds, such as polyphenols, triterpenes, and vitamins, which contribute to its therapeutic applications. Traditionally, dandelion has been utilized to support digestive health, enhance liver function, and promote overall well-being. It has adaptability to various environments and present widespread across Europe, Asia, and North America, and makes it a very interesting subject for modern studies of herbal therapies and their possible roles in modern healthcare [23].

This study explores the potential of *Taraxacum officinale* bioactive using virtual screening techniques to identify promising therapeutic candidates that can positively influence sirtuin activity, thereby potentially mitigating the effects of aging and chronic stress. Virtual screening techniques are an essential part of drug development, especially in targeting sirtuins, which are significant regulators in aging and stress resistance [24]. Virtual screening, by simulating the interactions between bioactive compounds and sirtuin proteins, identifies and prioritizes potential therapeutic candidates, accelerating the process of discovery [25]. This method provides insight into the mechanisms at the molecular level, increases cost-effectiveness by reducing the workload in the laboratory, and is integrated with experimental validation for confirming biological activity. Ultimately, the study aspires to contribute to the development of new strategies for enhancing longevity and improving health outcomes by targeting the sirtuin pathway through naturally derived bioactive compounds.

MATERIAL AND METHOD

System Configuration

Molecular docking was conducted using PyRx from The Scripps Research Institute, which incorporates AutoDock Vina as the docking engine [26]. Open Babel (integrated in PyRx) was used to prepared the ligand (compounds) which was imported in the form of SDF (structured data file), minimization of the energy, and convert them to PDB and further in AutoDock Vina to PDBQT format which stand for Protein Data Bank Partial Charges (Q) and Torsions (T).

The process predicted the binding affinity of *Taraxacum officinale* constituents within target proteins, with visualizations generated by Biovia Discovery Studio 2024 (Dassault Systèmes). Computations were performed on Personal Computer (PC) 11th Gen (Rocket Lake) Intel i9 processor with 8.00 GB RAM.

Ligand Structure Preparation

Three-dimensional structures of potential ligands were retrieved from the PubChem, an open chemistry database at The National Center for Biotechnology Information (NCBI), a division of the

National Library of Medicine (NLM) at the U.S. National Institutes of Health. The structures of 51 compounds were selected from a list of 280 bioactive compounds of Taraxacum officinale, taken from Dr. Duke's Phytochemical and Ethnobotanical Databases of the U.S. Department of Agriculture [27]. The selection process involves retrieving three-dimensional structures of potential ligands from PubChem. Systematic evaluation includes bioactivity, structural characteristics, and relevance to the research objectives. This careful selection aims to focus on the most promising candidates for further investigation into their therapeutic potential.

Table 1. Classification of bioactive compounds and potential biological activities

Category	Compound Name	CID	Notes
Amino Acids	Aspartic Acid	5960	Essential for neurotransmission and metabolic functions
	Glutamic Acid	33032	
Vitamins	Thiamine	1130	Plays crucial roles in metabolic processes
	Riboflavin	493570	
	Alpha-Tocopherol	14985	
Organic Acids	4-Hydroxyphenylacetic Acid	127	Antioxidant and anti-inflammatory properties
	Nicotinic Acid	938	
	Vanillic Acid	8468	
	Ferulic Acid	445858	
	p-Coumaric Acid	637542	
	Caffeic Acid	689043	
	Chlorogenic Acid	1794427	
	D-Glucuronic Acid	94715	
	2-Carboxyarabinitol	5165850	
	4-Oxopentanoate	5177120	
	L-Tartaric Acid	444305	
Sterols and Triterpenes	Beta-Sitosterol	222284	Contributes to membrane stability and signaling
	Campesterol	173183	
	Beta-Amyrin	73145	
	Taraxerol	92097	
	Cycloartenol	92110	
	Stigmasterol	5280794	
	Taraxasterol	115250	
	Faradiol	9846222	
	Arnidiol	10478550	
	Cycloartanol	12760132	
	31-Norcycloartenol	14524546	
	(+)-Taraxerol	42608290	
Flavonoids	Quercetin	5280343	Modulates oxidative stress and inflammation
	Luteolin	5280445	
	Isoquercetin	5280804	
	Luteolin 7-O-glucoside	5280637	
	Apigetrin	5280704	
	Isorhamnetin	5281654	
	Neoxanthin	5282217	
	Jasmonic Acid	5281166	
	Antheraxanthin	5281223	
	Cryptoxanthin	5281235	
	Flavoxanthin	5281238	
	Lutein	5281243	
	Lutein 5,6-epoxide	5281244	
Other Compounds	Scopoletin	5280460	Significant for health promotion
	Esculetin	5281416	
	Coumestrol	5281707	
	Quercimeritrin	5282160	
	Sitogluside	5742590	
	Androsterol	7056608	
	Germacranolide	91694425	
	Taraxacoside	131750952	
	Taraxacin	5241825	
	Violaxanthin	448438	

The classification of bioactive compounds highlights a diverse array of chemical structures, each with potential biological activities that are crucial for various physiological functions. These compounds were organized into several categories based on their chemical nature and biological roles, and divided into 6 category group as presented in Table 1. The category includes amino acids, vitamins, organic acids, sterols and triterpenes, flavonoids, and other compounds that do not belong to the previous categories. The table organizes the compounds by category, along with their CID numbers and brief notes on their significance.

The energy of the ligand structure was minimized using Open Babel with the Universal Force Field (UFF) parameters. UFF calculates the energy of a molecular system by considering various interactions, including bond stretching, angle bending, torsional (dihedral) angles, and non-bonded interactions such as van der Waals forces and electrostatics. Optimization algorithm in minimization of energy was used with 1000 number of steps. This step was essential to ensure that the ligands were in their most stable conformations prior to docking. Proper energy minimization helps improve the accuracy of subsequent docking simulations.

Sirtuin Macromolecules

This research focused on proteins associated with Sirtuin activity, crucial for regulating aging and stress resistance [28]. Sirtuin protein structures (Table 2) were retrieved from the Research Collaboratory for Structural Bioinformatic (RCSB) Protein Data Bank, prepared for docking by removing water and adding hydrogen atoms using Biovia Discovery Studio.

Table 2. Key functions of sirtuins related to aging and stress resistance

Protein Name	Function
Sirtuin 1 (PDB code: 4i5i) [29]	Involved in lifespan extension, DNA repair, and cellular senescence.
Sirtuin 2 (PDB code: 8tgp) [30]	Regulates the cell cycle.
Sirtuin 3 (PDB code: 3gls) [31]	Manages mitochondrial activity and oxidative stress; linked to longevity-associated SNPs.
Sirtuin 4 (PDB code: 5ojn) [32]	Engages in fatty acid oxidation and apoptosis.
Sirtuin 5 (PDB code: 2nyr) [33]	Contributes to fatty acid oxidation and oxidative stress management.
Sirtuin 6 (PDB code: 3k35) [34]	Supports lifespan extension, DNA repair, and telomere maintenance.
Sirtuin 7 (PDB code: 5iqz) [35]	Involved in epigenetic regulation and stress resistance.

ADMETox Prediction

ADMETox encompasses the evaluation of a compound's Absorption, Distribution, Metabolism, Excretion, and Toxicity, all critical for assessing its pharmacokinetic profile and safety in drug development. Absorption measures the compound's ability to cross biological membranes, while distribution evaluates its spread throughout the body [36]. Metabolism examines how the compound is chemically altered, affecting efficacy and safety, and excretion focuses on its elimination pathways, primarily via urine or bile. Toxicity assesses potential harmful effects on biological systems [37]. Together, these parameters provide vital insights into a drug candidate's viability and guide further development.

In-silico ADME prediction was performed using the SwissADME platform [38] to evaluate the drug-like properties of identified compounds, focusing on key pharmacokinetic parameters: absorption, distribution, metabolism, and excretion. This analysis prioritized candidates for further investigation. In addition, bioactive toxicity was predicted using ADMETlab 3.0 application developed by Computational Biology & Drug Design Group (CBDD) [39].

Lipinski's Rule of Five, one of the fundamental chemoinformatics filters for bioavailability, is central among property filters. It focuses chemical space toward drug-like narratives and avoids ADMET problems through a set of rules: (molecular weight (MW) $\leq$ 500 Da, logP $\leq$ 5, hydrogen bond donor (HBD) $\leq$ 5, hydrogen bond acceptor (HBA) $\leq$ 10, and number of rotatable bonds ($\leq$ 10) [40]. The Ghose filter identifies pharmacokinetic parameters for drug-likeness: 160 < molecular weight < 480 Da, -0.4 < logP < 5.6, 20 < number of atoms < 70 and 40 < molar refractivity < 130. The Veber filter

contains two simple rules (total polar surface area (TPSA) ≤ 140 Å2, bond rotation ≤ 10) that compounds must obey to optimize bioavailability. According to the Egan filter, compounds are likely to permeate cell membranes if they meet the following two criteria: a logP value of 5.88 or less and a topological polar surface area (TPSA) of 131.6 Å² or less [41]. Finally, Muegge's rule states that the soft mass should be less than 600 and the topological polar area should not exceed 150 [42]. Collectively, these rules guide the evaluation of a compound's drug-likeness and potential bioavailability.

Calculation of Binding Affinity

Ligand and receptor structures were imported into PyRx for molecular docking simulations using the AutoDock Vina algorithm. Parameters such as grid box (size and dimensions) and exhaustiveness (set to 8) were fine-tuned for both efficiency and accuracy in vina search space. The grid box defines the area in which ligands are allowed to dock, and refers to the 3D space around the target protein where the docking calculations are performed. The grid box is differed for each sirtuin protein (Table 3). Exhaustiveness affects the depth of the search in the conformational space, which is differ with iterations (number of optimization attempts). Higher exhaustiveness value gives more extensive search, exploring more conformations and orientations of the ligand.

Table 3. Grid box parameter of sirtuin

Sirtuin	PDB Code	Center coordinate			Dimensions (Å)		
		X	Y	Z	X	Y	Z
SIRT1	4i5i	26.59	15.43	22.69	73.94	79.68	51.05
SIRT2	8tgp	-8.16	-9.41	-6.73	37.32	56.28	59.76
SIRT3	3gls	-25.75	-25.01	20.57	140.93	106.02	100.13
SIRT4	5ojn	11.60	22.32	-14.90	48.00	42.36	63.25
SIRT5	2nyr	10.36	-15.41	11.00	58.02	66.79	66.00
SIRT6	3k35	8.54	21.69	-11.03	111.23	84.44	109.08
SIRT7	4iqz	-1.73	18.16	15.41	74.85	75.48	66.68

The resulting docking poses were assessed based on binding affinity values, with lower values indicating stronger interactions. To ensure the reliability of the docking protocol, a validation process was implemented. This involved re-docking known binders to the target protein and comparing the predicted binding modes and affinity values with previously published data [43,44].

Data Analysis and Visualization

Binding affinities of compounds derived from *Taraxacum officinale* to Sirtuin proteins were calculated in PyRx and exported in CSV format. Compounds with binding energies below a certain threshold (e.g., -7.0 kcal/mol) were considered for further analysis [45]. Values below this threshold suggest a high likelihood of significant binding affinity, making compounds more promising candidates for further biological evaluation. Interactions were visualized using Discovery Studio, offering insights into binding mechanisms and structure-activity relationships.

RESULT AND DISCUSSION

Compound Library Characterization

To explore potential therapeutic agents for Sirtuin modulation, a diverse compound library was assembled. The library comprises 51 bioactive compounds with a range of chemical structures, which is contain of 2 amino acids, 3 vitamins, 11 organic acids, 12 sterols and triterpenes, 13 flavonoids, and 10 other compounds.

Molecular weights vary from 115.11 g/mol (4-Oxopentanoate) to 600.87 g/mol (Neoxanthin), while logP values range from -3.59 (Aspartic Acid) to 7.07 (Cycloartanol), indicating diverse solubility profiles. Several compounds align with Lipinski's Rule of Five criteria, suggesting favorable

pharmacological properties, which positions the library as a promising source for new therapeutic agents aimed at Sirtuin modulation.

Bioavailability Assessment and Lipinski's Rule of Five

This study evaluated bioactive compounds from *Taraxacum officinale* for their drug-likeness properties, which are demonstrated by the SwissADME Bioavailability Radar, showing assesses key physicochemical properties essential for bioavailability: Lipophilicity, Size, Polarity, Solubility, Flexibility, and Saturation. Analysing these characteristics aids in identifying strengths and weaknesses, thereby informing the development of effective therapeutic agents. Table 4 summarizes the findings of this assessment, providing insights into the bioavailability profiles of the selected compounds.

The boiled egg model in SwissADME is a visual tool used to evaluate drug candidates' pharmacokinetic properties, specifically their ability to penetrate the blood-brain barrier (BBB) and achieve human intestinal absorption (HIA) [46]. In this model, the yolk represents compounds that can effectively cross the BBB due to their lipophilicity, while the egg white symbolizes those that are well-absorbed in the intestine. Additionally, compounds are classified based on their interaction with P-glycoprotein (PGP): PGP+ compounds are substrates that may face reduced absorption and BBB penetration, whereas PGP- compounds are less likely to interact with PGP, enhancing their bioavailability and efficacy. This model helps researchers optimize drug design for better therapeutic outcomes. Figure 1 shows ADME profile of *Taraxacum officinale* bioactive in the form of boiled-egg that shows absorption probability of the bioactive.

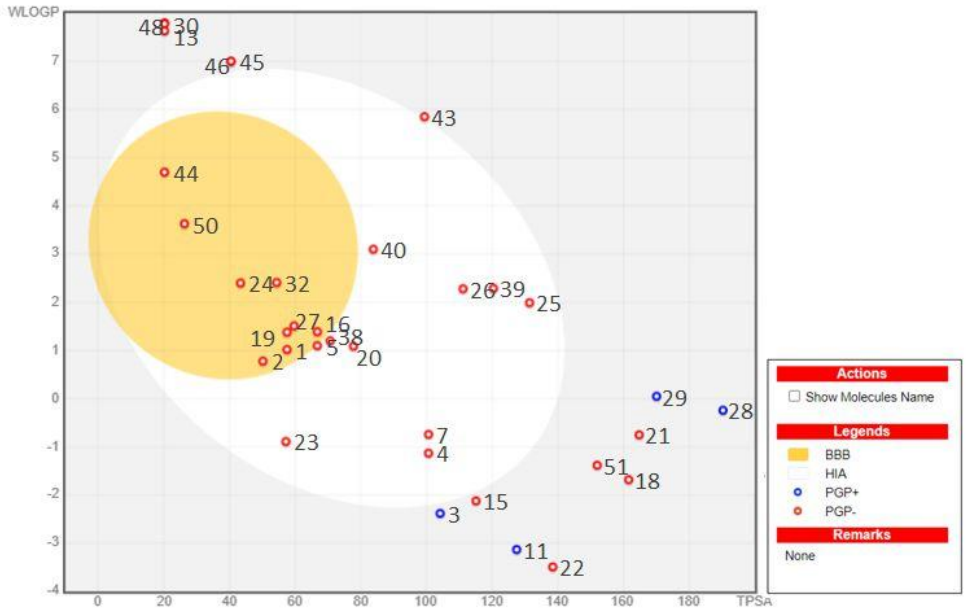
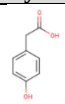
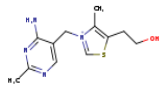
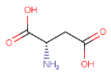
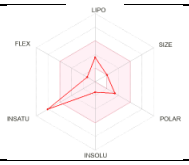
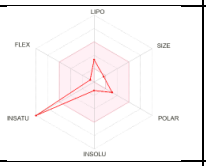
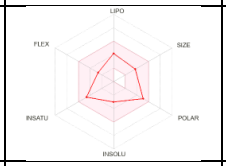
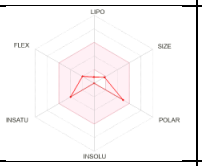
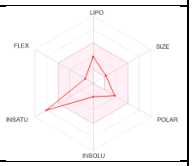
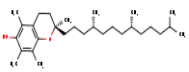
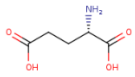
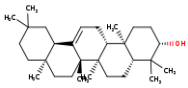
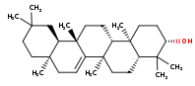
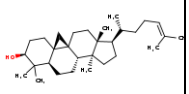
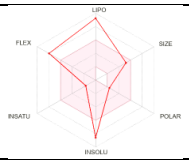
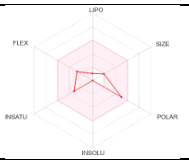
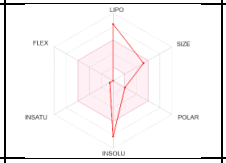
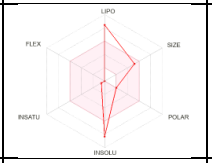
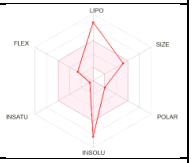


Figure 1. SwissADME Boiled-egg of *Taraxacum officinale* bioactive (Numbers represent *Taraxacum officinale* bioactive as listed in Table 4)

Table 4. Assessment of bioactive compounds for drug-likeness

No	1	2	3	4	5
Chem. Name	**4-Hydroxy-phenylacetic**	**Nicotinic acid**	**Vitamin B1**	**Aspartic Acid**	**Vanillic Acid**
Structure					
Bioavail-ability radar					
Lipinski #vio.	**0***	**0***	**0***	**0***	**0***
Ghose #vio	**2**	**3**	**1***	**4**	**0***
Veber #vio	**0***	**0***	**0***	**0***	**0***
Egan #vio	**0***	**0***	**0***	**0***	**0***
Muegge #vio	**1***	**1***	**0***	**3**	**1***
HIA	High×	High×	Low	High×	High×
BBB perm.	Yes×	Yes×	No	No	No
Bioav. Score	0.85×	0.85×	0.55	0.56	0.85×
Hep. Tox.	Med	Med	Low°	Med	Med
Neu. Tox.	Low°	Med	Low°	Low°	Med
Geno Tox.	Med	Low°	Poor	Low°	Low°

No	6	7	8	9	10
Chem. Name	**Alpha-Tocopherol**	**Glutamic Acid**	**Beta-Amyrin**	**Taraxerol**	**Cycloartenol**
Structure					
Bioavail-ability radar					
Lipinski #vio.	**1***	**0***	**1***	**1***	**1***
Ghose #vio	**3**	**4**	**3**	**3**	**3**
Veber #vio	**1***	**0***	**0***	**0***	**0***
Egan #vio	**1***	**0***	**1***	**1***	**1***
Muegge #vio	**1***	**2**	**2**	**2**	**2**
HIA	Low	High×	Low	Low	Low
BBB perm.	No	No	No	No	No
Bioav. Score	0.55	0.56	0.55	0.55	0.55
Hep. Tox.	Poor	Low°	Med	Med	Med
Neu. Tox.	Low°	Low°	Low°	Low°	Low°
Geno Tox.	Low°	Low°	Med	Low°	Low°

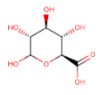
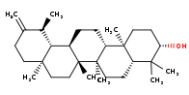
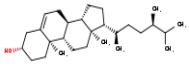
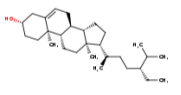
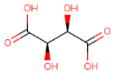
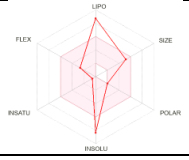
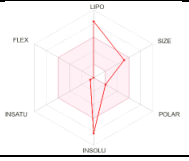
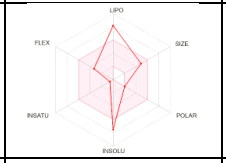
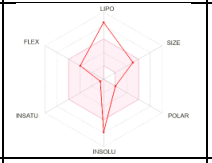
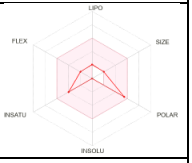
No	11	12	13	14	15
Chem. Name	**D-Glucuronic Acid**	**Taraxasterol**	**Campesterol**	**Beta-Sitosterol**	**L-Tartaric acid**
Structure					
Bioavail-ability radar					
Lipinski #vio.	**0***	**1***	**1***	**1***	**0***
Ghose #vio	**2**	**3**	**2**	**3**	**4**

Table 4 *(continue)*. Assessment of bioactive compounds for drug-likeness

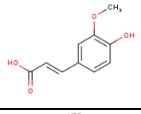
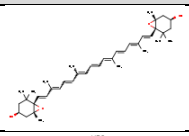
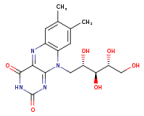
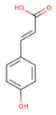
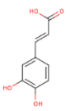
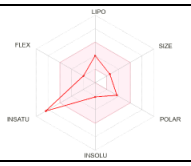
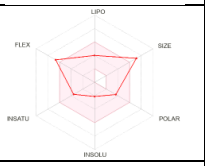
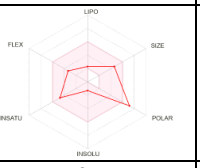
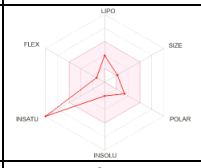
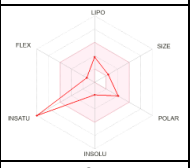
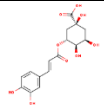
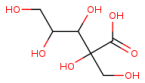
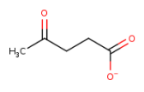
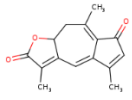
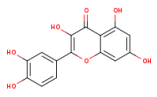
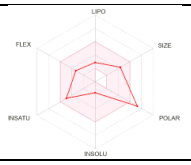
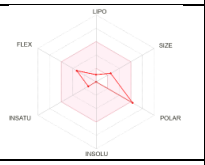
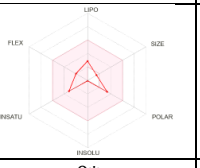
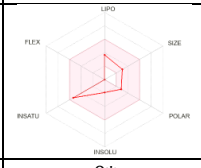
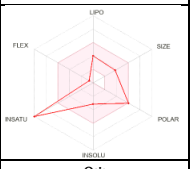
No	11	12	13	14	15
Chem. Name	**D-Glucuronic Acid**	**Taraxasterol**	**Campesterol**	**Beta-Sitosterol**	**L-Tartaric acid**
Veber #vio	**0***	**0***	**0***	**0***	**0***
Egan #vio	**0***	**1***	**1***	**1***	**0***
Muegge #vio	**2**	**2**	**2**	**2**	**2**
HIA	Low	Low	Low	Low	Low
BBB perm.	No	No	No	No	No
Bioav. Score	0.56	0.55	0.55	0.55	0.56
Hep. Tox.	Med	Med	Med	Med	Med
Neu. Tox.	Low°	Low°	Low°	Low°	Low°
Geno Tox.	Low°	Med	Low°	Low°	Low°
No	16	17	18	19	20
Chem. Name	**Ferulic acid**	**Violaxanthin**	**Riboflavin**	**p-Coumaric acid**	**Caffeic Acid**
Structure					
Bioavail-ability radar					
Lipinski #vio.	**0***	**0***	**0***	**0***	**0***
Ghose #vio	**0***	**0***	**1***	**0***	**0***
Veber #vio	**0***	**0***	**1***	**0***	**0***
Egan #vio	**0***	**0***	**1***	**0**	**0***
Muegge #vio	**1***	**1***	**1***	**1***	**1***
HIA	High×	High×	Low	High×	High×
BBB perm.	Yes×	Yes×	No	Yes×	No
Bioav. Score	0.85×	0.85×	0.55	0.85×	0.56
Hep. Tox.	Poor	Low°	Poor	Poor	Med
Neu. Tox.	Low°	Low°	Poor	Low°	Low°
Geno Tox.	Low°	Low°	Poor	Low°	Med
No	21	22	23	24	25
Chem. Name	**Chlorogenic Acid**	**2-Carboxy-arabinitol**	**4-Oxopentanoate**	**Taraxacin**	**Quercetin**
Structure					
Bioavail-ability radar					
Lipinski #vio.	1*	1*	0*	0*	0*
Ghose #vio	**1***	**2**	**4**	**0***	**0***
Veber #vio	**1***	**0***	**0***	**0***	**0***
Egan #vio	**1***	**1***	**0***	**0***	**0***
Muegge #vio	**2**	**3**	**1***	**0***	**0***
HIA	Low	Low	High×	High×	High×
BBB perm.	No	No	No	Yes×	No
Bioav. Score	0.11	0.56	0.85×	0.55	0.55
Hep. Tox.	Med	Low°	Med	Poor	Med
Neu. Tox.	Low°	Low°	Low°	Poor	Low°
Geno Tox.	Low°	Low°	Low°	Poor	Poor

Table 4 *(continue)*. Assessment of bioactive compounds for drug-likeness

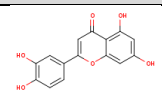
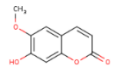
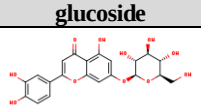
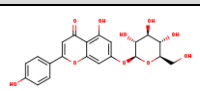
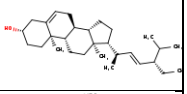
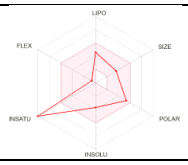
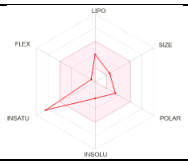
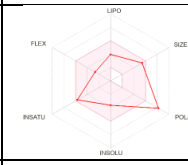
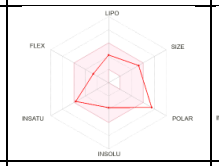
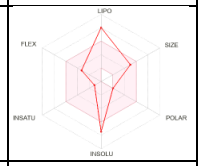
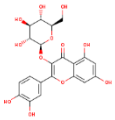
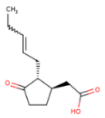
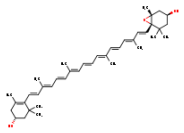
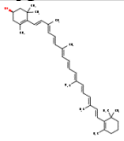
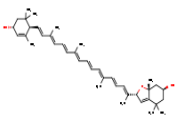
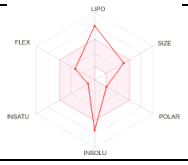
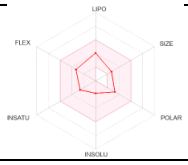
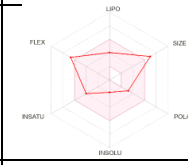
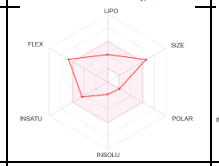
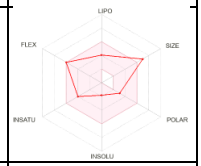
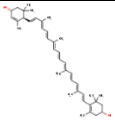
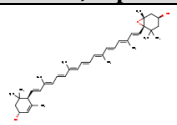
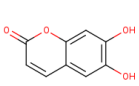
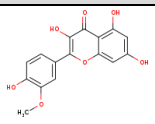
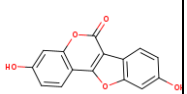
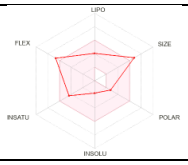
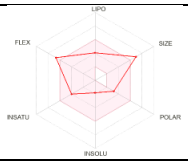
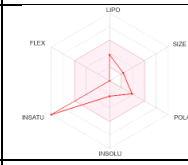
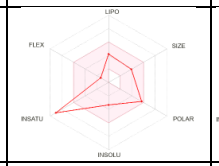
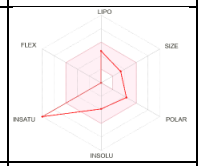
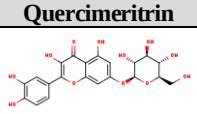
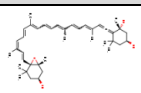
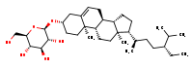
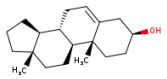
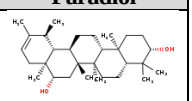
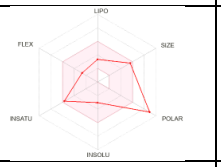
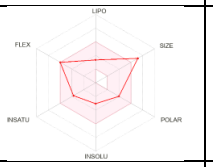
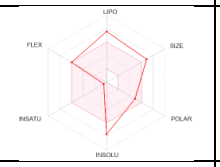
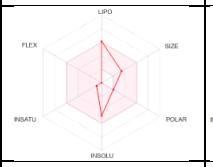
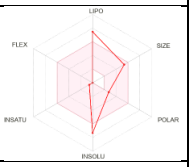
No	26	27	28	29	30
Chem. Name	**Luteolin**	**Scopoletin**	**Luteolin 7-O-glucoside**	**Apigetrin**	**Stigmasterol**
Structure					
Bioavail-ability radar					
Lipinski #vio.	0*	0*	**2**	1*	1*
Ghose #vio	**0***	**0***	**0***	**0***	**3**
Veber #vio	**0***	**0***	**1***	**1***	**0***
Egan #vio	**0***	**0***	**1***	**1***	**1***
Muegge #vio	**0***	**1***	**3**	**2**	**2**
HIA	High×	High×	Low	Low	Low
BBB perm.	No	Yes×	No	No	No
Bioav. Score	0.55	0.55	0.17	0.55	0.55
Hep. Tox.	Med	Med	Med	Med	Med
Neu. Tox.	Low°	Low°	Low°	Low°	Low°
Geno Tox.	Poor	Poor	Poor	Poor	Low°
No	31	32	33	34	35
Chem. Name	**Isoquercetin**	**Jasmonic Acid**	**Antheraxanthin**	**Cryptoxanthin**	**Flavoxanthin**
Structure					
Bioavail-ability radar					
Lipinski #vio.	**2**	0*	0*	0*	0*
Ghose #vio	**1***	**0***	**0***	**0***	**0***
Veber #vio	**1***	**0***	**0***	**0***	**0***
Egan #vio	**1***	**0***	**0***	**0***	**0***
Muegge #vio	**3**	**0***	**0***	**0***	**0***
HIA	Low	High×	High×	High×	High×
BBB perm.	No	Yes×	Yes×	Yes×	Yes×
Bioav. Score	0.17	0.85×	0.85×	0.85×	0.85×
Hep. Tox.	Med	Med	Med	Med	Med
Neu. Tox.	Low°	Med	Med	Med	Poor
Geno Tox.	Poor	Low°	Med	Low°	Poor
No	36	37	38	39	40
Chem. Name	**Lutein**	**Lutein 5,6-epoxide**	**Esculetin**	**Isorhamnetin**	**Coumestrol**
Structure					
Bioavail-ability radar					
Lipinski #vio.	**0***	**0***	**0***	**0***	**0***

Table 4 *(continue).* Assessment of bioactive compounds for drug-likeness

No	36	37	38	39	40
Chem. Name	**Lutein**	**Lutein 5,6-epoxide**	**Esculetin**	**Isorhamnetin**	**Coumestrol**
Ghose #vio	**0***	**0***	**1***	**0***	**0***
Veber #vio	**0***	**0***	**0***	**0***	**0***
Egan #vio	**0***	**0***	**0***	**0***	**0***
Muegge #vio	**0***	**0***	**1***	**0***	**0***
HIA	High×	High×	High×	High×	High×
BBB perm.	Yes×	Yes×	No	No	No
Bioav. Score	0.85×	0.85×	0.55	0.55	0.55
Hep. Tox.	Med	Med	Med	Med	Med
Neu. Tox.	Poor	Poor	Low°	Low°	Low°
Geno Tox.	Med	Poor	Poor	Poor	Poor

No	41	42	43	44	45
Chem. Name	**Quercimeritrin**	**Neoxanthin**	**Sitogluside**	**Androsterol**	**Faradiol**
Structure					
Bioavail-ability radar					
Lipinski #vio.	**2**	**2**	**1***	**1***	**1***
Ghose #vio	**1***	**1***	**4**	**0***	**3**
Veber #vio	**1***	**1***	**0***	**0***	**0***
Egan #vio	**1***	**1***	**0***	**0***	**1***
Muegge #vio	**3**	**3**	**1***	**2**	**1***
HIA	Low	Low	Low	High×	Low
BBB perm.	No	No	No	Yes×	No
Bioav. Score	0.17	0.17	0.55	0.55	0.55
Hep. Tox.	Med	Med	Med	Med	Med
Neu. Tox.	Low°	Med	Low°	Low°	Low°
Geno Tox.	Poor	Med	Low°	Low°	Med

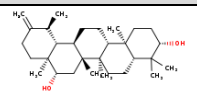
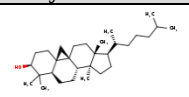
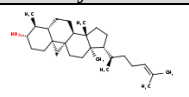
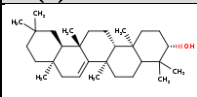
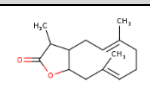
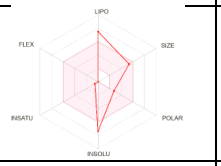
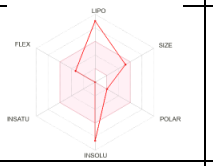
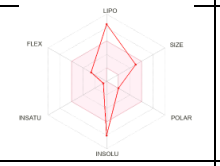
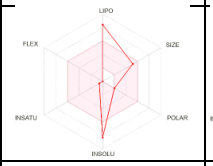
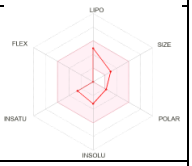
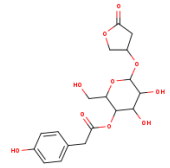
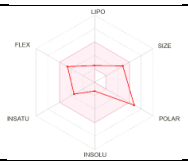
No	46	47	48	49	50
Chem. Name	**Arnidiol**	**Cycloartanol**	**31-Norcycloartenol**	**(+)-Taraxerol**	**Germacranolide**
Structure					
Bioavail-ability radar					
Lipinski #vio.	**1***	**1***	**1***	**1***	**0***
Ghose #vio	**3**	**3**	**3**	**3**	**0***
Veber #vio	**0***	**0***	**0***	**0***	**0***
Egan #vio	**1***	**1***	**1***	**1***	**0***
Muegge #vio	**1***	**2**	**2**	**2**	**0***
HIA	Low	Low	Low	Low	High×
BBB perm.	No	No	No	No	Yes×
Bioav. Score	0.55	0.55	0.55	0.55	0.55
Hep. Tox.	Med	Med	Med	Med	Med
Neu. Tox.	Low°	Low°	Low°	Low°	Med
Geno Tox.	Med	Low°	Low°	Low°	Low°

Table 4 *(continue).* Assessment of bioactive compounds for drug-likeness

No	51
Chem. Name	**Taraxacoside**
Structure	
Bioavail-ability radar	
Lipinski #vio.	**0***
Ghose #vio	**1***
Veber #vio	**1***
Egan #vio	**1***
Muegge #vio	**1***
HIA	Low
BBB perm.	No
Bioav. Score	0.55
Hep. Tox.	Poor
Neu. Tox.	Poor
Geno Tox.	Poor

Note:

1. * rule violation is preferable (0-1); ˣ bioavailability high result is preferable; bioavailability score (oral) indicator as: 0.0 – 0.3 = Low; 0.3 – 0.7 = Moderate; 0.7 – 1.0 = High; ° toxicity prefered (low value)
2. Abbreviation: #vio. = violation; HIA = Human intestine absorption; BBB = Blood-brain barrier; Bioavail.Score = Bioavailability score; Hep. Tox. = Hepatotoxicity; Neu. Tox. = Neurotoxicity; Geno. Tox. = Genotoxicity

Table 4 presents the results of drug similarity and toxicity assessments, covering several important metrics. The values highlighted in color are preferred and align with the ADMETox drug similarity rules, namely Lipinski's rule, Ghose's filter, Veber's rule, and Egan's rule, each of which evaluates drug similarity with different emphases. Lipinski's rule focuses on molecular descriptors for drug-like candidates, Ghose's filter assesses molecular descriptors relevant to drug efficacy, Veber's rule considers the number of rotatable bonds and polar surface area, and Egan's rule examines the balance of molecular properties for oral bioavailability, along with additional criteria for drug-likeness [47,48].

Intestinal Absorption (HIA) measures how well a compound is absorbed in the human gut, with higher values (typically above 30% for good absorption) indicating better absorption, which is important for oral efficacy [49]. Blood-Brain Barrier (BBB perm.) permeability is critical for central nervous system (CNS) drugs, with values above 0.3 indicating good permeability and the ability to cross this selective barrier to exert a therapeutic effect [50]. Bioavailability scores assess how effectively a drug reaches its target, with scores above 50% reflecting better absorption and overall bioavailability. Hepatotoxicity (Hep. Tox.) evaluates the potential for liver damage, with lower values (generally below 1 μM for safety) being preferred to ensure safety during drug development [51]. Neurotoxicity (Neu. Tox.) indicates the risk of toxicity to nerve cells; values should ideally be below 10 μM to minimize risk, which is critical for drugs targeting the CNS. Finally, Genotoxicity (Geno Tox.) assesses the potential for genetic damage; values indicating low genotoxicity should be below 1 for a good safety profile in drug candidates, as they help prevent mutations and cancer risk [52].

Docking Analysis of *Taraxacum officinale* Bioactive with Sirtuin

Table 5 represents the best 10 docking results, showing the binding affinities of various bioactive compounds from *Taraxacum officinale* with the sirtuin family which having higher interaction (less than -7.0 kcal/mol) [53], highlighting their potential interactions based on binding affinity. Binding free

energy, expressed in kcal/mol, quantifies the thermodynamic favorability of binding interactions; however, the term binding affinity is often used interchangeably in molecular docking studies, including in PyRx, to describe these interactions.

Table 5. Docking result (best 10) of *Taraxacum officinale* bioactive with sirtuins target showing its binding free energy (kcal/mol)

Rank	Sirtuin 1	Sirtuin 2	Sirtuin 3	Sirtuin 4	Sirtuin 5	Sirtuin 6	Sirtuin 7
1	Quercetin (-9.60)	31-Norcyclo artenol (-11.60)	Campesterol, (-10.70)	Taraxerol (-11.60)	Antheraxan-thin (-11.80)	Taraxerol (-11.50)	Beta-Amyrin (-11.50)
2	Coumestrol (-9.50)	Taraxasterol (-11.30)	(+)-Taraxe-rol (-11.00)	Taraxasterol (-11.30)	Flavoxanthin (-11.70)	Taraxasterol (-11.50)	(+)-Taraxe-rol (-11.00)
3	Apigetrin (-9.40)	Beta-Amyrin (-11.10)	Taraxerol (-10.70)	Beta-Amyrin (-11.10)	Lutein (-11.70)	Beta-Amyrin (-11.00)	Taraxerol (-10.70)
4	Flavoxanthin (-9.30)	(+)-Taraxe-rol (-11.00)	Taraxasterol (-10.70)	(+)-Taraxe-rol (-11.00)	Cryptoxan-thin (-11.40)	(+)-Taraxe-rol (-10.70)	Taraxasterol (-10.70)
5	Taraxacin (-9.20)	Faradiol (-10.90)	Cycloartanol (-10.40)	Faradiol (-10.90)	Taraxerol, (-11.00)	Faradiol (-10.30)	Cycloartanol (-10.40)
6	Chlorogenic Acid (-9.10)	Flavoxanthin (-10.70)	Cycloartenol (-9.80)	Flavoxanthin (-10.70)	Violaxanthin (-11.00)	Arnidiol (-10.30)	Cycloartenol (-9.80)
7	Germacrano lide (-9.10)	Sitogluside (-10.60)	31-Norcyclo-artenol (-9.70)	Sitogluside (-10.60)	Taraxasterol (-10.90)	Sitogluside (-10.00)	31-Norcyclo-artenol (-9.70)
8	Cryptoxan-thin (-9.00)	Cryptoxan-thin (-10.40)	Apigetrin (-9.60)	Cryptoxan-thin (-10.40)	Arnidiol (-10.90)	Apigetrin (-9.90)	Apigetrin (-9.60)
9	Isorhamnetin (-9.00)	Antheraxan-thin (-10.40)	Faradiol (-9.60)	Antheraxan-thin, (-10.40)	Faradiol (-10.80)	Campesterol (-9.90)	Faradiol (-9.60)
10	Lutein (-8.80)	Cycloartanol (-10.40)	Arnidiol (-9.60)	Cycloartanol (-10.40)	(+)-Taraxe-rol (-10.60)	Stigma-sterol (-9.90)	Arnidiol (-9.60)

Basically, all these affinities are highly important to explain the role of sirtuins in mediating the cellular action of the bioactive substances. Affinities refer to the strength of the interactions between bioactive substances and sirtuin proteins.

The sirtuins can interact with *Taraxacum officinale* bioactive ligands, although the nature of this interaction is somewhat different from that of traditional receptor-ligand binding. Unlike traditional receptors, sirtuins do not undergo conformational changes upon ligand binding; however, certain ligands can bind to the active or allosteric sites of sirtuins. This binding may enhance or inhibit the deacetylase activity of the enzymes [54]. Sirtuins contain a catalytic domain that binds NAD+ (nicotinamide adenine dinucleotide), which is essential for their enzymatic activity [55]. Additionally, they have binding sites for various small molecules, including natural compounds and synthetic drugs that can modulate sirtuin activity [56].

Analysis of Protein-Ligand Interactions

Following the docking analysis in Figure 2, the interactions between ligands (the best three bioactive from Table 5) and Sirtuin protein targets (Sirt1 to Sirt7) vary significantly for each sirtuin, which demonstrate high binding affinities and compliance with Lipinski's Rule, suggesting good oral bioavailability and therapeutic potential. Other ligands with favorable binding affinity values further indicate their promising role in modulating Sirtuin activity, warranting further investigation into their applications in anti-aging and stress resistance. The binding affinities of these ligands reflect their efficacy and specificity, with lower values suggesting more optimal interactions with key amino acid residues in the binding sites, crucial for understanding their mechanisms of action and therapeutic effects [57].

Protein-ligand interactions typically include hydrogen bonds, hydrophobic interactions, ionic bonds, and van der Waals forces. The types of interactions-such as hydrogen bonds, which stabilize binding through electrostatic attraction; hydrophobic interactions, which occur when nonpolar regions of the ligand and protein come together to avoid water; ionic bonds, which form between charged groups; and van der Waals forces, which arise from transient dipoles-contribute to the overall strength

and specificity of the binding, which ultimately influences the biological activity of the ligand [58]. The observed protein-ligand interactions indicate the potential to modulate sirtuins, influencing pathways associated with aging and stress resistance. The interaction including van der Waals and conventional hydrogen bonds, π interactions and Pi-Sigma, alkyl with carbon-hydrogen bonds, and hydrophobic interactions.

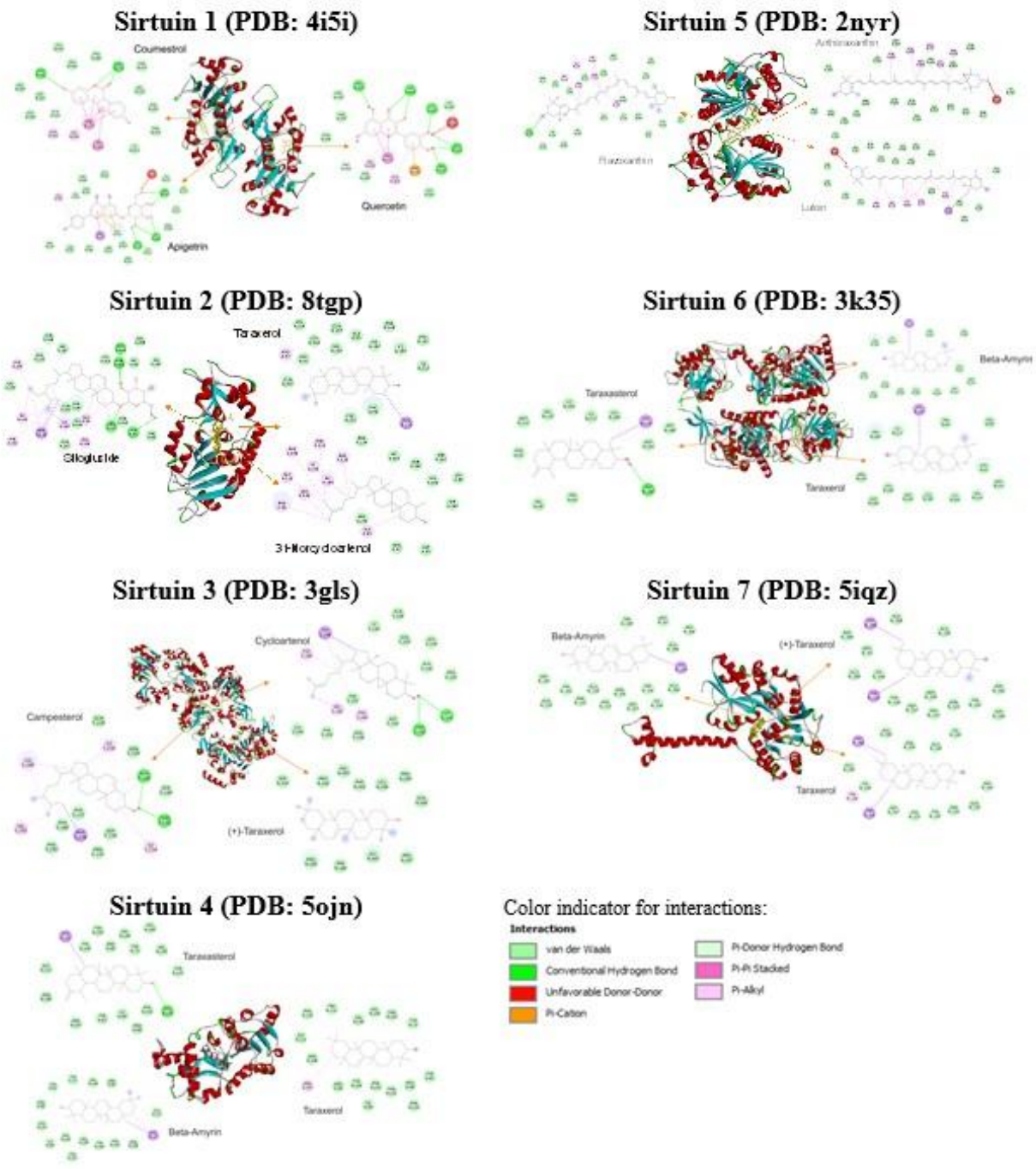


Figure 2. Visualization of Sirtuin protein – ligand interactions

Sirtuins, functioning as enzymes, are pivotal regulators within the cell, significantly influencing the acetylation state of various proteins and thereby impacting a multitude of biological processes. Their

enzymatic activities underscore their critical roles in metabolism, aging, and disease [59]. Specifically, sirtuins are integral to key processes such as: 1) metabolism and energy homeostasis, 2) cellular stress responses and survival, 3) DNA repair and maintenance of genomic stability, and 4) aging and longevity [60]. This essential involvement in vital cellular functions positions sirtuins as promising therapeutic targets for a spectrum of conditions, including metabolic disorders, neurodegenerative diseases, and age-related illnesses [61]. The findings align with these established roles, demonstrating that bioactive compounds from *Taraxacum officinale* can modulate sirtuin activity, thereby influencing metabolic pathways crucial for energy homeostasis. Specifically, compounds like taraxerol and taraxasterol exhibited favorable binding affinities with sirtuins, suggesting that they may enhance sirtuin-mediated regulation of metabolic functions. This is particularly relevant in the context of aging, where sirtuins are known to promote cellular resilience and longevity by activating stress response pathways.

Taraxerol has been shown to possess significant anti-inflammatory effects. Research indicates it can inhibit the production of pro-inflammatory cytokines and reduce inflammation in various models [62]. Taraxerol acetate was found to activate the antioxidant defense system in murine intestinal epithelial cells, effectively reducing H_2O_2-induced oxidative stress and modulating key metabolic pathways, suggesting its potential to promote health. These findings provide valuable insights for the further development of taraxerol as a therapeutic agent [63]. Taraxasterol reported has potential as an anticancer agent, with studies indicating it can inhibit the growth and proliferation of cancer cells in certain contexts, and as wound healing with its effects on certain enzyme activities [64,65]. The potential impact of these findings in the realm of anti-aging is significant; by modulating sirtuin activity, these compounds could promote improved metabolic health, enhance cellular resilience, and ultimately contribute to delayed aging and reduced risk of age-related diseases.

Beta-amyrin together with its anomer alpha-amyrin, effectively inhibited trinitrobenzene sulfonic acid-induced colitis in mice by suppressing inflammatory cytokines and COX-2 levels, likely through the inhibition of NF-κB and cAMP response element-binding protein (CREB)-signaling pathways, suggesting its potential for controlling inflammatory responses in bowel disease. This indicates that β-amyrin could be a valuable agent in managing inflammatory bowel conditions [66]. Beta-amyrin demonstrated significant anti-fibrotic effects in dimethylnitrosamine-induced hepatic fibrosis in rats by attenuating oxidative stress, inflammation, apoptosis, and fibrogenesis, suggesting its potential as a natural compound to treat liver fibrosis. These properties indicate that beta-amyrin could halt the progression of liver fibrosis to chronicity [67].

The utilization of molecular docking studies in this research has provided valuable insights into the binding free energies and ligand-target interactions of bioactive compounds from *Taraxacum officinale*. These studies reveal how effectively ligands fit into the sirtuin structure, guiding the design of novel compounds capable of modulating sirtuin activity. Notably, the ability of *Taraxacum officinale* bioactive compounds to influence sirtuin activity indicates that these ligands can significantly alter sirtuin functions, potentially affecting various biological pathways linked to aging and cellular stress responses [68]. The quantification of binding free energies further elucidates the strength of these interactions, suggesting that compounds exhibiting favorable binding profiles may enhance sirtuin activity, thereby promoting beneficial effects such as improved metabolic health and increased cellular resilience [69], although the actual binding affinity needs to determined experimentally through techniques like surface plasmon resonance (SPR), isothermal titration calorimetry (ITC), or other biophysical methods [70].

This study highlights the promising bioactive potential of *Taraxacum officinale* as a rich source of natural compounds with significant antioxidant, anti-inflammatory, and antimicrobial properties. Particularly noteworthy are compounds like taraxerol, taraxasterol, and beta-amyrin, which rank among the top ligands for several sirtuin proteins. These compounds demonstrate the capacity to enhance cellular health and modulate sirtuin activity, making them crucial players in the processes of aging and the management of chronic diseases. Furthermore, the potential of these compounds to impact sirtuin activity may have significant implications for managing age-related diseases, including metabolic disorders and neurodegenerative conditions. By placing our findings within the broader framework of sirtuin biology, the research highlights the therapeutic potential of *Taraxacum officinale* as a source of

natural compounds that may contribute to improved health outcomes through modulation of sirtuin function.

The rich phytochemical profile of *Taraxacum officinale*, combined with its historical applications in traditional medicine, underscores its value as a candidate for further research and therapeutic development. Ultimately, this plant offers new avenues for discovering natural therapies that may improve health outcomes and enhance quality of life, particularly through the modulation of sirtuin activity and its associated biological pathways. Further research, including experimental validation and clinical studies, which include in-vitro studies: 1) to validate the target binding receptor and evaluate its binding affinity, 2) to perform cellular activity assays, including dose-response studies, mechanism of action, and cytotoxicity assays, 3) using cellular or 4) assessment of metabolic stability; or in-vivo (animal model) to assess its ADME-Tox properties, is warranted to fully explore and harness the therapeutic potential of *Taraxacum officinale* in the development of novel anti-aging strategies.

AUTHOR CONTRIBUTIONS

Concept: M.R.S., T.S.; Design: M.R.S., M.C.S.; Control: M.R.S., I.A.I.W., H.L., T.S.; Sources: M.R.S., I.A.I.W., H.L., T.S.; Materials: M.C.S.; Data Collection and/or Processing: M.R.S., T.S.; Analysis and/or Interpretation: M.R.S.; Literature Review: I.A.I.W., H.L., T.S.; Manuscript Writing: M.R.S., T.S.; Critical Review: M.R.S., I.A.I.W., H.L., T.S.; Other: -

CONFLICT OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

The authors declare that the ethics committee approval is not required for this study.

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J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 49(3): 809-816, 2025
Doi: 10.33483/jfpau.1660133

ORIGINAL ARTICLE / ÖZGÜN MAKALE

A VALIDATED ADSORPTIVE STRIPPING DIFFERENTIAL PULSE VOLTAMMETRIC METHOD FOR THE QUANTIFICATION OF LINAGLIPTIN FROM URINE SAMPLE

İDRARDAN LİNAGLİPTİN TAYİNİ İÇİN ADSOPRTİF SIYIRMA DİFERANSİYEL PULS VOLTAMETRİK YÖNTEM GELİŞTİRİLMESİ VE VALİDASYONU

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ABSTRACT

Objective: *In this study, a low-cost, sensitive and practical electroanalytical method was developed and validated for the determination of Linagliptin (LNG), a therapeutic agent used in the treatment of Diabetes Mellitus, one of the most common chronic diseases worldwide, characterized by high blood glucose levels and directly affecting millions of people.*
Material and Method: *Electroanalytical studies were completed using screen-printed carbon electrodes in pH: 8 Britton - Robinson buffer solution. Mechanistic studies were carried out by cyclic voltammetry technique. Adsorptive stripping differential pulse voltammetry technique was used for the electrochemical determination of LNG.*
Result and Discussion: *The developed method exhibited linearity in the range of 0.1 - 7.5 µM and the limit of detection value was calculated as 37 nM. The developed method was applied for the determination of LNG from urine and a recovery value of 100.02% was obtained.*
Keywords: *Adsorptive stripping differential pulse voltammetry, antidiabetic drug, electrochemical determination, linagliptin*

ÖZ

Amaç: *Bu çalışmada, dünya çapında en yaygın kronik hastalıklardan biri olan, yüksek kan şekeri ile karakterize, ve milyonlarca insanı doğrudan etkileyen bir rahatsızlık olan Diabetes Mellitus tedavisinde kullanılan terapötik bir ajan olan Linagliptin (LNG) tayini için düşük maliyetli, hassas ve pratik bir elektroanalitik yöntem geliştirilmiş ve valide edilmiştir.*
Gereç ve Yöntem: *Elektroanalitik çalışmalar pH: 8 Britton - Robinson tampon çözelti ortamında perde baskılı carbon elektrotlar kullanılarak tamamlanmıştır. Mekanistik çalışmalar dönüşümlü voltametri tekniği ile yapılmıştır. LNG'nin elektrokimyasal tayini için de adsorptif sıyırma diferansiyel puls voltametri tekniği kullanılmıştır.*
Sonuç ve Tartışma: *Geliştirilen yöntem 0.1 – 7.5 µM aralığında doğrusallık sergilemiş ve gözlenebilme sınırı 37 nM olarak hesaplanmıştır. Geliştirilen yöntem idrardan LNG tayini için uygulanmış ve % 100.02'lik bir geri kazanım değeri elde edilmiştir.*
Anahtar Kelimeler: *Adsorptive sıyırma diferansiyel puls voltametri, antidiyabetik ilaç, elektrokimyasal analiz, linagliptin*

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Submitted / Gönderilme : 19.03.2025
Accepted / Kabul : 12.05.2025
Published / Yayınlanma : 19.09.2025

INTRODUCTION

Diabetes Mellitus (DM) is one of the most prevalent chronic metabolic diseases on earth that is characterised by high glucose levels because of reduced insulin production from pancreas or insufficient usage of produced insulin. It causes serious problems to the many parts of the body such as heart, kidney, eyes and blood vessels [1-3]. According to data presented by the World Health Organization (WHO), 830 million people were living with DM in 2022, up from 200 million in 1990 [4]. This significant rise in DM prevalence underscores the growing importance of effective treatment strategies. Although factors such as regular and balanced nutrition, exercise and lifestyle are effective in the controlling of glucose level, medications bear the majority of the treatment burden. Linagliptin (LNG), the first member of dipeptidyl peptidase-4 inhibitors, is an antidiabetic drug used for treatment of patients with type II DM (Figure 1) [5,6]. Since it was first licensed in the US, Europe, Japan and elsewhere in 2011 [5,7] it is commonly used to improve glycaemic control in adults with the type II DM.

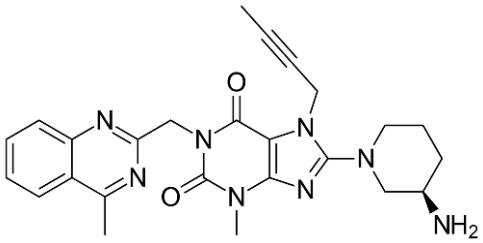


Figure 1. Chemical structure of LNG

Drug analysis is one of the most important fields of analytical chemistry. Drug analysis is performed from different matrices such as pharmaceutical forms, blood, urine and tissue for clinical, quality control, forensic studies, etc. [8]. Different analytical techniques including chromatography [9], spectroscopy [10], and electroanalysis [11] are used in this context. Electroanalytical techniques stand out in the field of analytical methods due to their quick response time, portability, affordability, simple procedures, high sensitivity, and minimal interference from drug excipients. As a result, they have become a crucial tool in pharmaceutical analysis [12-17]. Moreover, these methods offer several benefits over conventional techniques, such as enabling in-situ and real-time analysis without the need for complex sample preparation. This not only accelerates the analysis but also minimizes the risk of sample contamination and loss. Although limited in number, electroanalytical methods developed for LNG analysis can be found in the literature [18-21]. However, these methods are often laborious and costly, involving material synthesis, sensor fabrication and characterization steps. In this study, a rapid and easily applicable adsorptive stripping differential pulse voltammetric (AdSDPV) method for the analysis of LNG, which does not require any material synthesis or sensor fabrication processes, was developed and validated. To evaluate the applicability of the developed method, LNG was analysed from spiked urine.

MATERIAL AND METHOD

Chemicals

LNG as a standard was kindly gifted from Nobel Pharmaceuticals (Türkiye). All chemicals used in the experimental steps were of analytical grade and were used without any purification. Ultra-pure water (Millipore, Merck, Germany) was used for the preparation of aqueous solutions. LNG was dissolved in methanol to obtain stock solution.

Apparatus

All voltammetric measurements were performed using the AUT204NFRA32M model Metrohm-Autolab (Netherlands) potentiostat-galvanostat system controlled with NOVA 2.1 software. Screen-printed carbon (SPC) electrodes (LifeSense, Türkiye) were used during the study.

Voltammetric Measurements

During the study, differential pulse voltammetry (DPV), adsorptive stripping differential pulse voltammetry (AdSDPV) and cyclic voltammetry (CV) were used. Before measurements, SPC electrode surfaces were rinsed with water and ethanol, respectively, to remove dust or any other particles that stuck on to electrode surface. All measurements were carried out in the 0.25 M Britton – Robinson buffer solution (BRbs) as an electrolyte solution. The pH value of BRbs was adjusted with 2 M NaOH and 2 M HCl.

Real Sample Preparation

Firstly, artificial urine sample was filtered from 0.45 PTFE syringe filter. Afterwards, it was diluted 10-fold with BRbs. Finaly, it was spiked with LNG at 1.0 µM concentration.

RESULT AND DISCUSSION

Electrochemical Behaviour of LNG

Electrochemical behaviour of 100 µM LNG on the SPC electrode surface was investigated by DPV in pH: 2 BRbs medium. As seen in Figure 2, a well-defined oxidation peak was observed around 0.95 V with the anodic peak current (I_{pa}) of ~1.8 µA.

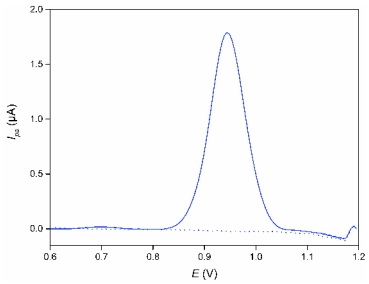


Figure 2. Electrochemical response of LNG on the SPC electrode surface – baseline corrected DPVs of 100 µM LNG in 0.25 M pH: 2 BRbs (Dotted line; 0.25 M pH: 2 BRbs in the absence of LNG)

Optimization of pH

The pH value of the electrolyte solution directly affects both electrodes and methods performance. Therefore, pH optimization of electrolyte solution is one of the most important step in the electroanalytical method development process. In this context electrochemical behaviour of LNG was evaluated in the BRbs at different pH values changing from 2 – 10. Figure 3 shows the I_{pa} of 100 µM LNG in the different pH media. As is seen, increasing analytical responses were observed up to pH: 8. However, after pH: 8 I_{pa} started to decrease. Thus, pH: 8 was chosen as the optimum experimental condition for further experiments.

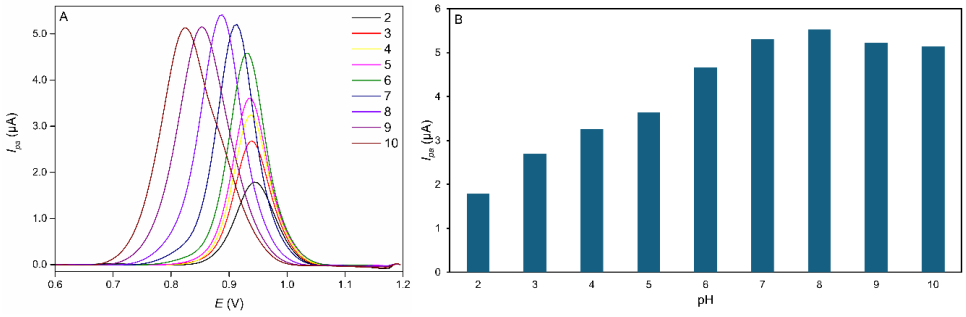


Figure 3. (A) Baseline corrected DPVs of 100 µM LNG in 0.25 M BRbs at different pH values, (B) Electroanalytical response of 100 µM LNG at different pH media

Influence of Scan Rate

In electroanalytical studies, it is important to determine the reaction mechanism by which the target analyte moves on the sensor surface. For this purpose, CVs of 100 µM LNG in 0.25 M pH: 8 BRbs on the SPC electrode surface at different scan rates (v) from 0.01 – 0.50 $V \cdot s^{-1}$ were recorded (Figure 4). A linear relationship between the logarithm of the scan rate (log v) and logarithm of the I_{pa} (log I_{pa}) was observed. The slope of this plot indicates the type of reaction that occurs on the electrode surface. The slope of 0.6685 (Seen in Figure 4B) which is between 0.5 and 1 indicates that the electro-oxidation of LNG on the SPC electrode surface is adsorption as well as diffusion controlled.

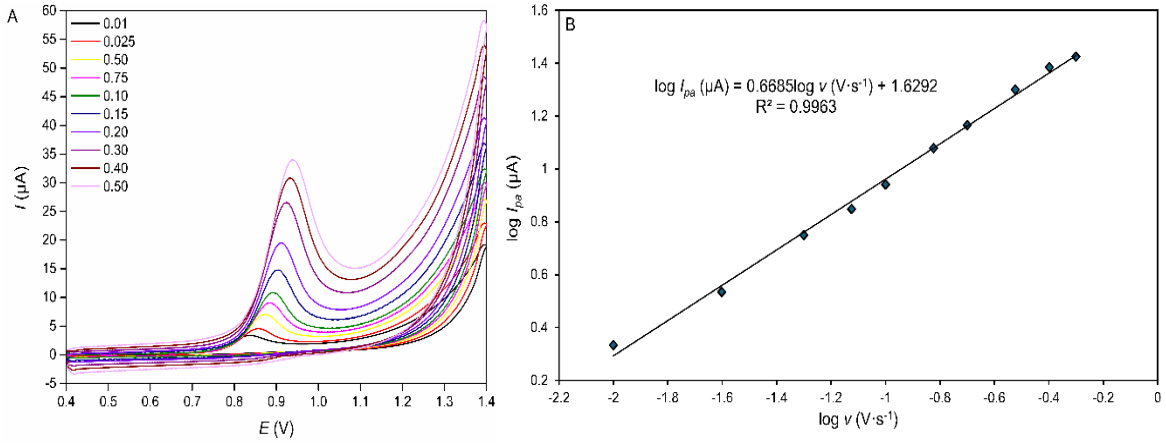


Figure 4. (A) CVs of 100µM LNG in 0.25 M, pH: 8 BRbs at different scan rates, (B) plot of log v vs. log I_{pa}

Optimization of Accumulation Step

Since the electro-oxidation mechanism of LNG on the SPC electrode surface is affected by adsorption as well as diffusion, the accumulation steps need to be optimized to get the best performance from the developed method. In this context, voltammetric responses of 100 µM LNG in 0.25 M pH: 8 BRbs at different accumulation potentials (E_{acc}) and different accumulation times (t_{acc}) were compared. Obtained results are exhibited in Figure 5. As can be seen, an increase in I_{pa} was observed with an increase in potential up to 0.4 V, after which the I_{pa} started to decrease. For this reason, 0.4 V was chosen as the optimum E_{acc}. Similarly, it is also observed that the I_{pa} increases with increasing t_{acc}. However, after 480 s, the rate of increase decreased very much and became insignificant. Therefore, 480 s was chosen as the optimum t_{acc} in order not to keep the analysis time too long.

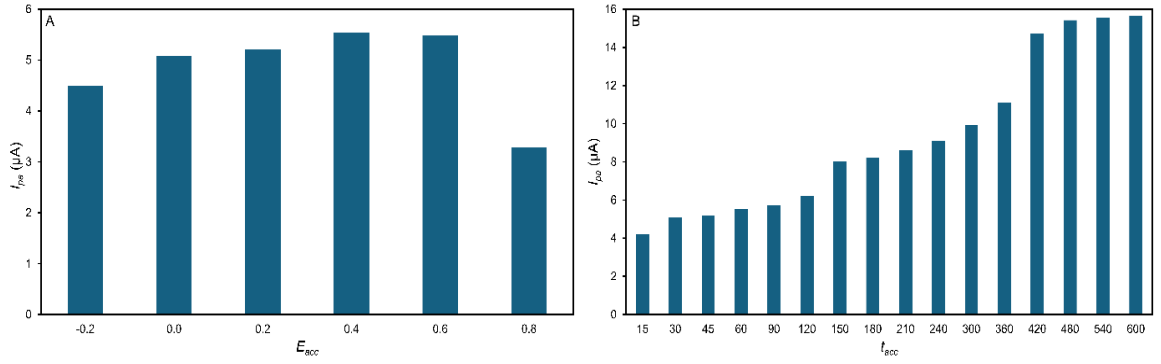


Figure 5. (A) I_{pa} of 100 µM LNG in 0.25 M pH: 8 BRbs at different E_{acc}, (B) I_{pa} of 100 µM LNG in 0.25 M pH: 8 BRbs at different t_{acc}

Analytical Performance

Before the electroanalytical measurements, the experimental parameters that affect the AdSDPV responses were optimized as shown in Table 1. Under the optimized conditions, the I_{pa} values was found to be linear against the increasing concentration of LNG from 0.1 to 7.5 µM with the related regression equation of I_{pa} (µA) = 0.3524C_{LNG} (µM) + 0.2163 (R^2 = 0.9961) (Figure 6). The limit of detection (LOD) and limit of quantification (LOQ) values were calculated according to the formula *k.SS/m*, where *k* is 3 for LOD and 10 for LOQ, *SS* is the standard deviation of the I_{pa} of 6 consecutive measurements recorded at the lowest concentration of the linear working range, and *m* is the slope of calibration curve. Using this formula, LOD and LOQ values were calculated as 37 nM and 123 nM, respectively. Table 2 shows the results of previously reported studies in the literature for LNG analysis. As it is seen, even without any modification of the SPC electrode, highly sensitive results were obtained compared to existing studies, which proves the superiority of this study.

Table 1. Optimized experimental parameters affecting the AdSDPV responses

Parameter	Value
Start potential (V)	0.4
Stop potential (V)	1.4
Deposition potential (V)	0.4
Deposition time (s)	480
Step (V)	0.005
Modulation amplitude (V)	0.025
Modulation time (s)	0.05
Interval time (s)	0.1
Scan rate ($V \cdot s^{-1}$)	0.05

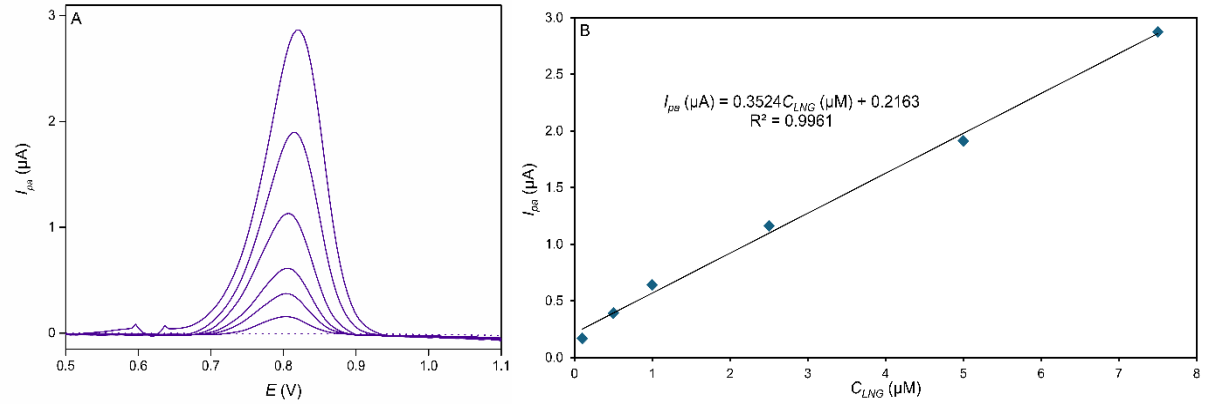


Figure 6. (A) AdSDPVs of LNG at increasing concentrations (Dotted line; 0.25 M pH: 8 BRbs in the absence of LNG), (B) Calibration plot of LNG

Table 2. Comparison of previously reported electroanalytical methods for LNG detection

Method	Sensor	Linear range (µM)	LOD (nM)	Reference
SWV	Co_3O_4NPs/MWCNT	39.8 – 1530	11300	[18]
DPV	gCN-βCD/CPE	0.01 – 50	3	[19]
SWV	Fe_2O_3NPs/CPE	0.063 – 182	17	[20]
DPV	L-cys-MoS_2/GCE	1 – 153.4	190	[21]
SWV	PGE	0.5 – 11	210	[22]
SWV	GCE	0.4 – 4.8	110	[23]
AdSDPV	SPC electrode	0.1 – 7.5	37	This study

SWV; square wave voltammetry, DPV; differential pulse voltammetry, NPs; nanoparticles, MWCNT; multi-walled carbon nanotube, CPE; carbon paste electrode, PGE; pencil graphite electrode, GCE; glassy carbon electrode

Repeatability and Selectivity

In order to evaluate the repeatability of the method, 1 µM LNG was measured 6 times consecutively and the relative standard deviation (*RSD*%) of the electroanalytical responses was calculated. The *RSD*% was found to be 1.66%. This value proves that the method is valid in terms of repeatability. Another important parameter in analytical methods is selectivity. Thus, the changes on the electroanalytical response of LNG in the presence of possible 100-fold interference such as glucose, urea, Ca^{2+}, Na^{+}, K^{+}, CO_3^{2-}, Cl^{-}, and NO_3^{-} were evaluated. The difference of I_{pa} (ΔI_{pa}) and the difference of E_{pa} (ΔE_{pa}) between in the absence and presence of interferences were calculated as 0.71% and 0.003 V respectively (Presented in Table 3). These results are clear evidence that the developed method is able to selectively detect LNG in the presence of possible interferences.

Table 3. AdSDPV responses of 1 µM LNG in the absence and in the presence of various interferences (N = 3)

Analyte	C (µM)	I_{pa} (µA)	ΔI_{pa} (%)	E_{pa} (V)	ΔE_{pa} (V)
LNG	1	0.14203	0.71	0.815	0.003
LNG	1	0.14102		0.818	
Glucose	100				
Urea	100				
$CaCO_3$	100				
NaCl	100				
KNO_3	100				

Real Sample Analyses

The suitability of the method was evaluated by analysing the LNG content of urine sample using standard addition method [24]. The average recovery value was found 100.02% with the *SD* of 0.52 (N = 3). It is clearly seen that the developed method can detect LNG from complex biological matrix with high recovery values (Table 4).

Table 4. Recovery values of LNG from spiked urine sample

Added (µM)	Found (µM)	Recovery (%)
1.0000	0.9981	99.81
1.0000	1.0063	100.63
1.0000	0.9961	99.61
	Mean	100.02
	SD	0.54

Conclusion

DM is a chronic disease characterized by high blood glucose levels that directly affects more and more people in the world, is directly and indirectly responsible for many deaths, and has many social and economic impacts on society. Although it cannot be completely cured, it is possible for DM patients to keep their blood sugar levels under control with various treatment methods and lead a life like healthy individuals. Undoubtedly, medication plays the biggest role in these treatment methods. LNG is a dipeptidyl peptidase-4 inhibitor used in the treatment of DM.

Electroanalytical methods stand out among analytical methods because they are cheap, fast and easy to apply. They are increasingly preferred in drug analysis, especially because they can determine the target drug without the need to separate excipients in pharmaceutical forms. Screen-printed electrodes are very easy to procure and very practical to use systems that include traditional triple electrode systems consisting of working electrode, reference electrode and counter electrode on a single platform.

In this study, AdSDPV method was developed for LNG determination using SPC electrodes and

validated in terms of selectivity, repeatability and recovery parameters. The developed method exhibited linearity in the concentration range of 0.1 - 7.5 µM and reached a LOD of 37 nM. In repeatability studies, the RSD% value was calculated as 1.66%. The developed method was able to analyse LNG in the presence of possible interferences without being affected by these interferences. The method proved its suitability by determining LNG from urine with a recovery of 100.02%.

The developed method has been introduced to literature as a cheaper and practical method compared to existing methods involving material synthesis and characterization in terms of using the SPC electrode surface without any modification process. It also stands out as a more sensitive method than most of the existing methods. As a result, it is considered an innovative alternative in areas where both quality control and clinical analysis of LNG are needed.

ACKNOWLEDGEMENTS

This study was financially supported by The Scientific and Technological Research Council of Türkiye (TÜBİTAK) with the grant number of 124Z724 and Dicle University Scientific Research Project Coordination Unit with the grant number of ECZACILIK.23.001.

AUTHOR CONTRIBUTIONS

Concept: A.K.A.; Design: A.K.A.; Control: A.K.A.; Sources: A.K.A.; Materials: A.K.A.; Data Collection and/or Processing: A.K.A.; Analysis and/or Interpretation: A.K.A.; Literature review A.K.A.; Manuscript Writing: A.K.A.; Critical review: A.K.A.; Other: -

CONFLICT OF INTEREST

The author declares that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

The author declares that the ethics committee approval is not required for this study.

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J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 49(3): 817-826, 2025
Doi: 10.33483/jfpau.1657517

ORIGINAL ARTICLE / ÖZGÜN MAKALE

CHARACTERIZATION OF GREEN SYNTHESIZED SILVER AND ZINC NANOPARTICLES USING PROPOLIS EXTRACT AND THEIR ANTIMICROBIAL, ANTIBIOFILM, ANTIQUORUM SENSING ACTIVITIES

PROPOLİS EKSTRESİ KULLANARAK YEŞİL SENTEZLENMİŞ GÜMÜŞ VE ÇİNKO NANOPARTİKÜLLERİNİN KARAKTERİZASYONU VE ANTİMİKROBİYAL, ANTİBİYOFİLM, ÇOĞUNLUĞU ALGILAMA İNHİBİSYONU AKTİVİTELERİ

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ABSTRACT

Objective: *AgNP and ZnO NP were synthesized by green synthesis method using propolis, the characterization of nanoparticles was examined and their antimicrobial, antibiofilm and antiquarum sensing activities were examined.*
Material and Method: *Nanoparticles were synthesized from the prepared propolis extract using 100 ml and 25 ml of extract for AgNP and ZnONP, respectively. Characterization was performed using UV-Visible (UV-VIS) spectra, XRD, EDX and SEM. Antimicrobial, antibiofilm and anti-quorum sensing activities of the nanoparticles were examined.*
Result and Discussion: *Looking at the characterization results, AgNP and ZnO NP with very small particle sizes were synthesized. According to the antibacterial activity results, the MIC values of ZnO NPs and AgNPs were determined as 0.0625 mg/ml and 0.0250 mg/ml against S. aureus ATCC 43300 (MRSA), respectively. The percentage biofilm inhibition value of ZnO NPs and AgNPs was found to be 38.94% and 38.67%, respectively. The synthesized AgNP and ZnO NPs did not show anti-quorum sensing activity.*
Keywords: *Antibiofilm, antimicrobial, antiquorum sensing, characterization, green synthesis, silver nanoparticle, zinc nanoparticle*

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Submitted / Gönderilme : 21.03.2025
Accepted / Kabul : 10.05.2025
Published / Yayınlanma : 19.09.2025

ÖZ

Amaç: *Propolis kullanılarak yeşil sentez yöntemi ile AgNP ve ZnO NP sentezlendi, nanopartiküllerin karakterizasyonu incelendi ve antimikrobiyal, antibiyofilm ve çoğunluğu algılama inhibisyonu aktiviteleri incelendi.*

Gereç ve Yöntem: *Hazırlanan propolis ekstresinden AgNP ve ZnONP için sırasıyla 100 ml ve 25 ml ekstre kullanılarak nanopartiküller sentezlendi. Karakterizasyon, UV-Görünür bölge (UV-VIS) spektroskopisi, XRD, EDX ve SEM kullanılarak gerçekleştirildi. Nanopartiküllerin antimikrobiyal, antibiyofilm ve çoğunluğu algılama inhibisyonu aktiviteleri incelendi.*

Sonuç ve Tartışma: *Karakterizasyon sonuçlarına bakıldığında oldukça küçük partikül boyutuna sahip AgNP ve ZnO NP sentezlendi. Antibakteriyel aktivite sonuçlarına göre ZnO NP'leri ve AgNP'leri S. aureus ATCC 43300 (MRSA)'ya karşı sırasıyla 0.0625 mg/ml ve 0.0250 mg/ml MİK değerleri gösterdi. ZnO NP'leri ve AgNP'lerinin biyofilm inhibisyon yüzdesi değeri sırasıyla %38.94 ve %38.67 olarak bulundu. Sentezlenen AgNP ve ZnO NP çoğunluğu algılama inhibisyonu aktivitesi göstermedi.*

Anahtar Kelimeler: *Antibiyofilm, antimikrobiyal, çinko nanopartikül, çoğunluğu algılama inhibisyonu, gümüş nanopartikül, karakterizasyon, yeşil sentez*

INTRODUCTION

Propolis is a natural bee product produced by *Apis mellifera* L. (honey bee) to clean, repair and protect the hive from invaders. Propolis is used among the public due to its pharmacological properties and low toxicity. Studies have found that propolis collected from different regions has different effects such as antiviral, anticancer and antioxidant. The reason for the different effects is that the chemical compounds found in propolis vary depending on the region where it is collected [1-3]. Generally, approximately 50% of raw propolis contains resin, i.e. phenolic acid, flavonoids and their polyphenolic compounds [4]. This high phenolic substance content increases the potential of propolis for nanoparticle synthesis with green methods.

Propolis has been proven to have antioxidant, anticancer, antimicrobial and anti-inflammatory effects [5]. Propolis has also been reported to have antifungal, antibacterial, hepatoprotective, antiviral, immunostimulating, cytotoxic, anti-trypanosomal and antiprotozoal therapeutic effects [2].

Nanotechnology is the science and technology of small, delicate objects with dimensions of 1 to 100 nm [6]. Developments made with this technology in the last decade have created a widespread use in many fields such as health, biomedical, pharmaceutical industry, magnetic, electronics, sensors, space science. There are two main approaches to the methods used in the production of nanoparticles; top-down and bottom-up synthesis approaches. Each approach includes a unique characterization and application stage. In the top-down approach, the targeted material is separated into nano-sized particles by mechanical milling, alloying and spraying methods. However, this method must be applied with extreme care and control, as it can damage the surface structure of the material and therefore its surface chemistry and physical properties. In the bottom-up approach, atoms or molecules are first combined into nuclei during the synthesis phase and then nano-sized particles are synthesized by precipitation, sol-gel and atomic condensation techniques. In this method, both synthetic and living materials (plants and microorganisms) can be used [7].

The risk of environmental pollution increases as the chemical materials used in the application of new physical or chemical techniques developed for the synthesis of nanoparticles produce harmful by-products [8]. Green synthesis method refers to the production of nanoparticles by a process involving the use of metal salts and biological materials as reductants [9]. In this way, the green synthesis of silver and zinc nanoparticles is affordable, reliable, environmentally friendly and does not aggregate over time [10,11]. In the synthesis of nanoparticles by green method, substances rich in phenolic acids are frequently used. Phenolic acids act as both reducing agents and stabilizers in the NP synthesis process. The use of phenolic acids represents a simple, reproducible, cost-effective and profitable strategy to synthesize metal NPs [7].

The use of green nanotechnology is an impeccable solution to struggle all these negative effects in terms of the commercial infeasibility of chemical and physical methods and the environmental

pollution caused by the byproducts released during synthesis. It is a safe, non-toxic and environmentally friendly method for nanoparticle synthesis using green synthesis [12].

The employment of silver nanoparticles (AgNPs) has witnessed a considerable surge in recent years, with these particles finding application in a plethora of fields. The primary reasons for their prevalence are attributable to their superior surface-enhanced Raman scattering, electrical and thermal conductivity, high catalytic activity, chemical stability, and antimicrobial properties. Zinc oxide nanoparticles (ZnO NPs) have a variety of applications, including use in superconducting materials, gas sensors, catalysts, drug delivery systems and cancer treatment. In addition, they exhibit antibacterial effects [7].

Antibiotic resistance is one of our most pressing global health challenges. The rise in multidrug-resistant (MDR) bacterial strains has significantly reduced the effectiveness of conventional antibiotics, leading to increased morbidity, mortality, and healthcare costs [13]. Furthermore, the sluggish rate of new antibiotic discovery has worsened the crisis, making it essential to explore alternative therapeutic strategies [14]. Biofilms are structured microbial communities encased in an extracellular polymeric matrix that adheres to surfaces and protect bacteria from host immune responses and antimicrobial agents [15]. Within biofilms, bacteria exhibit enhanced tolerance to antibiotics, making infections more difficult to eradicate. Quorum sensing (QS) is a bacterial cell-cell communication mechanism that regulates virulence factors including toxin production, motility and biofilm formation [16]. Given the critical role of QS in pathogenicity, recent studies have focused on developing alternative antibiofilm and anti-QS agents to combat antibiotic resistance. Emerging evidence indicates that nanoparticles show significant potential as antimicrobial and anti-QS agents [17,18].

In this study, ethanolic extract with high phenolic content was prepared from propolis collected from Giresun region, AgNPs and ZnO NPs were synthesized by green method using this extract and these synthesized nanoparticles were characterized using different techniques. After characterization, antimicrobial, antibiofilm and antiquorum detection activities of the synthesized nanoparticles were examined.

MATERIAL AND METHOD

Chemicals

Silver nitrate ($AgNO_3$) and Zinc nitrate ($Zn(NO_3)_2$) was prepared from Sigma Aldrich (Hamburg, Germany). Sodium hydroxide (NaOH) and ethanol (CH_3OH) were purchased from İsolab Chemicals (Eschau, Germany). The raw propolis used in this study originated from Giresun region of Turkey. Distilled water was provided by Ankara University Central Laboratory-3.

Instrumentation

Spectroscopic measurements were carried out using a Shimadzu 1601 model double-beam spectrophotometer (Kyoto, Japan). Rigaku XRD MiniFlex 600 was used for X-ray diffraction (XRD) analysis, Oxford Instruments, INCA Energy for Energy dispersive X-ray spectroscopy (EDX) analysis and EISS, Supra 50 V for Scanning electron microscope (SEM) analysis and these devices were used to obtain information about the size, shape and elemental content of the nanoparticles. A Lab Comparation SIF500R (Seoul, South Korea) model incubator shaker was used for incubation. An ISOLAB Laborgeräte GmbH (Eschau, Germany) digital pH meter was used for pH measurement. Stirring was carried out on a magnetic stirrer from ISOLAB Laborgeräte GmbH (Eschau, Germany). Weighing was carried out using a Shimadzu ATX224 model weighing device (Kyoto, Japan).

Preparation of Propolis Extract

Raw propolis samples were obtained from the Giresun region. To prepare the ethanolic extract of propolis, 500 ml of water:ethanol (30:70, v/v) mixture was added to 20 g of crude propolis samples and mixed in a magnetic stirrer at 450 rpm for 5 hours at room temperature. The solution was then filtered and extract was stored at +4^{o}C in the dark until analysis or use [19].

Green Synthesis of Silver and Zinc Nanoparticles

Initially, 100 ml of 50 mmol metal salt solutions were prepared in water. Subsequently, 100 ml of propolis extract was added to the aqueous solution of $AgNO_3$, in order to initiate the synthesis of AgNP. In a similar fashion, 25 ml of propolis extract was added to the aqueous solution of $Zn(NO_3)_2$, in order to initiate the synthesis of ZnO NP. The extract was then added to the prepared metal salt solutions, and the pH was adjusted to 10. The resultant mixture was then subjected to incubation at 60 °C for a period of 24 hours. Subsequently, the mixture was subjected to three cycles of centrifugation at 5000 rpm for a duration of 30 minutes. Following centrifugation, the solution was filtered and the precipitate was subjected to incubation in a porcelain crucible with the lid closed at 60 °C for a further 24 h [20].

Antimicrobial Activity

In the antimicrobial activity tests, *Staphylococcus aureus* ATCC 25923 (methicillin-susceptible, MSSA), *S. aureus* ATCC 43300 (methicillin-resistant, MRSA), *Klebsiella pneumoniae* ATCC 13883, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853were used as test bacteria. *Candida albicans* ATCC 10231 was used as a test fungus. The MIC (Minimum Inhibitory Concentration) values of the nanoparticles were determined by the broth microdilution method [21,22]. The nanoparticles were dissolved in sterile water. Serial dilutions of nanoparticles were prepared in Mueller-Hinton Broth (MHB) ranging from 0.25 mg/ml to 0.002 mg/ml.

Antibiofilm Activity

Before performing the antibiofilm activity test, the MIC values of the nanoparticles against P. aeruginosa PAO1 were determined. The sub-MIC values were tested for antibiofilm activity. Antibiofilm activity was evaluated using the crystal violet method. The antibiofilm activity test was performed using the method in the study by Junejo et al. (2023) [18].

Antiquorum Sensing Activity

Before performing the antiquorum sensing activity test, the MIC values of the nanoparticles against *Chromobacterium violaceum* ATCC 12472 were determined. The antiquorum sensing activity test was conducted using the disc diffusion method described by Junejo et al. (2023) with the subMIC values [18].

RESULT AND DISCUSSION

Characterization of AgNP and ZnO NP

Checking that the synthesis of nanoparticles has taken place is first done by looking at the color change. For AgNP, it showed that the color quickly changed from light yellow to black upon pH adjustment of the silver nitrate solution. For ZnO NP, after adjusting to the pH of the zinc nitrate solution, the color quickly changed from light yellow to dark yellow.

In the synthesis of nanoparticles, two different temperatures (60 ^{o}C and 90 ^{o}C) were studied. The color change observed during the synthesis was observed in the synthesis performed at 60 ^{o}C. Therefore, the synthesis temperature was selected as 60 ^{o}C.

In the synthesis of AgNP and ZnO NP green, the amount of extract was first optimized. For AgNP, 75 ml, 100 ml and 125 ml extracts were used and the best UV-Vis result was found for 100 ml extract (Figure 1a). For ZnO NP, 15 ml-, 25ml and 35 ml extracts were used and the best UV-Vis results were obtained for NPs synthesized using 25 ml extract (Figure 1b). The absorption peaks of ZnO NPs and AgNPs were detected at 320 and 420 nm, respectively.

XRD measurements of AgNP and ZnO NP synthesized using propolis extract were taken between 5° and 70° with a scanning speed of 2° per minute, operating at 40 kV and 15 mA. The crystallite size of AgNP and ZnO NP was calculated using the Debye-Scherrer formula, where λ is the X-ray wavelength (~0.15 nm for Cu), D is the crystallite size in nm, K is 0.95, and β is the full width at half maximum (FWHM) in equation 1.

$$D = \frac{K\lambda}{\beta Cos\theta} \qquad \textit{(eq. 1)}$$

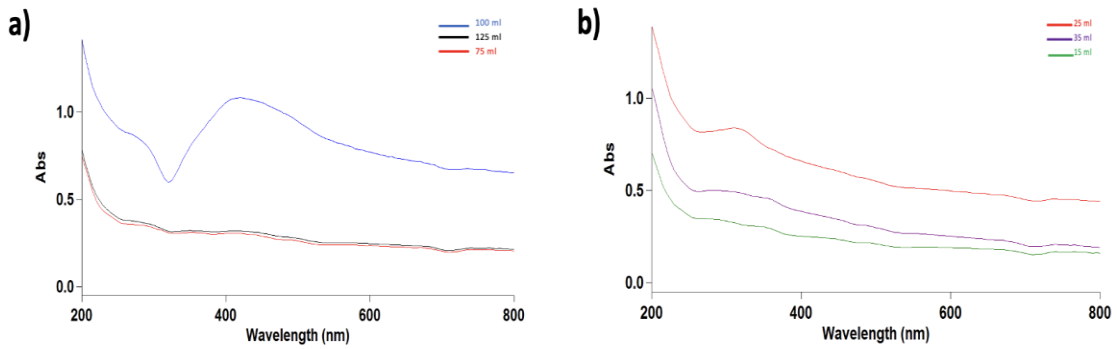


Figure 1. UV-Vis spectroscopy analysis of a) AgNP, and b) ZnO NP green synthesized by propolis extract

The XRD spectrum clearly show that AgNPs and ZnO NPs are in crystalline structure given Figure 2. The XRD peaks of AgNPs are observed at ~38° (111), ~45° (200) and ~66° (220) respectively, and calculated crystallite sizes of the peaks are ~15 nm, ~9 nm ~10 nm orderly. The average crystal line size is calculated as ~12 nm shown in Table 1. The XRD specra shows that AgNPs have the polycrystalline structure of silver and mainly have face-centered cubic (fcc) crystal structure [23]. For the first XRD peak of ZnO NPs is observed at ~36° (002) and a calculated crystallite size of ~5 nm. The second peak is determined at ~38° (101) and a calculated crystallite size of ~6 nm. The third peak is found at ~58° (110) and a calculated crystallite size of ~4 nm respectively [24]. Although the calculated average crystal size is ~5 nm, it is not sufficient for a mono/polycrystalline structure shown in Table 1.

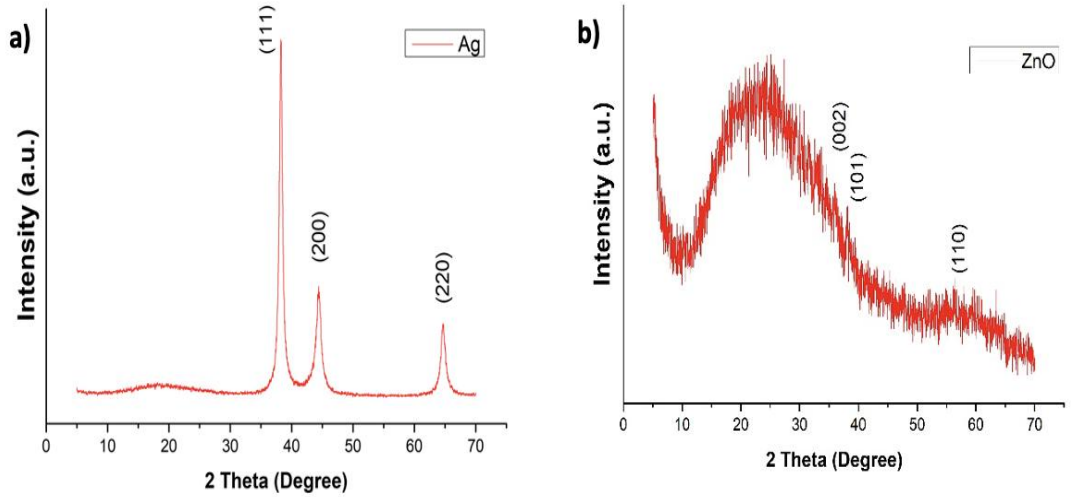


Figure 2. XRD analysis of green synthesized a) AgNP, and b)ZnO NP by using propolis

Table 1. Analysis of XRD peaks of AgNP and ZnO NP by using propolis

Nanoparticle	**Reflection Plane**	**Peak position (2θ°)**	**FWHM β_{size} (2θ°)**	**Dp (nm)**	**Average Dp (nm)**
Ag	(111)	~38	~0.57	~15	**~12**
	(200)	~45	~0.97	~9	
	(220)	~66	~1.02	~10	
Nanoparticle	**Reflection Plane**	**Peak position (2θ°)**	**FWHM β_{size} (2θ°)**	**Dp (nm)**	**Average Dp (nm)**
ZnO	(002)	~36	~1.6	~5	**~5**
	(101)	*~38*	*~1.4*	*~6*	
	(110)	*~58*	*~2*	*~4*	

SEM analyses provide information about the shapes of green synthesized nanoparticles. AgNP mostly has very small spherical nanoparticles and ZnO NP has similarly spherical nanoparticles (Figure 3). When EDX analysis is examined, it is observed that 85.85% of the weight of AgNP consists of Ag element and 49.33% of the weight of Zn element consists of Zn element for ZnO NP (Table 2).

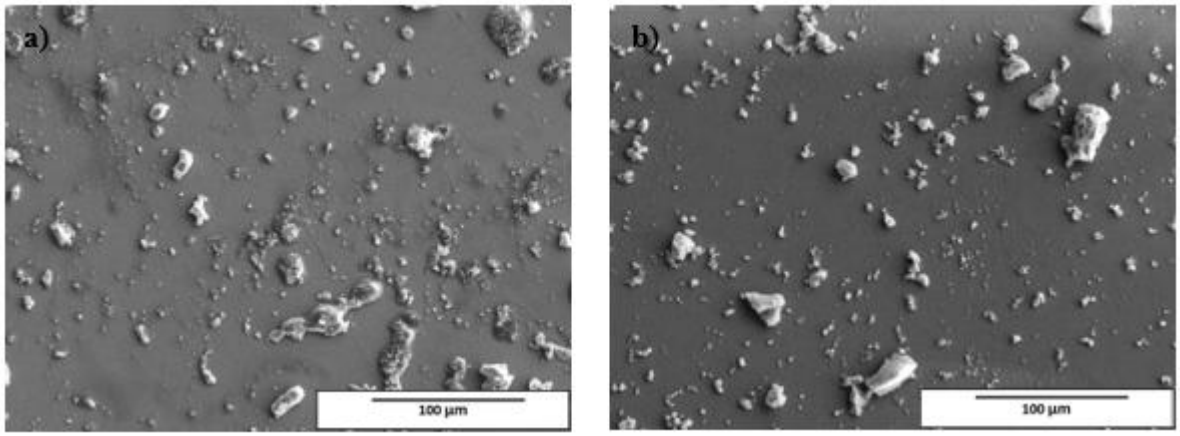


Figure 3. SEM image of synthesized a) AgNPs and b) ZnO NPs by using propolis

Table 2. EDX analysis of synthesized AgNPs and ZnO NPs by using propolis

AgNPs			**ZnO NPs**		
Element	Weight %	Atomic %	Element	Weight %	Atomic %
C K	4.91	24.24	C K	13.25	36.53
O K	5.33	19.75	O K	11.06	22.89
F K	2.40	7.51	Cl K	4.49	4.20
Si K	0.17	0.37	Cu K	21.86	11.39
Br L	0.29	0.22	Nd L	0.00	0.00
Zr L	1.05	0.68	Zn L	49.33	24.99
Ag	85.85	47.23			
Totals	100.00		Totals	100.00	

Antimicrobial Activity

The MIC values of AgNPs and ZnO NPs against test microorganisms are presented in Table 3. It was observed that none of the tested nanoparticles demonstrated antibacterial activity against *P.*

aeruginosa ATCC 27853. AgNP exhibited antibacterial activity exclusively against the Gram-positive bacteria tested. In contrast, ZnO NP exhibited antibacterial activity against both Gram-positive and Gram-negative bacteria. The strongest antibacterial activity of ZnO NP was observed against *S. aureus* ATCC 43300, with an MIC value of 0.0625 mg/ml. Among the nanoparticles tested, ZnONP was the only one to demonstrate antifungal activity, with an MIC value of 0.5 mg/ml. ZnO NP was found to exhibit stronger antimicrobial activity than AgNP.

Table 3. MIC values (mg/ml) of the AgNPs and ZnO NPs

	Gram-positive test bacteria		**Gram-negative test bacteria**			**Fungus**
Sample	*S. aureus* ATCC 25923 (MSSA)	*S. aureus* ATCC 43300 (MRSA)	*E. coli* ATCC 25922	*K. pneumoniae* ATCC 13383	*P. aeruginosa* ATCC 27853	*C. albicans* ATCC 10231
ZnO NP	0.125	0.0625	1	1	-	0.5
AgNP	0.25	0.25	-	-	-	-
Ciprofloxacine	<0.00025	0.0005	<0.00025	0.0625	<0.00025	NT
Fluconazole	NT	NT	NT	NT	NT	0.00156

NT: not tested, (-): no activity

Antibiofilm Activity

The biofilm inhibition percentages of the synthesized metal oxide nanoparticles (NPs) are presented in Figure 4. The percentage of biofilm inhibition of ZnO NP is 38.94%, while AgNP exhibited 38.67% biofilm inhibition.

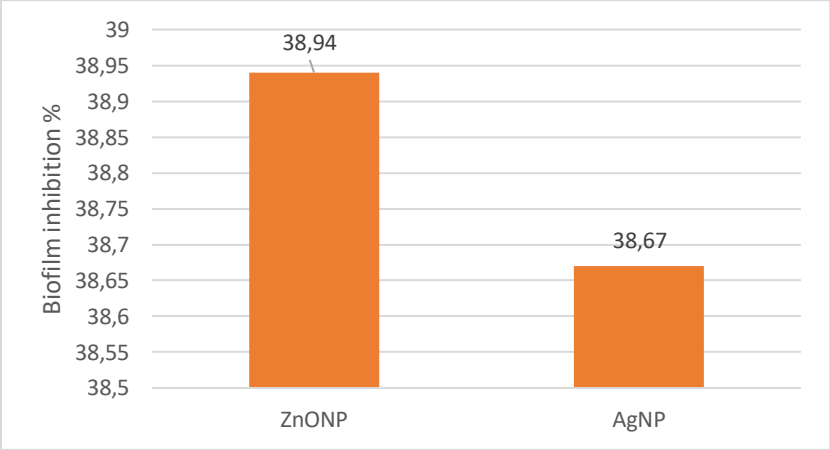


Figure 4. Antibiofilm activity of AgNPs and ZnO NPs

Antiquorum Sensing Activity

The formation of what may be described as a 'translucent inhibition zone' in the proximity of the disc has been shown to be an indication of the presence of potential quorum sensing inhibitory activity. However, none of the AgNPs and ZnO NPs tested showed antiquorum sensing activity, as shown in Figure 5.

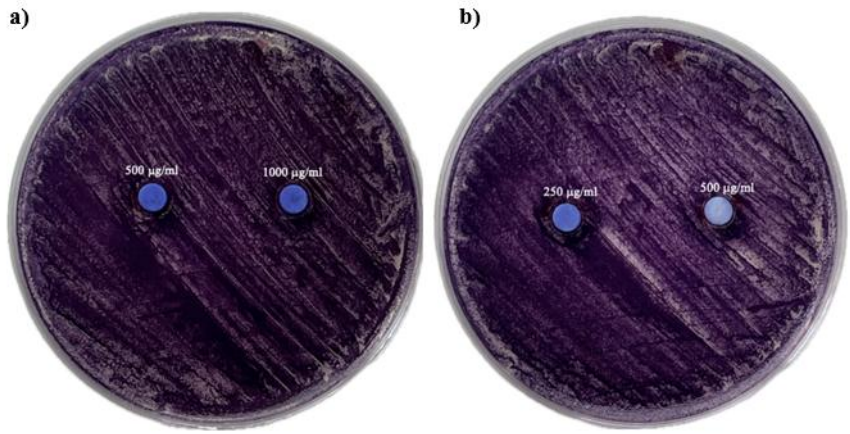


Figure 5. Quorum sensing inhibitory activity of a) silver and b) zinc nanoparticles

Conclusion

Nowadays, one of the most fundamental needs regarding the medical and pharmaceutical applications of nanotechnology is its environmental compatibility and its use as an antimicrobial. This study represents a new way to synthesize silver and zinc nanoparticles that are both antimicrobial and environmentally compatible by a green synthesis method without using chemicals and materials previously used to stabilize particles from raw propolis samples specific to a particular region.

In this study, ZnO NP and AgNP were synthesized using the extract prepared from raw propolis. As a result of the examination of the characterization studies, it was revealed that a successful synthesis was achieved. UV-visible spectra are compatible with the literature. XRD spectra show that AgNP has a face-centered cubic (fcc) crystal structure. It was also confirmed that the synthesized nanoparticles were free of impurities. The sizes of the nanoparticles were calculated from the XRD results and were found to be smaller than 15 nm. SEM images showed that the nanoparticles had a spherical shape. When we look at the characterization results, they seem to be compatible with the literature. This green procedure features high efficiency and easy operations without involving toxic reagents. Environmental compatibility, low cost and easy synthesis are some of the advantageous aspects of this method compared to other reported methods.

As a result of the study, AgNP and ZnO NP with very small particle sizes (maximum 15 nm) were synthesized and when EDX analysis was examined, it was seen that the majority of the particles were synthesized as the desired nanoparticles by weight. In this way, it has the potential to be used in areas where physically and chemically synthesized nanoparticles are used, especially in sensors. In addition, since it can pass the blood-brain barrier due to its very small size, it can also be used in drug delivery systems. However, nanoparticles synthesized with the green method settle after a short time because they remain suspended in the liquid.

In this study, ZnO NPs and AgNPs were found to be more effective against Gram positive bacteria. The size and shape of the metal NP are factors affecting its antibacterial activity. Since they have the same shape structure, it is thought that their antimicrobial effects are due to their different sizes. It has been demonstrated that smaller NPs possess larger specific surface areas, which results in a higher probability of contacting the bacterial cell membrane and penetrating into the bacteria than larger NPs [18]. ZnO NP showed stronger antimicrobial activity due to its smaller size compared to AgNP. In this study, the synthesized silver and zinc nanoparticles showed a low antibiofilm activity. Also, the synthesized nanoparticles did not exhibit antiquorum sensing activities.

AUTHOR CONTRIBUTIONS

Concept: N.V., N.G.; Design: N.V., N.G., S.S.R., M.B., İ.M.P.; Control: N.V., N.G., M.B.,

İ.M.P.; Sources: N.V., N.G., S.S.R., M.B., İ.M.P.; Materials N.V., N.G., S.S.R., M.B., İ.M.P.; Data Collection and/or Processing: N.V., N.G., S.S.R., M.B., İ.M.P.; Analysis and/or Interpretation: N.V., N.G., S.S.R., M.B., S.B.A., M.E., N.K.P., İ.M.P.; Literature Review: N.V., N.G.; Manuscript Writing: N.V., N.G., S.S.R., M.B., S.B.A., M.E., N.K.P., İ.M.P.; Critical Review: N.V., N.G., S.S.R., M.B., S.B.A., M.E., N.K.P., İ.M.P.; Other: -

CONFLICT OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

The authors declare that the ethics committee approval is not required for this study.

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J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 49(3): 827-837, 2025
Doi: 10.33483/jfpau.1666211

ORIGINAL ARTICLE / ÖZGÜN MAKALE

SPECTROPHOTOMETRIC CO-ESTIMATION OF ATORVASTATIN AND EZETIMIBE IN A BINARY MIXTURE USING PRINCIPLE COMPONENT REGRESSION AND PARTIAL LEAST SQUARES REGRESSION MODELS

TEMEL BİLEŞEN REGRESYONU VE KISMİ EN KÜÇÜK KARELER REGRESYONU MODELLERİ KULLANILARAK ATORVASTATİN VE EZETİMİBİN İKİLİ KARIŞIMLARDA EŞZAMANLI TAYİNİ

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ABSTRACT

Objective: *Fixed-dose formulations, such as combination of atorvastatin (AT) and ezetimibe (EZ), present analytical challenges due to overlapping spectral features in UV-Vis spectroscopy. Although traditional chromatographic methods are effective in these cases, they are not cost-efficient, and the required instrumentation is not easily accessible. This study aims to develop a simpler, cost-effective spectrophotometry-based approach for the simultaneous quantification of AT and EZ in fixed-dose formulations.*
Material and Method: *Principal component regression (PCR) and partial least squares (PLS) regression models were used in combination with UV-Vis spectroscopy. A calibration set of binary mixtures was prepared in the working range of 4–36 µg/ml for both drugs. PCR and PLS models were constructed using three latent variables. The developed methods were evaluated for accuracy, precision, and selectivity using laboratory-prepared samples. The applicability of the methods was demonstrated by analyzing commercial tablet samples.*
Result and Discussion: *Both models achieved high recovery rates (98–102.3%) and low relative standard deviations (<2.0%), confirming their accuracy and precision. Standard addition studies confirmed the selectivity of the methods. Then, the proposed PCR and PLS methods successfully quantified AT and EZ in commercial film-coated tablets without extensive sample preparation, offering a simple, cost-effective, and efficient alternative to conventional techniques.*
Keywords: *Atorvastatin, chemometrics, ezetimibe, PCR, PLS, spectrophotometry*

ÖZ

Amaç: *Atorvastatin (AT) ve ezetimibin (EZ) kombinasyonu gibi sabit dozlu formülasyonlardaki etken maddelerin spektral örtüşmeleri, UV-GB spektroskopi temelli analiz yöntemlerinin geliştirilmesini zorlaştırır. Geleneksel kromatografik yöntemler bu tür durumlarda etkili olsa da, maliyet açısından verimli değildir ve gerekli cihazlara erişim kolay olmayabilir. Bu çalışma, sabit dozlu formülasyonlarda AT ve EZ'in eş zamanlı miktar tayini için daha basit ve ekonomik bir spektrofotometri temelli yaklaşım geliştirmeyi amaçlamaktadır.*
Gereç ve Yöntem: *Bu çalışmada, temel bileşen regresyonu (PCR) ve kısmi en küçük kareler regresyonu (PLS) modelleri, UV-GB spektroskopisi ile birlikte kullanılmıştır. Her iki ilaç için de 4–*

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Submitted / Gönderilme : 26.03.2025
Accepted / Kabul : 12.05.2025
Published / Yayınlanma : 19.09.2025

36 µg/ml çalışma aralığında ikili karışımlardan oluşan bir kalibrasyon seti hazırlanmıştır. PCR ve PLS modelleri üç gizli değişken kullanılarak oluşturulmuştur. Geliştirilen yöntemler, laboratuvarda hazırlanan örnekler kullanılarak doğruluk, kesinlik ve seçicilik açısından değerlendirilmiştir. Yöntemlerin uygulanabilirliği, ticari tablet örnekleri analiz edilerek gösterilmiştir.

Sonuç ve Tartışma: *Her iki modelle yüksek geri kazanım oranları (%98–102.3) ve düşük bağıl standart sapma değerleri (<%2.0) elde edilmiş, yöntemlerin doğruluk ve kesinliklerini doğrulamıştır. Yöntemlerin seçiciliği, standart ekleme çalışmaları ile teyit etmiştir. Kapsamlı numune hazırlama gerektirmeden, PCR ve PLS yöntemleri kullanılarak film kaplı tabletlerdeki AT ve EZ'in eş zamanlı miktar tayinleri başarıyla gerçekleştirilmiştir. Geliştirilen yöntemler, geleneksel tekniklere kıyasla basit, ekonomik ve verimli alternatifler sunmuştur.*

Anahtar Kelimeler: *Atorvastatin, ezetimib, kemometri, PCR, PLS, spektrofotometri*

INTRODUCTION

Fixed-dose formulations are commonly used in cardiovascular diseases due to their advantages such as improved patient adherence, better clinical outcomes, and reduced health-care costs [1,2]. However, they present significant challenges for analytical chemists due to the complexity. The pharmaceutical industry relies heavily on chromatographic techniques to address this challenge, but these techniques require long periods of method development, sophisticated instrumentation, trained personnel, and higher costs [3]. Consequently, developing reliable, accurate, precise yet simple, low-cost and environmentally sustainable analytical methods for fixed-dose formulations is an important task.

UV-Vis spectroscopy offers simple, straightforward, and cost-effective analytical procedures for quality control and pharmaceutical research. However, it often becomes inadequate when the analytes exhibit overlapping spectral features. Since univariate spectroscopic methods cannot resolve spectral interferences, the use of chemometric and multivariate calibration techniques becomes necessary to analyze the overlapping spectra of active pharmaceutical ingredients in fixed-dose formulations [3-5]. In particular, principal component regression (PCR) and partial least squares (PLS) regression techniques provide effective solutions by extracting latent variables and modelling the relationship between analyte concentrations and the spectral data with overlapping features [6-8].

Atorvastatin (AT) and ezetimibe (EZ), two widely prescribed lipid-lowering agents, are frequently combined in fixed-dose formulations to enhance therapeutic efficacy, improve patient adherence, and reduce side effects [9]. AT is a statin that inhibits 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, thereby decreasing hepatic production of low-density lipoprotein cholesterol. EZ, an inhibitor of intestinal cholesterol absorption, is usually combined with a statin such as AT [10]. However, the spectrophotometric analysis of this combination presents a challenge because their absorbance spectra overlap almost completely in the region 200-300 nm. Indeed, a literature survey reveals that the quantitative estimation of AT and EZ has been mostly studied by using chromatographic techniques [11-24]. Conversely, some studies have proposed UV-Vis spectroscopy combined with simple mathematical algorithms as an alternative for analyzing AT and EZ. Techniques such as Vierordt's method [25], H-point standard additions method [26], difference absorbance method, [27,28], Q-absorbance method [29], ratio spectra method [28], first derivative spectroscopy [26,30], and ratio spectra derivative spectrophotometry [26,31] have provided relatively simple and cost-effective approaches. However, these techniques rely on the accurate wavelength selection, are sensitive to noise, and have limited applicability in multi-component mixtures [32-34]. In contrast, multivariate calibration methods, such as PCR and PLS uses full spectral data, reduce collinearity, and improve signal-to-noise ratios [35-36].

The objective of this study was to demonstrate the applicability of PCR and PLS models combined with UV-Vis spectrophotometry as reliable alternatives to conventional methods for the co-estimation of AT and EZ in fixed-dose formulations. PCR and PLS regression models were developed, and their performance was evaluated through a series of validation studies, including accuracy, precision, selectivity, and robustness. Finally, the proposed methods were successfully applied to the analysis of film-coated tablet samples, demonstrating their applicability in pharmaceutical analysis.

MATERIAL AND METHOD

Instruments and Software

UV-Vis spectra of the samples were recorded using a Shimadzu UV-2550 double-beam spectrophotometer (Kyoto, Japan), equipped with UVProbe Software (Shimadzu, Kyoto, Japan). Measurements were conducted using a quartz cuvette with a 1 cm optical path length, and the slit width was set to 2 nm. Spectra were recorded between 210–340 nm, with a spectral resolution of 0.1 nm against methanol as blank. The spectra of samples were exported into an Excel sheet (Microsoft, USA) as a matrix with each column as a different sample. The development of PCR and PLS models, as well as validation and prediction steps were operated in Matlab (MathWorks, USA). The same software was also used to plot the figures as well.

Chemicals and Reagents

Standard materials of AT and EZ were generously provided by Neutec Pharmaceuticals (Sakarya, Türkiye). Analytical-grade methanol was obtained from Carlo Erba (Milan, Italy). The commercial sample of AT and EZ fixed-dose combination was procured from a local pharmacy, produced by Neutec Pharmaceuticals, Sakarya, Türkiye. Label claim was 10 mg AT and 10 mg EZ per tablet.

Standard Solutions

Stock solutions of AT and EZ were individually prepared by dissolving 10 mg of each compound in 100 ml of methanol. The working concentration ranges were 4–36 µg/ml for AT and EZ. A calibration set comprising 25 solutions was generated by factorial design at five concentration levels. Solutions in calibration set were prepared by mixing the required amount of stock solutions and diluting them with methanol. The specific concentrations of each standard material in calibration sample set are listed in Table 1.

Table 1. Calibration set used to construct PCR and PLS models

	AT	EZ		AT	EZ		AT	EZ		AT	EZ		AT	EZ
C1	4	4	**C6**	12	4	**C11**	20	4	**C16**	28	4	**C21**	36	4
C2	4	12	**C7**	12	12	**C12**	20	12	**C17**	28	12	**C22**	36	12
C3	4	20	**C8**	12	20	**C13**	20	20	**C18**	28	20	**C23**	36	20
C4	4	28	**C9**	12	28	**C14**	20	28	**C19**	28	28	**C24**	36	28
C5	4	36	**C10**	12	36	**C15**	20	36	**C20**	28	36	**C25**	36	36

Concentration values are given in µg/ml

To assess the performance of the chemometric models, an independent test set of 11 samples with varying concentrations of both analytes was prepared following the same procedure. The concentration values of these test samples, listed in Table 2, fell within the defined working ranges. As can be seen in Table 2, the concentration of the analytes in the test set was different than those in the calibration set. Another set of synthetic binary mixtures at three distinct concentration levels (Table 3) were prepared in triplicate to evaluate both intra-day and inter-day precision. Additionally, a set of standard addition samples were prepared in triplicate to evaluate the selectivity. For this purpose, the required volumes of standard stock solutions of AT and EZ were added onto a fixed volume of commercial sample solutions and were diluted with methanol up to the final volume. The final concentration of added standards were 10 µg/ml, 20 µg/ml, and 30 µg/ml for both drugs. One extra solution was prepared by diluting the fixed volume of commercial sample to the final to be used for subtraction in standard addition studies.

Commercial Sample Solutions

Ten film-coated tablets were grounded into a powder in a mortar. A portion of the powder, equivalent to 0.5 tablet was placed in a 25 ml volumetric flask, then filled to volume with methanol. The solution was magnetically stirred for 20 minutes and, filtered from a syringe filter with a pore size of 0.45 µm. Final sample solution to be analyzed was prepared by diluting 2 ml filtrate to 10 ml, with a theoretical concentration of 20 µg/ml of each analyte. This procedure was repeated 10 times.

RESULT AND DISCUSSION

Overlapping spectra of analytes is a common challenge for spectroscopic analyses. Multivariate calibration methods such as PCR and PLS have the ability to extract latent variables that can be used for simultaneous analysis in spite of overlapping. However, these methods still rely on the assumption that absorbance-concentration relationship follows Lambert-Beer's law because the deviations from linearity can cause the models to loose their predictive power [37-38]. Hence, the first step was to define the linear concentration range of the analytes. For this purpose, individual standard solutions were prepared by diluting the stock solution. A working range of 4-36 µg/ml, with five calibration points with equal steps was studied for both drugs. The absorbance spectra of these solutions were recorded between 200-360 nm. The absorbance values of analytes at their λmax (246 nm for AT and 233 nm for EZ) were plotted against the concentration values. Linearity was evaluated by inspecting the mentioned calibration graph, as well as studentized residual plots [39]. Linearity was confirmed as calibration plots showed a linear trend and residuals were randomly distributed (See supplemental data). The spectra of the individual analytes at calibration levels 4, 12, 20, 28 and 36 µg/ml are illustrated in Figure 1. As can be seen in this figure, conventional univariate calibration would not be useful in to determine AT and EZ in their mixtures because of the heavily overlapping spectra. In order to address this challenge the most popular multivariate models, PCR and PLS, were used to develop new analytical methods to quantify AT and EZ in their commercial samples.

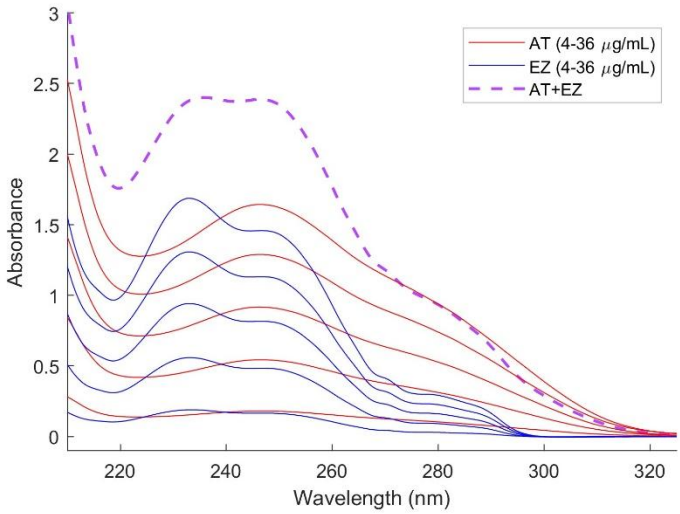


Figure 1. Absorption spectra of 4-36 µg/ml AT, 4-36 µg/ml EZ, and their binary mixture in methanol

Multivariate Calibration Models

PCR and PLS regression models were applied for the quantification of AT and EZ in their mixtures. In both models, the concentration matrix and the absorbance matrix between 210-340 nm with $\Delta\lambda$=0.1 were used after mean-centering. Both methods are multivariate calibration techniques based on latent variables but differ in how these variables are computed. PCR combines principal component analysis (PCA) with inverse least squares regression, using only spectral data to extract latent variables. The latent variables describe the variance of the spectral dataset, independent of the analyte concentrations. These latent variables, also referred to as scores, are used as predictors to build a regression model that relates them to the concentration data [40].

The implementation of the PCR model was started by decomposing absorbance matrix of the calibration set into score matrix T and loading matrix. After this step, cross-validation was performed to find the number of components to be used in the predictive phase. As can be seen in Figure 2, three principal components were adequate for both drugs. Then, the truncated score matrix T with three

columns was regressed on the concentration matrix to calculate the regression coefficient matrix. Finally, the predicted concentration matrix was computed by matrix multiplication of regression coefficient matrix with the absorbance matrix of samples [40-41].

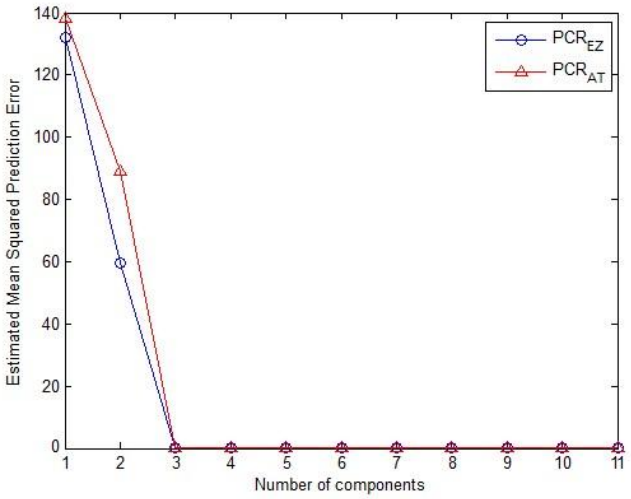


Figure 2. Cross validation plot of the PCR model for AT and EZ

Conversely, PLS includes both spectral and concentration data during decomposition to describe the covariance between the two datasets, resulting in analyte-dependent latent variables. In PLS implementation, absorbance and concentration matrices were simultaneously decomposed by PCA, with an internal equation relating the two score matrices P, and Q. Then, weight matrix, W, that maximizes the covariance between scores and the concentration matrix was computed. Similar to the PCR, cross validation was performed to decide on the number of latent variables as three for AT and EZ drugs (See Figure 3). Later, the regression coefficient matrix B was computed using the truncated matrices by the equation $B = W(P^TW)^{-1}Q^T$ to be used in the prediction step [38,41,42].

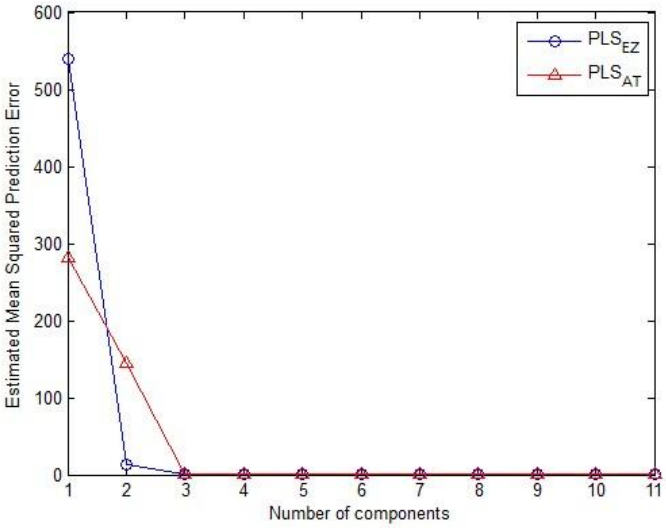


Figure 3. Cross validation plot of the PLS model for AT and EZ

Actual and predicted concentration values by the PCR and PLS models were plotted to evaluate the outliers and to visualize the predictive power as given in Figure 4.

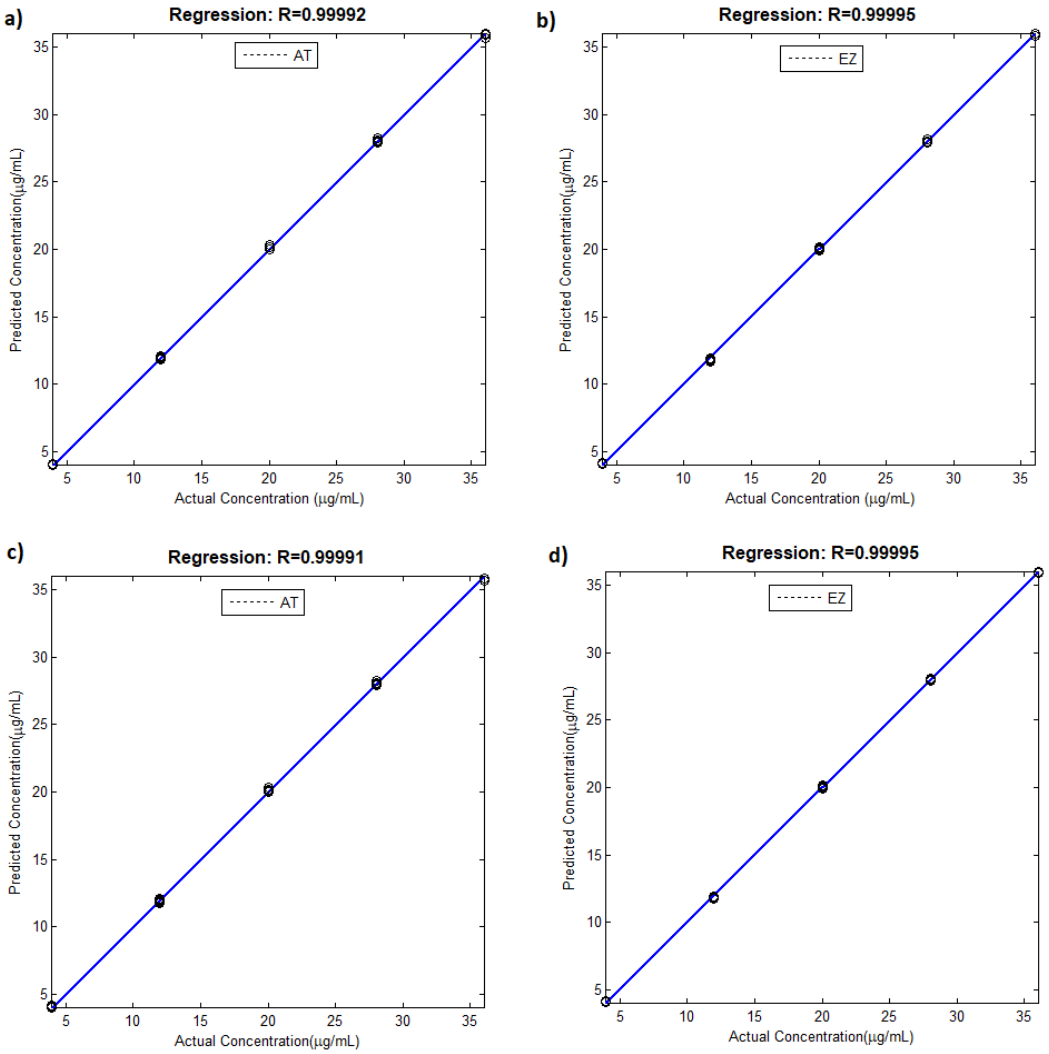


Figure 4. Actual and predicted concentration plots of AT and EZ obtained by the developed PCR (a and b) and PLS (c and d) models

Analytical Validation

Analytical validation of the developed PCR and PLS methods were carried out by analyzing different sets of laboratory-made solutions and assessing their results. A test set of 11 binary mixtures were prepared by diluting stock solutions to reach various concentration levels. Actual and predicted concentration values by the PCR and PLS methods are given in Table 2. The recovery value of each sample, as well as their mean, standard deviation and relative standard deviation were also listed in the same table. Mean recovery values were reported to be between 102.3% and 98 %, with relative standard deviation values smaller below 2.0, indicating sufficient accuracy and precision of the models in spite of overlapping spectral features. Additionally, to compare how well PCR and PLS models performs on independent data, standard error of prediction (SEP) was used. As an estimation of the average deviation of predicted values from the actual values, standard deviation of residuals obtained by PCR and PLS models for both drugs were calculated and reported as SEP values in Table 2.

Table 2. Recovery results of independent test samples by PCR and PLS methods

			PCR		PLS		PCR		PLS	
	Added (µg/µl)		Found (µg/µl)		Found (µg/µl)		Recovery (%)		Recovery (%)	
Code	**EZ**	**AT**	**EZ**	**AT**	**EZ**	**AT**	**EZ**	**AT**	**EZ**	**AT**
M1	4	30	4.10	30.08	4.14	30.08	102.4	100.3	103.6	100.3
M2	12	30	12.09	29.47	12.37	29.50	100.7	98.2	103.1	98.3
M3	20	30	20.12	29.62	20.38	29.66	100.6	98.7	101.9	98.9
M4	28	30	27.96	29.81	28.26	29.86	99.9	99.4	100.9	99.5
M5	36	30	36.41	29.59	36.63	29.61	101.1	98.6	101.8	98.7
M6	30	4	31.51	3.98	31.17	3.96	105.0	99.5	103.9	99.1
M7	30	12	30.73	11.25	30.49	11.30	102.4	93.7	101.6	94.2
M8	30	20	30.72	19.55	30.80	19.61	102.4	97.7	102.7	98.0
M9	30	28	30.44	27.31	30.54	27.38	101.5	97.5	101.8	97.8
M10	30	36	30.44	35.00	30.59	35.07	101.5	97.2	102.0	97.4
M11	30	30	30.41	29.15	30.51	29.22	101.4	97.2	101.7	97.4
				Mean:			101.7	98.0	102.3	98.1
			Standard deviation:				1.37	1.74	0.92	1.59
			Relative standard deviation:				1.35	1.77	0.90	1.62
			Standard error of prediction:				0.64	0.60	0.63	0.55

Table 3. Analysis results of intra-day and inter-day samples (n=3)

	Added (µg/ml)		Found (µg/ml)			
			PCR		PLS	
	AT	**EZ**	**AT**	**EZ**	**AT**	**EZ**
Inter-day	10	10	10.22	9.70	10.35	9.69
	20	20	19.79	19.45	19.67	19.44
	30	30	30.41	29.45	30.56	29.51
Intra-day	10	10	10.18	9.77	10.08	9.63
	20	20	20.43	19.61	20.31	19.54
	30	30	30.86	29.34	30.90	29.41
			Mean recovery (%)			
			PCR		**PLS**	
			AT	EZ	AT	EZ
Inter-day			102.23	97.03	103.54	96.86
			98.96	97.24	98.35	97.21
			101.38	98.16	101.87	98.36
Intra-day			101.81	97.67	100.83	96.31
			102.17	98.04	101.56	97.72
			102.86	97.81	102.98	98.05
			Relative Standard Deviation			
			PCR		**PLS**	
			AT	EZ	AT	EZ
Inter-day			3.69	2.34	2.41	3.22
			2.15	2.17	1.98	1.83
			1.38	0.85	0.68	0.76
Intra-day			3.01	0.37	2.21	2.03
			2.51	2.99	1.83	2.63
			1.51	0.61	1.08	0.57
			Relative Error			
			PCR		**PLS**	
			AT	EZ	AT	EZ
Inter-day			2.23	-2.97	3.54	-3.14
			-1.04	-2.76	-1.66	-2.79
			1.38	-1.84	1.87	-1.64
Intra-day			1.81	-2.33	0.83	-3.69
			2.17	-1.97	1.56	-2.29
			2.86	-2.19	2.98	-1.95

Precision and accuracy of the methods were also evaluated by inter-day and intra-day studies. Validation samples at three concentration levels (10 µg/ml, 20 µg/ml, 30 µg/ml) of both drugs was analyzed three times in one day (intra-day) and on three consecutive days (inter-day). The analysis results were summarized in Table 3, presented as mean percent recovery, relative standard deviation, and percent relative error.

The selectivity of the proposed methods was investigated by standard addition studies. The predicted concentration of AT and EZ in the sample prepared without the addition of standard was subtracted from the predicted concentration of AT and EZ in the remaining samples. The experiments were performed in triplicates. The average of added amounts, mean recoveries and relative standard deviations were listed in Table 4. The standard addition studies demonstrated that tablet excipients had no interferent effect on the predicted concentrations.

Table 4. Analysis results of standard addition studies

	Added		**Found (µg/µl)**			
	(µg/µl)		**PCR**		**PLS**	
	EZ	**AT**	**EZ**	**AT**	**EZ**	**AT**
Sample solution +	10	10	10.07	9.65	9.85	9.71
Sample solution +	20	20	20.41	20.14	20.16	20.21
Sample solution +	30	30	30.27	29.74	30.05	29.81
			Recovery (%)			
			PCR		**PLS**	
			EZ	AT	EZ	AT
			100.7	96.5	98.5	97.1
			102.1	100.7	100.8	101.0
			100.9	99.1	100.2	99.4
			Relative standard deviation			
			PCR		**PLS**	
			EZ	AT	EZ	AT
			0.04	0.04	0.04	0.04
			0.11	0.07	0.12	0.07
			0.03	0.10	0.08	0.09

Assay Results of Commercial Samples

The developed PCR and PLS methods were applied to the quantitative analysis of commercial sample solutions. The assay results of commercial tablets are presented in Table 5 as milligrams per tablet. The average assay results confirmed well with the label claim. The assay results from using PCR and PLS were compared using the F-test and t-test. The computed F- and t-statistic values were smaller than the critical values, indicating comparable results in terms of variance and mean. The analysis results obtained by PCR and PLS models did not significantly differ for either AT or EZ.

In this study, two new spectrophotometric methods based on multivariate regression were developed for the co-estimation AT and EZ in fixed-dose tablet formulations. The regression methods, namely PCR and PLS, were used to overcome the spectral overlapping problem by using the latent variables. The analytical performance of the proposed methods was confirmed by analytical validation studies. The developed PCR and PLS models were applied to the simultaneous estimation of EZ and AT in film-coated tablet formulations. Both methods showed good agreement with the label claim, with no significant difference between them. The proposed methods were directly applicable for the pharmaceutical analysis and did not require laborious sample preparation, extraction, nor separation steps. This study shows PCR and PLS models can be combined with spectrophotometry to ensure accurate, precise, reliable, and efficient analysis of pharmaceuticals containing two active pharmaceutical ingredients.

Table 5. Assay results of commercial tablets containing AT and EZ

	mg/tablet*			
	PCR		PLS	
	EZ	AT	EZ	AT
T1	9.71	10.19	10.03	10.10
T2	10.25	10.07	10.17	9.81
T3	10.25	10.13	10.22	9.87
T4	10.17	9.85	10.11	9.90
T5	10.18	9.82	10.26	9.97
T6	10.26	9.80	10.13	9.95
T7	10.44	10.04	10.18	9.79
T8	10.30	9.99	10.72	10.14
T9	10.45	9.95	10.08	9.71
T10	10.50	9.76	10.09	9.82
Mean	10.25	9.96	10.20	9.91
Standard deviation	0.22	0.15	0.19	0.14
Relative standard deviation	2.16	1.48	1.90	1.38
F-stat	1.29	1.18	**F-crit** =3.18 (p=0.05)	
t-stat	0.56	0.85	**t-crit** =2.10 (p=0.05)	

*Label claim: 10 mg AT, 10 mg EZ per tablet

AUTHOR CONTRIBUTIONS

Concept: Z.C.E., E.D.; Design: Z.C.E., E.D.; Control: E.D.; Sources: E.D.; Materials: E.D.; Data Collection and/or Processing: Z.C.E., E.D.; Analysis and/or Interpretation: Z.C.E., E.D.; Literature Review: Z.C.E.; Manuscript Writing: Z.C.E., E.D.; Critical Review: Z.C.E., E.D.; Other: -

CONFLICT OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

The authors declare that the ethics committee approval is not required for this study.

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Ankara Ecz. Fak. Derg. / J. Fac. Pharm. Ankara, 49(3): 838-851, 2025
Doi: 10.33483/jfpau.1662695

ÖZGÜN MAKALE / ORIGINAL ARTICLE

IRE1α İNHİBİTÖRÜ GSK2850163'ÜN NÜKLEOZİT METABOLİK İNHİBİTÖRÜ KAPESİTABİN ÜZERİNDEKİ GÜÇLENDİRİCİ ROLÜNÜN KOLON KANSERİ HÜCRELERİNDE DEĞERLENDİRİLMESİ

EVALUATION OF THE BOOSTER ROLE OF IRE1α INHIBITOR GSK2850163 ON NUCLEOSIDE METABOLIC INHIBITOR CAPECITABINE IN COLON CANCER CELLS

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ÖZ

Amaç: *Kapesitabin kolon kanseri tedavisine yönelik yaygın olarak kullanılan etkili ajanlardan birisidir. Ancak sistemik yan etkiler ve direnç gelişimi gibi olumsuz durumlar tedavi etkinliğini sınırlandırabilmektedir. Çalışmamızın amacı, UPR sinyal yolunun IRE1α/XBP-1 dalının seçici inhibitör olan GSK2850163 aracılı inhibe edilmesinin kolon kanseri hücrelerinde kapesitabine olan duyarlılığa olan etkilerinin araştırılmasıdır.*
Gereç ve Yöntem: *Çalışmalarımızda insan kolon kanseri hücreleri olan Caco-2 ve HT-29 hücreleri kullanılmıştır. Kapesitabin ve GSK2850163'ün hücre canlılığı üzerine olan etkileri WST-1 testi ile incelendi. Takiben belirlenen etkin konsantrasyonlarda kombinasyon çalışmaları ile canlılık analizlerine devam edildi. GSK2850163'ün IRE1α/XBP-1 yolu üzerindeki inhibe edici etkisi immünoblotlama çalışmaları ile doğrulandı. Kapesitabin ve GSK2850163 eş uygulama tedavisinin hücrelerin migrasyon ve invazyon yeteneği üzerine olan etkileri yara iyileşme ve matrijel-kaplı Boyden-chamber invazyon testi ile değerlendirildi.*
Sonuç ve Tartışma: *Çalışmamızda IRE1α/XBP-1'in GSK2850163 aracılı inhibisyonunun kolon kanseri hücrelerinin canlılığını, migrasyon ve invazyon kapasitesini önemli ölçüde sınırladığı belirlendi. Kapesitabin ve GSK2850163'ün eş uygulama tedavisinin yalnız başına kapesitabin veya GSK2850163 uygulamalarına kıyasla kolon kanseri hücreleri üzerinde daha etkili olduğunu gözlemlendi. Araştırma bulgularımız, kolon kanserine yönelik IRE1α/XBP-1 sinyal yolunun farmakolojik olarak hedeflenmesinin etkili tedavi yaklaşımları sunabileceğini önermektedir.*
Anahtar Kelimeler: *GSK2850163, IRE1α, kapesitabin, kolon kanseri, UPR*

ABSTRACT

Objective: *Capecitabine is one of the widely used potent agents for the treatment of colon cancer. However, adverse events, such as systemic side effects and resistance development, may limit the effectiveness of the treatment. Our study aimed to investigate the impact of GSK2850163-mediated selective inhibition of the IRE1α/XBP-1 branch of the UPR signaling pathway on the sensitivity of colon cancer cells to capecitabine.*

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Gönderilme / Submitted : 21.03.2025
Kabul / Accepted : 14.05.2025
Yayınlanma / Published : 19.09.2025

Material and Method: *Human colon cancer cells Caco-2 and HT-29 were used in our studies. The effects of capecitabine and GSK2850163 on cell viability were examined by WST-1 test. Viability analyses were continued with combination studies to determine effective concentrations. The inhibitory effect of GSK2850163 on the IRE1α/XBP-1 pathway was confirmed by immunoblotting studies. The impact of capecitabine and GSK2850163 co-administration treatment on the migration and invasion ability of cells was evaluated by wound healing and matrigel-coated Boyden-chamber invasion test.*

Result and Discussion: *Our study determined that GSK2850163-mediated inhibition of IRE1α/XBP-1 significantly limited the viability, migration and invasion capacity of colon cancer cells. We observed that co-administration of capecitabine and GSK2850163 was more effective on colon cancer cells compared to capecitabine or GSK2850163 alone. Our research findings suggest that pharmacological targeting of the IRE1α/XBP-1 signaling pathway for colon cancer may offer effective treatment approaches.*

Keywords: *Capecitabine, colon cancer, GSK2850163, IRE1α, UPR*

GİRİŞ

Kolorektal kanser kolonda ve/veya rektumdaki dokuların kanserleşmesi nedeniyle ortaya çıkan ve günümüzde özellikle değişen beslenme alışkanlıkları nedeniyle sıklıkla teşhis edilen kanser türlerinden birisidir [1]. Amerika Birleşik Devletleri'nde kolorektal kanser, erkeklerde kanserle ilişkili ölümlerin üçüncü, kadınlarda ise dördüncü önde gelen nedenidir. Amerikan Kanser Derneği'nin 2025 yılı tahminlerine göre Amerika Birleşik Devletleri'nde 107.320 yeni kolon kanseri vakası gözleneceği ön görülmektedir. 2025 yılında yaklaşık 52.900 bireyin yaşamını yitirmesine neden olması beklenmektedir [2]. Günümüzde cinsiyet, yaş, diyet, obezite, sigara kullanımı, alkol tüketimi, sedanter yaşam tarzı, bağırsak mikrobiyotası ve kalıtsal hastalıklar gibi çeşitli risk faktörlerinin kolorektal kanserle ilişkili olduğu tespit edilmiştir [3]. Kolon kanseri tedavisinde çoğunlukla lokal tedavi olarak cerrahi girişim, ablasyon ve embolizasyon, radyoterapi, sistemik tedavi olarak ise kemoterapi, hedefe yönelik tedavi ve immünoterapi gibi yaklaşımlardan faydalanılmaktadır [4].

Kapesitabin oral olarak kullanılan pirimidin analoğu olarak bilinen 5-Fluorourasil (5-FU)'in ön ilacıdır ve 5-FU ile aynı hücresel etki mekanizmasını sergileyerek metabolitlerinin DNA ve RNA'ya dahil edilmesi yoluyla antikanser etkiler sergilemektedir [5,6]. Kapesitabin, yaygın olarak kolon kanseri tedavisinde monoterapi, adjuvan tedavi ve metastatik veya ilerlemiş kanserler için diğer ajanlarla kombinasyon tedavisi olarak kullanılmaktadır [7]. Kapesitabin doz-sınır toksisiteleri arasında diyare, karın ağrısı, bulantı, stomatit ve el-ayak sendromu bulunmaktadır [8]. Bununla birlikte kapesitabin'in yarattığı yan etkiler ve hastalarda gelişen ilaç direnci gibi olumsuz durumlar tedavideki kullanımını sınırlandırabilmektedir [6,9]. Son 10 yılda yapılan çalışmalar, hücrelerdeki endoplazmik retikulum (ER) stresi, katlanmamış protein cevabı (UPR) ve proteostaz kontrolünün kanser ilerlemesi ve kazanılmış ilaç direncinde önemli mekanizmalar olduğuna işaret etmektedir [10,11]. Bu nedenle fizyolojik öneme sahip bu mekanizmaların farmakolojik olarak hedeflenmesi kanser araştırmalarında daha da ilgi çekici hale gelmiştir.

ER; lipit, karbonhidrat ve kolesterol metabolizması gibi önemli fizyolojik biyokimyasal süreçlere ev sahipliği yapmasının yanı sıra ökaryotik hücrelerde protein sentezi için önemli merkezlerden birini teşkil etmektedir [12,13]. Bununla birlikte ER, proteinlerin katlanması, ko- ve post-translasyonel modifikasyonların gerçekleştirilmesi ve bu proteinlerin olgun formlarına ulaştırılmasının hassas bir şekilde denetlendiği protein kalite kontrolü süreçlerine ilişkin sofistike mekanizmalara ev sahipliği yapan hayati bir organeldir [14]. ER'nin kapasitesinin aşılması, genotoksik etkiler, oksidatif stres gibi çeşitli etkenler proteinlerin katlanmasında aksamalara ve bozulmalara neden olarak yanlış katlanmış ve katlanmamış proteinlerin ER lümeninde birikmesiyle karakterize olan ER stresi adı verilen bir hücresel süreci tetikleyebilmektedir [15]. ER stresinin aşılmasında ve bu yol ile ER homeostazisinin yeniden kurulmasındaki başlıca mekanizma UPR sinyalizasyonudur [15].

Proteostaz koşulları altında moleküler şaperon BiP/GRP78 proteini, ER-membranında lokalize transmembranal özellikteki üç ER stres sensör proteini olan; inozitol gerektiren enzim 1 alfa (IRE1α), protein kinaz RNA benzeri ER kinaz (PERK) ve aktive edici transkripsiyon faktörü 6 alfa (ATF6α)'yı inaktif olarak tutmaktadır. ER stresi altında hatalı katlanan proteinler için daha yüksek bağlanma afinitesine sahip olan BiP/GRP78, bağlı olduğu sensör proteinlerinden ayrışarak UPR

sinyalizasyonunun uyarımına aracılık eder [16]. Bu süreç bir seri sinyal kaskatını aktive ederek transkripsiyonel ve translasyonel düzeydeki adaptif sinyal yollarını uyarmaktadır [16-18]. Son yıllardaki çalışmalar, UPR'nin IRE1α/XBP-1 sinyal kolunun aktivitesindeki değişimlerin kanser de dahil olmak üzere birçok patoloji ile ilişkili olduğunu ortaya koymuştur [17,18].

IRE1α, sitozolde bir kinaz domainine ve ER'de bir endoribonükleaz domainine sahiptir. IRE1α'nın uyarımı oligomerizasyonuna ve oto-fosforilasyonuna neden olmaktadır. Aktive olan IRE1α endoribonükleaz aktivitesi ile X-box-binding protein 1 (XBP-1u) mRNA'sından 26 bp'lik bir intron splice edilerek aktif bir transkripsiyon faktörü, XBP1'in splice edilmiş formu (XBP-1s) oluşur. Bu oluşum UPR hedef genlerinin ekspresyonunu seçici olarak modüle etmektedir [19,20]. Yapılan çalışmalarda IRE1α/XBP-1 sinyal yolu modülasyonunun prostat kanseri, meme kanseri ve kolorektal kanser ile ilişkilendirilmiş olup birçok kanser türünde kanser gelişimi ve metastaz ile ilişkisi gösterilmiştir. IRE1α/XBP-1 kolunun kanser hücre biyolojisindeki süreçlerle ilişkili olması kanser tedavileri için umut verici bir hedef olabileceğinden bu yolağa özgü birçok terapötik ajan geliştirilmiştir [19-23].

Literatürde gerek deneysel açıdan ve gerekse klinik uygulamalarda kanser tedavisine yönelik birçok olası ilaç kombinasyonlarının kullanımları mevcuttur [24,25]. Bu bağlamda antikanser ilaçların kombine kullanımlarının sistemik sitotoksik etkilerden kaçınılmasında, daha etkili yanıtların elde edilmesinde ve gelişen ilaç direncinin üstesinden gelmesinde yaygın olarak araştırılmaktadır [25]. Bu açıdan UPR'nin IRE1α sinyal kolunun modülasyonu kansere yönelik geliştirilecek yeni terapötik yaklaşımlar açısından önemli bir hedef haline gelmektedir. GSK2850163 fosforile IRE1α'ya bağlanarak IRE1α'nın endoribonükleaz aktivitesinin inhibisyonu yolu ile XBP-1 splicing sürecini inhibe eder [26]. 2022 yılında yayınlanan bir çalışmada GSK2850163 kullanılarak IRE1α/XBP-1 yolunun inhibe edilmesinin meme kanseri hücrelerinde tamoksifene karşı duyarlılığı geliştirdiği raporlanmıştır [27].

Bu çalışmada, GSK2850163'ün Caco-2 ve HT29 insan kolon kanseri hücrelerinde kapesitabin üzerindeki iyileştirici etkisinin test edilmesi amaçlanmıştır. Bu kapsamda kolon kanseri hücrelerinin proliferasyon, migrasyon ve invazyon gibi kanser ile ilişkili önemli özellikleri üzerindeki etkileri çeşitli *in vitro* deney modelleri ile test edilmiştir. Ek olarak apoptotik hücre ölümü yolu ile ilişkili bazı genlerin ifade düzeyleri üzerindeki etkileri değerlendirilmiştir. Sonuçlarımız, GSK2850163'ün kapesitabin ile birlikteki eş uygulanmasının, kapesitabin'in Caco-2 ve HT29 hücrelerindeki antikanser etkilerini önemli ölçüde geliştirdiğini göstermiştir. Bulgularımız kolon kanseri hücrelerinde kapesitabin uygulaması ile UPR sinyalinin de farmakolojik olarak eş zamanlı modülasyonunun etkili yeni tedavi protokollerinin geliştirilmesinde umut verici bir yaklaşım olabileceğini düşündürmektedir.

GEREÇ VE YÖNTEM

Materyaller

GSK2850163 (#SML1684) ajanı Sigma-Aldrich, kapesitabin (sc-205618) ise Santacruz Biotechnology firmasından temin edildi. Hücre kültürü çalışmalarda kullanılan plastik malzemeler Corning firmasından temin edildi. Hücre kültürü çalışmalarındaki büyüme ortamı, fetal sığır serumu (FBS) ve diğer büyüme takviyeleri iler hücre kültürü sıvı materyallerinin tamamı Capricorn Scientific firmasından satin alındı. Tavşan poliklonal antikorlar XBP-1u (25997-1-AP) (1:2500) ve XBP-1s (24868-1-AP) (1:2500) Proteintech firmasından temin edildi. Fare monoklonal beta-aktin antikoru (#A5316) (1:10000) Sigma-Aldrich'ten satın alındı. HRP-konjuge anti-fare (#31430) (1:500) veya anti-tavşan (#31460) (1:500) IgG (H+L) ikincil antikorlar Thermo Fisher Scienctific'ten temin edildi. Thapsigargin (#12758) ajanı Cell Signaling Technology firmasından temin edildi. Hücre kültürü kalitesindeki dimetil sülfoksit (DMSO) (#20385.01) SERVA firmasından satın alındı.

Hücre Kültürü

Caco-2 (HTB-37TM) ve HT29 (HTB-38TM) insan epitelyal kolorektal adenokarsinoma hücre hatları American Type Culture Collection (ATCC)'dan temin edildi. Caco-2 hücreleri %10 FBS ve 2 mM L-glutamin içeren Eagle's Minimum Essential Medium (EMEM), HT29 hücreleri ise %10 FBS ve 2 mM L-glutamin içeren McCoy's 5a medium kullanılarak konvansiyonel hücre kültürü şartları olan 37 ^{o}C ve %5 CO_2 bulunan hücre kültürü inkübatöründe kültüre edildi. Hücrelerin mikoplazma içermediği

Biological Industries firmasından temin edilen EZ-PCR™ Mikoplazma Tespit Kiti (#20-700-20) kullanılarak rutin olarak doğrulandı.

Hücre Canlılık Analizi

Hücreler 96 kuyucuklu hücre kültür kaplarına her bir kuyucukta 10.000 hücre konsantrasyonunda ekildi. 24 saat sonra hücrelere 48 saat süre ile kapesitabin ve GSK2850163 veya kombinasyonları uygulandı. Kontrol uygulaması olarak hücrelere eş hacimde çözgen uygulaması gerçekleştirildi. Hücrelere uygulanan GSK2850163 DMSO'da kapesitabin ise steril dH_2O'da 1000x konsantre olarak hazırlandı. Hücrelere uygulanan DMSO'nun final konsantrasyonu % 0.05'i aşmamaktadır. 48 saat sonra hücre canlılık düzeyleri WST-1 hücre proliferasyon reaktifi (#MK400) (Takara) kullanılarak üreticinin önerdiği protokol adımları takip edilerek gerçekleştirildi. Her bir kuyucuğa 20 µl WST-1 ajanı eklendi ve 2 saat süre ile 37 ^oC'de inkübasyon işlemi gerçekleştirildi. Takiben mikroplaka okuyucu (BioTek, Epoch 2)'da 450nm dalga boyunda absorbans okuması gerçekleştirildi. Her bir örnek 3 teknik ve 3 biyolojik tekrar şeklinde çalışıldı ve sonuçlar grafikte % canlılık olarak sunuldu. IC_{50} konsantrasyonları GraphPad Prism 8.0 kullanılarak hesaplandı.

İmmünoblotlama

İmmünoblotlama çalışmaları daha önceki çalışmalarımızda rapor edildiği gibi yürütülmüştür [28]. Basitçe, deney sonlandığında pellet haline getirilen hücreler %1 memeli proteaz inhibitörü içeren radyoimmünopresipitasyon testi (RIPA) tamponu ile lizatlandı. Takiben örnekler 4 °C'de 20 dakika boyunca 14.000 r.p.m' de santrifüj edildi ve total protein içeriğini barındıran süpernatant sonraki adımlarda kullanılmak üzere saklandı. Örneklerdeki total protein içeriğinin belirlenmesi amacıyla bikinkoninik asit protein tahlil yöntemi (BCA) (#23225) (Thermo Scientific) kullanıldı. Çalışmalarda 30 µg protein örneği kullanıldı. Protein örnekleri 4x Laemmli tamponu içerisinde 95 ^oC'de 5 dakika kaynatılarak proteinler denatüre edildi. El dökümü olarak hazırlanan sodyum dodesil sülfat-poliakrilamid jel elektroforezi (SDS-PAGE) jellerine protein örnekleri yüklenerek 2 saat süre ile elektroforez işlemine tabi tutuldu. Takiben ayrıştırılan protein örnekleri Immun-Blot® poliviniliden diflorür (PVDF) (#1620177) (Bio-Rad) membrana transfer edildi. Bu işlem sonrasında membran sırasıyla bloklama, birincil antikor uygulanması, yıkama işlemi, HRP-konjuge ikincil antikor uygulanması, yıkama işlemi ve kemiluminesans görüntüleme işlemlerine tabi tutuldu. Protein bantlarının görüntülenmesi için Clarity™ Western ECL substrat (#1705061) (Bio-Rad) solüsyonu kullanıldı ve protein bantları Fusion Pulse (Vilber Lourmat) sisteminde görüntülendi.

Yara İyileşme Deneyi

Hücreler 12 kuyucuklu hücre kültür kaplarına $3.5x10^5$ hücre/kuyucuk yoğunluğunda ekildi. 24 saat sonra steril 200 µl mikropipet ucu yardımıyla yara alanları oluşturuldu. Besi ortamı uzaklaştırılarak hücreler steril 1xPBS ile iki kez yıkandı ve taze besiyeri eklendi. Hücreler 72 saat boyunca bileşiklerle tedavi edildi. Yara alanları 0. günde ve 72. günde kamera sistemine sahip olan faz-kontrast mikroskop ile fotoğraflandı (Sunny SopTop microscope). % yara kapanma düzeyleri ImageJ yazılımı (http://imagej.nih.gov/ij/) ile analiz edildi. Her bir örnek 3 teknik ve 3 biyolojik tekrar şeklinde çalışıldı ve sonuçlar grafikte % yara kapanma alanı olarak sunuldu.

İnvazyon Analizi

İnvazyon analizleri daha önce tanımlandığı şekilde gerçekleştirildi [33]. Bu kapsamda Matrigel (BD Biosciences) ve serumsuz besiyeri bir tüpte bir araya getirildi (1:8). Karışımdan 45 µl alınarak 8 µm por boyutuna sahip transwell (Sarstedt) yüzeyine uygulandı ve 37 °C'de inkübe edildi. Takiben transwell'in üst bölümüne 100 µl serumsuz besiyeri eklendi ve 30 dakika yeniden inkübe edildi. Her bir transwell'de 10.000 hücre olacak şekilde ekim işlemi gerçekleştirildi. Transwell'in alt bölümü ise %20 FBS içeren besiyeri tamamlandı. Hücrelere ajan uygulaması gerçekleştirildi ve 72 saat sonra göç eden hücreler fikse edilip kristal viyole ile boyanarak hücre sayımı gerçekleştirildi. Her bir örnek 3 teknik ve 3 biyolojik tekrar şeklinde çalışıldı ve sonuçlar % invazyon olarak grafikte sunuldu.

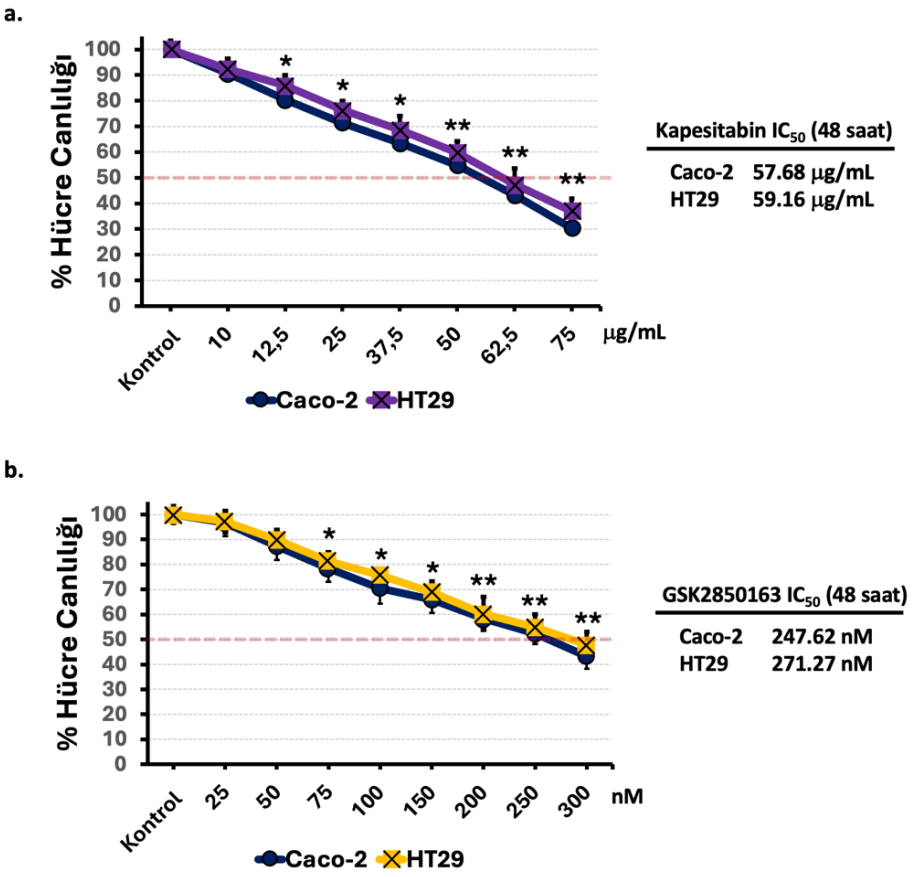
İstatistiksel Analiz

Gruplar arasındaki farkların istatistiksel anlamlılığı GraphPad Prism 8.0 kullanılarak en az %95 güven aralığı ile iki taraflı eşit varyanslı Student t-testi veya tek yönlü ANOVA analizleri ile belirlendi. $p<0.05$ değeri istatistiksel olarak anlamlı kabul edildi. Sonuçlar ortalama ± standart sapma (SD) olarak sunuldu.

SONUÇ VE TARTIŞMA

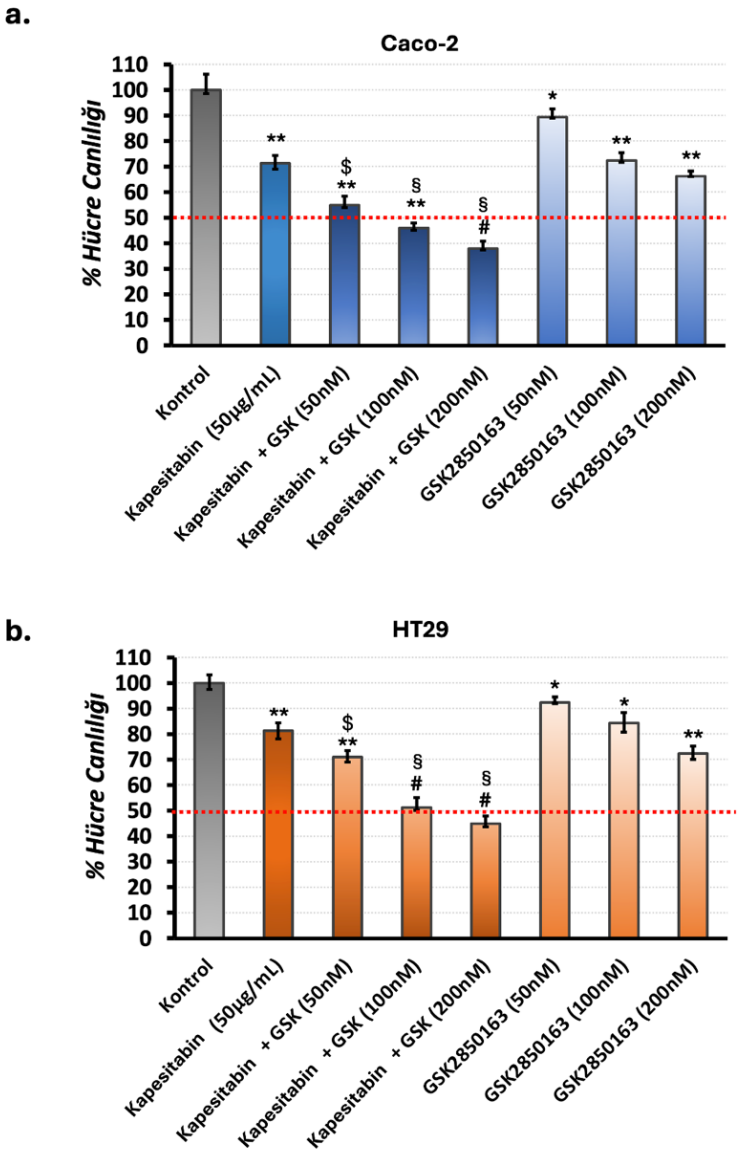
GSK2850163, Kapesitabin'in Kolon Kanseri Hücrelerinin Canlılığı Üzerindeki Etkisini Geliştirdi

Caco-2 ve HT29 hücrelerinde kapesitabin ve GSK2850163'ün hücre canlılığı üzerine olan etkilerini WST-1 temelli hücre canlılık testleri ile incelenmiştir. Bu amaçla literatür bilgiside dikkate alınarak hücreler 48 saat süreyle 10, 12.5, 25, 37.5, 50, 62.5 ve 75 µg/ml kapesitabin ve 25, 50, 75, 100, 150, 200, 250 ve 300 nM GSK2850163 ile muamele edildi. Takiben WST-1 canlılık analizi gerçekleştirildi. Ayrıca her iki ajan için de IC50 konsantrasyonları hesaplandı. Sonuçlarımız her iki ajanında konsantrasyon bağımlı olarak her iki kolon kanseri hücresinin hücre canlılığını baskıladığını gösterdi (Şekil 1a ve Şekil 1b). Caco-2 ve HT29 hücrelerinde kapesitabin için IC_{50} konsantrasyonları sırasıyla 57.8 ve 59.16 µg/ml olarak belirlendi. GSK2850163 için ise 247.62 ve 271.27 nM olarak hesaplandı.



Şekil 1. Kapesitabin ve GSK2850163'ün Caco-2 ve HT29 hücrelerinin canlılığı üzerindeki etkilerinin ve bu hücrelerdeki IC_{50} konsantrasyonlarının belirlenmesi. Caco-2 ve HT29 hücreleri **a.** 10, 12.5, 25, 37.5, 50, 62.5 ve 75 kapesitabin ve **b.** 25, 50, 75, 100, 150, 200, 250 ve 300 nM GSK2850163 ile 48 saat süre ile muamele edildi. Takiben hücre canlılığı WST-1 testi ile belirlendi. Her deney üç bağımsız biyolojik ve üç teknik tekrar ile gerçekleştirildi. IC_{50} konsantrasyonları GraphPad Prism 8.0 kullanılarak hesaplandı (Kontrol ile kıyaslamalar * $p<0.05$, ** $p<0.001$)

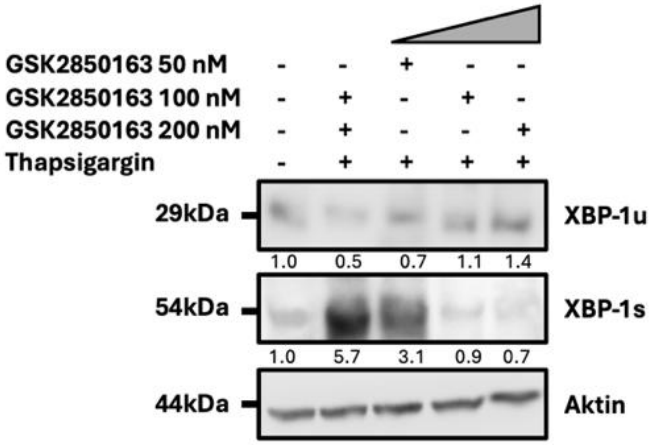
GSK28153'ün kolon kanseri hücrelerinde kapesitabin'in hücre canlılığı üzerindeki etkisine olan katkısını değerlendirmek üzere 50, 100 ve 200 nM konsantrasyonlarındaki GSK2850163 ve 50 µg/ml konsantrasyonundaki kapesitabin ile eş uygulamalarının etkilerini değerlendirdik. Kapesitabin için her iki hücre hattında belirlenen IC_{50} konsantrasyonu dikkate alınarak 50 µg/ml tercih edildi. Benzer şekilde GSK2850163 için de IC_{50} konsantrasyonunun altındaki değerler kat artışı olacak şekilde etkileri değerlendirilmek üzere tercih edildi. Sonuçlarımız yalnız başına kapesitabin veya GSK2850163 uygulamasına kıyasla GSK2850163 ile kapesitabin'in kombine uygulanmasının hücre canlılığını daha güçlü düzeyde baskıladığı belirlendi (Şekil 2a ve Şekil 2b). Bu sonuçlar GSK2850163 ile kapesitabin'in eş uygulamasının kolon kanseri hücreleri üzerinde daha güçlü antikanser etkiye sahip olduğunu göstermektedir.



Şekil 2. Kapesitabin ve GSK2850163'ün kolon kanseri hücrelerinin canlılığı zerine olan etkilerinin incelenmesi. **a.** Caco-2 ve **b.** HT29 hücreleri 50, 100 ve 200 nM GSK2850163 ve 50 µg/ml kapesitabin veya kombinasyonları ile 48 saat süre ile muamele edildi. Sonuçlar kontrol grubuna kıyasla % canlılık değişimi olarak grafikte sunuldu. Her deney üç bağımsız biyolojik ve üç teknik tekrar ile gerçekleştirildi (Kontrol ile kıyaslamalar; * p<0.05, ** p<0.005, # p<0.001, Kapesitabin ile kıyaslamalar; $ p<0.05, § p<0.01)

GSK2850163'ün IRE1α'nın Endonükleaz Aktivitesi Üzerindeki Etkinliğinin Doğrulanması

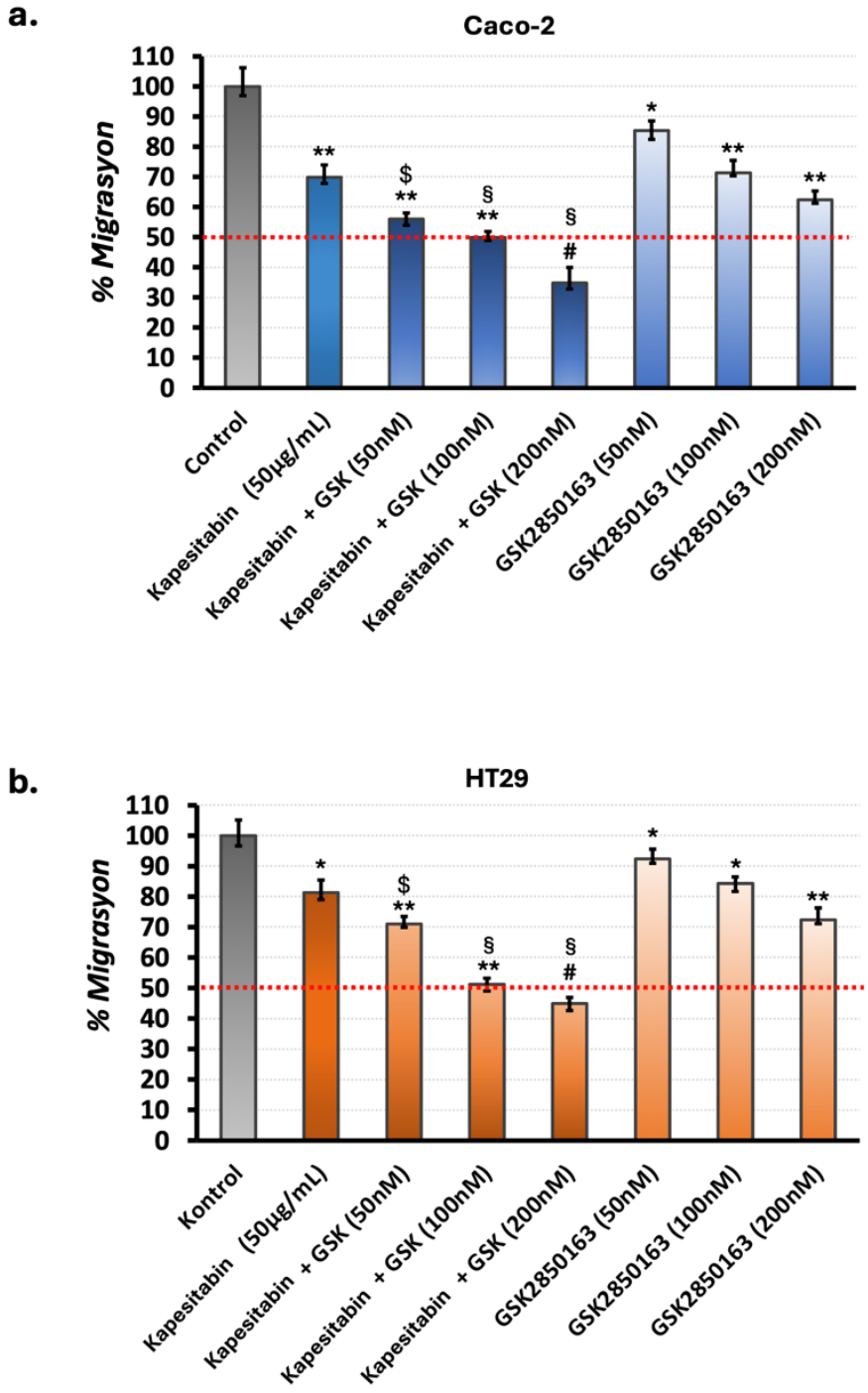
GSK2850163'ün RE1α sinyalizasyonu üzerindeki baskılayıcı etkisini doğrulamak amacıyla XBP-1u ve XBP-1s'nin test edildiği immünoblotlama çalışmaları gerçekleştirildi. Bu çalışmalarda pozitif kontrol olarak ER stresini indüklediği iyi bilinen seskiterpen lakton olan thapsigargin ajanı 24 saat süre ile 50 nM konsantrasyonda hücrelere uygulandı [29]. Sonuçlarımız kontrol grubuna kıyasla thapsigargin uygulamasına bağlı olarak XBP-1s düzeylerinde artış gözlendiğini bu artışın GSK2850163 uygulaması ile önemli ölçüde konsantrasyon bağımlı olarak baskılandığını ortaya koydu. XBP-1u düzeylerinde ise XBP-1s sonuçlarının tersine thapsigargin uygulamasına bağlı olarak azalma olduğu, GSK2850163 uygulaması ile konsantrasyon bağımlı olarak arttığı belirlendi (Şekil 3). Bu sonuçlar, kolon kanseri hücrelerine GSK2850163 uygulamasının etkin bir şekilde IRE1α/XBP-1 sinyal yolunu baskılayabilme yeteneğinde olduğunu gösterdi.



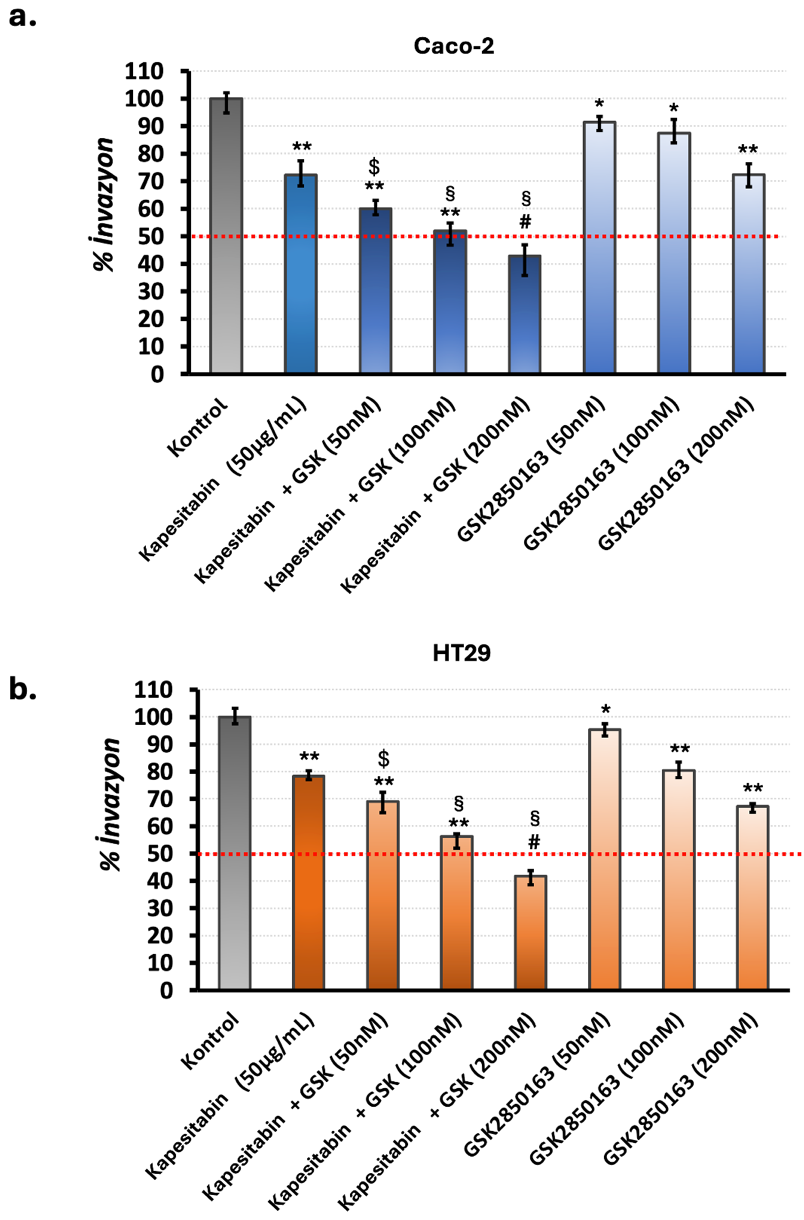
Şekil 3. GSK2850163'ün IRE1α/XBP-1 sinyali üzerindeki inhibitör etkisinin doğrulanması. Caco-2 hücreleri 50 nM thapsigargin ve 50, 100 ve 200 nM GSK2850163 ile kombinasyonu şeklinde 24 saat süre ile uygulamaya tabi tutuldu. Takiben immünoblotlama çalışmaları ile ilgili proteinlerin ifade seviyeleri incelendi. Beta-aktin yükleme kontrolü olarak kullanıldı. Thapsigargin pozitif kontrol olarak kullanıldı. Kontrol grubu olarak yalnızca çözgen ile muamele edilmiş hücreler kullanıldı. Aktin bantlarına göre normalize edilmiş XBP-1u ve XBP-1s proteinlerinin densitometrik verileri blot görüntülerinin altında sunuldu

GSK2850163 ve Kapesitabin'in Eş Uygulaması Kolon Kanseri Hücrelerinin Migrasyon ve İnvazyon Yeteneğini Önemli Ölçüde Baskıladı

GSK2850163'ün kolon kanseri hücrelerinde kapesitabin'in tümörijenik özellikleri üzerindeki etkisinin nasıl değiştiğini anlamaya yönelik gerçekleştirdiğimiz çalışmalarda kolon kanseri hücrelerinin migrasyon ve invazyon yeteneklerindeki değişimler sırasıyla yara iyileşme testi ve matrijel kaplı transwellerin kullanıldığı deney modelleri ile test edildi. Bu amaçla hücreler 50, 100 ve 200 nM GSK2850163 ve 50 μg/ml kapesitabin veya kombinasyonları ile migrasyon deneyleri için 72 saat invazyon deneyleri için ise 48 saat ile muamele edildi. Sonuçlarımız yalnız başına kapesitabin ve GSK2850163 uygulamalarının Caco-2 ve HT-29 hücrelerinin migrasyon ve invazyon yeteneklerinin kontrol grubuna kıyasla anlamlı düzeyde baskılandığını ortaya koydu (Şekil 4a ve Şekil 4b) (Şekil 5a ve Şekil 5b). Farklı konsantrasyonlardaki GSK2850163'ün kapesitabin ile kombinasyonel uygulamalarının ise yalnız başına uygulamalara kıyasla daha etkin bir düzeyde hücrelerin tümörijenik özelliklerini baskıladığını göstermektedir. Bu sonuçlar her iki ajanın eş uygulamasının kolon kanseri hücrelerinde daha güçlü antikanser etkilerin gözlendiğini önermektedir.



Şekil 4. Kapesitabin ve GSK2850163'ün kolon kanseri hücrelerinin migrasyon yeteneği üzerine olan etkilerinin incelenmesi. **a.** Caco-2 ve **b.** HT29 hücreleri 50, 100 ve 200 nM GSK2850163 ve 50 µg/mL kapesitabin veya kombinasyonları ile muamele edildi. Yara alanları 72 saat boyunca izlendi. Yara kapanma % değişimleri ImageJ yazılımı kullanılarak 0. saat ile 72. saat arasındaki kapanma alanları üzerinden belirlendi. Her deney üç bağımsız biyolojik ve üç teknik tekrar ile gerçekleştirildi (Kontrol ile kıyaslamalar; * $p<0.05$, ** $p<0.005$, # $p<0.001$, Kapesitabin ile kıyaslamalar; $ $p<0.05$, § $p<0.01$)



Şekil 5. Kapesitabin ve GSK2850163'ün kolon kanseri hücrelerinin invazyon yeteneği üzerine olan etkilerinin incelenmesi. **a.** Caco-2 ve **b.** HT29 hücreleri 50, 100 ve 200 nM GSK2850163 ve 50 µg/ml kapesitabin veya kombinasyonları ile muamele edildi. 72 saat sonra invazyon sergileyen hücreler kristal viyole ile boyanarak sayıldı. Her deney üç bağımsız biyolojik ve üç teknik tekrar ile gerçekleştirildi (Kontrol ile kıyaslamalar; * p<0.05, ** p<0.005, # p<0.001, Kapesitabin ile kıyaslamalar; $ p<0.05, § p<0.01)

ER stresi tümör büyümesi ve invazyonu ile ilişkili önemli bir faktördür [30]. ER stresinin hücrelerdeki regülasyonunda hayati rol oynayan UPR mekanizması, kanser hücrelerindeki progresyon, malignite ve ilaç direnci gelişmesi gibi kansere yönelik önemli özelliklerin süreci açısından sorumlu tutulmuştur. Kanser hücrelerinde UPR genellikle hücre sağkalım fonksiyonuna sahiptir [31]. Memelilerde, UPR'yi IRE1α, PERK ve ATF6 olmak üzere üç transmembran stres sensör proteini düzenlemektedir [32]. Çok sayıdaki çalışma yüksek derecede aktif IRE1α/XBP-1'in hepatoselüler karsinom, prostat, pankreas, meme ve kolon kanseri ile ilişkili olduğu bildirilmiştir [33,34]. Bu durum UPR'nin kanser hücreleri için bir çok açıdan adaptif mekanizma olduğunu desteklemektedir [31]. Bu

nedenle değişen UPR aktivitesinin farmakolojik olarak yeniden bazal düzeylere indirgenmesi kansere yönelik önemli bir yeni tedavi stratejisi olabileceğini düşündürmektedir.

Çalışmamızda klinik uygulamalarda kolon kanserinin tedavisine yönelik sıklıkla kullanılan kapesitabin'in hücreler üzerindeki antikanser etkinliğinin UPR'nin IRE1α kolunun farmakolojik olarak modülasyonu yolu ile desteklenmesine ilişkin denemeler gerçekleştirilmiştir. Bu amaçla GSK2850163 ajanından faydalanılmıştır. Yakın tarihte keşfedilen GSK2850163 ajanı UPR'nin IRE1α kolunu modüle eden sensör proteini IRE1α'nın kinaz ve RNaz aktivitelerini seçici olarak inhibe etmektedir [34]. Temelde IRE1α sinyali, IRE1α'nın ER stresine bir cevap olarak otofosforilasyona ve dimerizasyona uğraması ile aktive olur. Bu süreçte IRE1α'nın aktive olan endoribonükleaz aktivitesi aktif bir transkripsiyon faktörü olan XBP-1s'nin splicing ile oluşumuna izin verir. XBP-1s protein katlanmasını, üretimini veya bozunmasını destekleyen enzimleri ve proteinleri kodlayan genlerin transkripsiyonunu regüle etmektedir [35]. GSK2850163 ajanı seçici olarak IRE1α sinyalinin bu etkisini baskılayabilme yeteneğine sahiptir [26,36].

IRE1α/XBP-1'in kolon kanseri hücrelerinde kapesitabin'in hücre canlılığı üzerindeki sınırlayıcı rolünün GSK2850163 ile eş uygulamasına bağlı olarak değişimini araştırmak için, hücre canlılığı analizleri gerçekleştirdik. Kapesitabin özellikle metastatik tipteki meme ve kolorektal kanserlerin tedavisinde yaygın olarak kullanılan nükleozit metabolizmasına etki eden güçlü bir kemoterapötik ajandır [9,37,38]. Mekanistik olarak kapesitabin kanser hücrelerinde enzimatik olarak florourasile dönüşen bir ön ilaçtır. Güçlü şekilde hücrelerdeki DNA sentezini inhibe ederek, tümör dokusunun büyümesini ve yayılımını yavaşlatmaktadır [38]. Bu açıdan kapesitabin'in etkinliğinin geliştirilebilmesi son derece önemlidir. Sonuçlarımız beklendiği gibi kolon kanseri hücrelerine yalnız başına kapesitabin uygulamasının anlamlı düzeyde kolon kanseri hücrelerinin canlılığını sınırlandırdığını gösterdi (Şekil 1a). Benzer sonuçlar yalnızca GSK2850163 uygulamasına bağlı olarak da gözlendi. Eş uygulama çalışmalarımızda dikkat çekici bir şekilde kapesitabin ile artan konsantrasyonlarda GSK2850163 uygulamasının yalnızca kapesitabin veya GSK2850163 uygulamalarına kıyasla daha etkili şekilde kolon kanseri hücrelerinin canlılığını baskıladığını gözlemledik (Şekil 2a ve Şekil 2b). Bu sonuçlar kanser hücreleri için bir adaptasyon ve sağkalım mekanizması gibi çalışan UPR yolunun baskılanmasının kapesitabin'e karşı olan duyarlılığı geliştirdiğini önermektedir.

GSK2850163 tedavisinin verimliliğini test ettiğimiz çalışmalarda, thapsigargin ile uyarılan IRE1α sinyalinin uyarımı sonrasında oluşan XBP-1s'nin düzeylerinin GSK2850163 ile etkili bir şekilde baskılandığını gözlemledik (Şekil 3). Bu sonuç GSK2850163 uygulamasına bağlı olarak elde edilen sonuçların IRE1α sinyalinin negatif modülasyonu yolu ile elde edildiğini doğrulamaktadır.

Kanser hücrelerinin en iyi tanımlanan özelliklerinden birisi de bir hayli aktif olan migrasyon ve invazyon yeteneğidir [38]. Özellikle kanser hücrelerinin farklı doku ve organlara yayılmalarında ve yüksek sağkalım yeteneğine sahip olmalarında bu iki süreç son derece önemlidir. Hastalar açısından ise tedavi sürecinde üstesinden gelinmesi gereken önemli sınırlayıcı faktörler arasındadır [39,40]. Deney modelimizde GSK2850163'ün kapesitabin'in migrasyon ve invazyon üzerindeki sınırlayıcı olası etkilerini test etmek amacıyla matrigel modifiyeli Boyden Chamber testi ve yara iyileşme deney modelleri kullandık. Sonuçlarımız kapesitabin ile GSK2850163'ün eş uygulanmasının kolon kanseri hücrelerinin invazyon ve migrasyon yeteneğinin sınırlanmasına yönelik daha etkili olduğunu önermektedir (Şekil 4a ve Şekil 4b) (Şekil 5a ve Şekil 5b). Yapılan çalışmalarda ER stresi ve aktive olan UPR sinyalinin kanser hücrelerinin invazyon ve migrasyon da dahil olmak üzere çok sayıdaki karakteristik özelliğini desteklediği gösterilmiştir [41,42]. Bu açıdan GSK2850163 ile IRE1α/XBP-1 sinyal yolunun kolon kanseri hücrelerinde hedef alınması ile elde ettiğimiz bulgular literatür ile uyumludur.

Özellikle IRE1α'nın kanser tedavisine yönelik olarak geliştirilen tedavi stratejilerine getireceği yeni uygulamalara dikkat çekilmektedir [17,21]. *In vivo* ve *in vitro* çalışmalarda da umut verici sonuçlar elde edilmiştir [43-46]. Prostat ve meme gibi hormon bağımlı kanserlerde, kan kanserlerinde, glioblastoma ve pankreatik kanserlerinde önemli bir hedef olarak önerilmektedir [4,44,47-50]. Mevcut araştırma verilerimiz IRE1α'nın terapötik açıdan önemlini kolon kanseri hücreleri için de desteklemektedir.

Sonuç olarak araştırma bulgularımız kolon kanseri tedavisinde yoğun olarak kullanılan kapesitabin ile birlikte UPR'yi hedef alan farmakolojik yaklaşımların eş olarak kullanılmasının daha etkili terapötik sonuçların elde edilebilmesi açısından önemli bir yaklaşım olabileceğini önermektedir. Gelecek çalışmalarda GSK2850163'ün farklı kemoterapötik ajanlar ile kombinasyonuna ilişkin yeni deneysel çalışmaların gerçekleştirilmesi ve ilaç direnci üzerindeki uzun süreli etkilerinin test edilmesi, terapötik kullanımına ilişkin algımızın geliştirilmesi açısından oldukça değerli olacaktır.

Hücre kültürü temelli yürütülen *in vitro* çalışmaların gerçek tümör mikroçevresini tam olarak mimik edememesi, hücre hatlarının kanser hücrelerinin eşsiz özelliklerini yansıtmasındaki sınırlamalar, test edilen ajanların *in vivo* ve klinik uygulamalarındaki doz-zaman aralıklarının optimizasyonlarındaki zorluklar gibi önemli kısıtlılıklar, deneysel çalışmaların daha ileriye taşınmasının önündeki en önemli sınırlamalardır. Bu bağlamda araştırma bulgularımız *in vitro* koşullarda IRE1α/XBP-1'in hedeflenmesinin kolon kanserinin tedavisine yönelik sürdürülen mevcut uygulamalar üzerinde umut verici bir yaklaşım sunabileceğini gösterse de *in vivo* bulgular ile sonuçların desteklemesi gerekliliğini sürdürmektedir.

TEŞEKKÜR

Bu çalışmanın hayata geçirilmesinde Süleyman Demirel Üniversitesi Bilimsel Araştırma Projeleri birimi tarafından daha önce desteklenen projelerimizin (TSG-2021-8302, TAB-2020-8253) bütçesinden faydalanılmıştır. Bu çalışmanın bir bölümü 19-20 Ekim tarihlerinde düzenlenen "17th International Medicine and Heath Sciences Researches Congress" toplantısında sözlü bildiri olarak sunulmuştur.

YAZAR KATKILARI

Kavram: Y.E.; Tasarım: Y.E.; Denetim: Y.E.; Kaynaklar: Y.E.; Veri Toplama ve/veya İşleme: Y.E., Y.D.; Analiz ve/veya Yorumlama: Y.E.; Literatür Taraması: Y.E., Y.D.; Makalenin Yazılması: Y.E.; Kritik İnceleme: Y.E., Y.D.; Diğer: -

ÇIKAR ÇATIŞMASI BEYANI

Yazarlar bu makale için gerçek, potansiyel veya algılanan çıkar çatışması olmadığını beyan ederler.

ETİK KURUL ONAYI

Yazarlar bu çalışma için etik kurul onayının zorunlu olmadığını beyan etmektedir.

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J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 49(3): 852-858, 2025
Doi: 10.33483/jfpau.1629137

ORIGINAL ARTICLE / ÖZGÜN MAKALE

ANTIMICROBIAL AND ANTIOXIDANT ACTIVITY OF ENDEMIC *HAPLOPHYLLUM MYRTIFOLIUM* BOISS.

ENDEMİK HAPLOPHYLLUM MYRTIFOLIUM BOİSS.'UN ANTİMİKROBİYAL VE ANTİOKSİDAN AKTİVİTESİ

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ABSTRACT

Objective: *Haplophyllum myrtifolium Boiss. an endemic species for Türkiye, belongs to the Rutaceae family. In this study, antioxidant and antimicrobial effects of n-hexane and methanol extracts of the aerial parts plant were examined.*
Material and Method: *The total phenolic content of the extracts was examined by a spectrophotometric method using Folin-Ciocalteu reagent (FCR). The DPPH free radical scavenging and ABTS test were applied to determine the antioxidant effect. The antibacterial effects of H. myrtifolium extracts were assayed against Staphylococcus aureus ATCC 29213, Bacillus subtilis NRRL B478* and *Enterococcus faecalis ATCC 51299 by the broth microdilution method.*
Result and Discussion: *The total phenolic content was found to be 44.69±0.021 mg GAE/g for the methanol extract. The methanol extract of H. myrtifolium exhibited higher antioxidant activity (IC_{50} 0.120±0.0008 mg/ml) compared to the n-hexane extract (IC_{50} 0.256±0.015 mg/ml). ABTS test results, the data closest to the TEAC standard was seen in methanol extract (1.783±0.0062 mM). The minimum bactericidal concentrations of the H. myrtifolium methanol extracts varied between 0.125-2 mg/ml and the n-hexane extracts showed antimicrobial activity with the 3 mg/ml concentration.*
Keywords: *Antimicrobial activity, antioxidant activity, Haplophyllum myrtifolium, total phenolic content*

ÖZ

Amaç: *Haplophyllum myrtifolium Boiss. Türkiye'ye özgü bir tür olup, Rutaceae familyasına aittir. Bu çalışmada, toprak üstü kısımlarının metanol ve n-hekzan ekstrelerinin antimikrobiyal ve antioksidan etkileri araştırılmıştır.*
Gereç ve Yöntem: *Toplam fenol içeriği, gallik asit eşdeğeri (GAE) olarak Folin-Ciocalteu reaktifi (FCR) kullanılarak belirlenmiştir. Antioksidan etkiyi belirlemek için DPPH ve ABTS testi uygulanmıştır. H. myrtifolium ekstrelerinin antibakteriyel etkileri Stapylococcus aureus ATCC 29213, Bacillus subtilis NRRL B478 ve Enterococcus faecalis ATCC 51299'a karşı broth mikrodilüsyon yöntemiyle test edilmiştir.*
Sonuç ve Tartışma: *Metanol ekstresi için toplam fenolik içerik 44.69±0.021 mg GAE/g olarak bulundu. H. myrtifolium metanol ekstresi, n-hekzan ekstresine (IC_{50} 0.256±0.015 mg/ml) kıyasla*

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Submitted / Gönderilme : 29.01.2025
Accepted / Kabul : 16.05.2025
Published / Yayınlanma : 19.09.2025

daha yüksek antioksidan aktivite (IC_{50} 0.120±0.0008 mg/ml) gösterdi. ABTS test sonuçlarına göre TEAC standardına en yakın veri metanol ekstrelerinde (1.783±0.0062 mM) görüldü. H. myrtifolium metanol ekstrelerinin minimum bakterisidal konsantrasyonları 0.125-2 mg/ml arasında değişirken, n-hekzan ekstreleri 3 mg/ml konsantrasyonda antimikrobiyal aktivite göstermiştir.
Anahtar Kelimeler: *Antimikrobiyal aktivite, antioksidan aktivite, Haplophyllum myrtifolium, toplam fenolik içerik*

INTRODUCTION

The *Rutaceae* family is defined worldwide by 319 genera and 6659 species. It is distributed across Africa, the Arabian Peninsula, the Tropical Indian subcontinent of Asia, and Eastern Europe [1]. In Türkiye, the family is represented by 25 species and 4 genera: *Citrus* L., *Haplophyllum* A. Juss, *Dictamnus* L., and *Ruta* L. [2].

Haplophyllum genus was mentioned in the book Flora of Turkey and East Aegean Islands with a total of 19 taxa including 14 species, 3 varieties, and 2 subspecies, 8 of which are endemic [3]. In the book Vascular plants, Adil Güler mentioned 9 species, 6 of which are endemic [4]. Tugay and Ulukuş (2017) introduced *H. sahinii* Tugay & Ulukuş, and Ulukuş and Tugay (2018) introduced *H. ermenekense* Ulukuş & Tugay endemic species [5-6]. The genus with a high rate of endemism is an important gene center for Türkiye. It is popularly known as "Sedo" [2].

Townsend, 1967, who examined the plants of this genus botanically, defined it as a difficult genus in their book Flora of Turkey and, as many species have characteristic facies that are difficult to express in words. They are semi-shrub-like perennial plants with woody lower part and herbaceous upper part. It has 5 petals that range from white to yellow. It has a bracteate cymose flower state. There are 10 stamens with hairy filaments. It has capsule-shaped fruits. It is a plant that grows in sandy, stony and rocky areas at altitudes of 750-2150 m [7].

The genus, which has a wide distribution throughout the world, is widespread distributed from China to Spain, from the North Caucasus to Iran, Iraq, and Somalia. It has traditional use in various diseases in Oman, Sudan, Algeria, Iraq, Saudi Arabia, Egypt, Iran, Türkiye and Mongolia. It has been reported that plants in the *Haplophyllum* genus are used in a wide multiplicity of diseases such as malaria, diarrhea, fever, headache, arthritis, GIS disorders, anemia, skin disorders, gynecological disorders, diabetes, otitis, dermal wounds, allergic rhinitis, asthma [8-16]. According to the evaluation results, whole plants, leaves, fruiting or flowering parts of the plants were used as decoction, infusion, juice, and paste [10,12-14,16]. Additionally, ethnobotanical information on *Haplophyllum* species is crucial for future studies, and these plants are commonly used in traditional medicine in numerous countries.

According to the results of the phytochemical study, *Haplophyllum* species contain essential oils, alkaloids, lignans, coumarins, flavonoids, and terpenes as secondary metabolites [8-12,17-21]. The essential oil has been obtained by various extraction methods using herbs, flowers, leaves, and fruits of plants. According to the analysis results, the main compound classes were classified as terpenic compounds [8-10,20-22].

In a study, it was showed that except the water extract of *H. myrtifolium*, other extracts had anticholinesterase, antityrosinase, antiglucosidase and antiamylase activities [23]. The antitrichomonal effect of *H. myrtifolium* was investigated against *T. vaginalis trophozoite;* its ethanol extract and skimmianine were found effective against it [24]. Due to the chemical content, some *Haplophyllum* species such as *H. patavinum* L., *H. tuberculatum* Juss., *H. hispanicum* Spach, *H. suaveolens* and *H. buxbaumii* are used as antimicrobial, antimalarial, insecticidal in folk medicine [11,16,25,26].

Since many *Haplophyllum* species have important pharmacological properties, their use in traditional medicine has a long history in many countries. The antioxidant and antimicrobial activities of *Haplophyllum* species have been investigated by many researchers using different methods. However, studies on *H. myrtifolium*, an endemic species, are limited. It is crucial to assess its antibacterial and antioxidant activity based on knowledge on its internal and exterior applications related to its ethnobotanical features. Additionally, it is anticipated that the findings about *H. myrtifolium* will serve as the foundation for further pharmacological analyses. In this study, the antimicrobial and antioxidant effect of the endemic *H. myrtifolium* plant, considered as a potential natural resource, were searched.

MATERIAL AND METHOD

Plant Material

H. myrtifolium was collected from Seyitgazi, Eskişehir on May 30, 2022. Plant samples are kept in the Herbarium of Anadolu University Faculty of Pharmacy (ESSE 16362).

Preparation of Extracts

The aerial parts of the plant weighed 10 grams each. 100 ml of hexane (yield 10.1%) and methanol (yield 20.9%) were added and macerated at 150 rpm for 24 hours. This process was repeated three times. It was evaporated with an evaporator at 40ºC. The extracts were kept in the refrigerator at +4ºC.

Determination Total Phenolic Content

The total phenolic content of extracts was measured as gallic acid equivalent by Folin-Ciocalteu method [27].

Determination of DPPH Scavenging Effect

The method developed by Kumarasamy et al. was used for the determination of DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging effect [28]. Gen5 Data Analysis program (BioTek) was used for statistical evaluations. Results are given as mean ± standard deviation. For radical scavenging activity, 50% inhibition concentration value (IC_{50}) was estimated and the formula was used for the % inhibition value.

$IC_{50} = [(A_0-A_1) /A_0)] \times 100$

IC_{50}: 50% inhibitory concentration, A_0: control absorbance, A_1: sample absorbance

Determination of $ABTS^{\bullet+}$ Scavenging Effect

The experiment was performed using the standard Trolox equivalent antioxidant capacity method (TEAC) [29]. This method involves scavenging the $ABTS^{\bullet+}$ (2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid radical cation) and its comparison with Trolox.

Antimicrobial Activity

The antimicrobial broth microdilution method was employed to assess the Minimum Bactericidal Concentration (MBC) of extracts against standard bacteria species. According to the Clinical and Laboratory Standards Institute (CLSI) in 1999, the determination of MBCs involved identifying the lowest doses necessary to kill 99.9% of the bacterial inoculum [30].

The plant extracts were dissolved in DMSO and prepared by using a two-fold serial dilution technique in a 96-well microplate. The concentrations ranged from 4000 µg/ml to 1.95 µg/ml. Microorganisms were standardized against the McFarland 0.5 reference (1×10^8 cfu/ml) and then bacterial suspensions were diluted 1:100 [31]. Subsequently, firstly 100 µl of each microorganism culture and extracts were added to the plates, which incubated at 37°C for 24 hours. The microorganisms used in the experiment are given in Table 1. Chloramphenicol was used as the standard control, and MHB (Mueller Hinton Broth) medium was used for sterility control. After incubation, 20 µl of 0.01% resazurin dye solution was added to each well. This staining step was performed to differentiate between live and dead cells, with the color change from blue (or green) to pink indicating cell viability. The experiment was conducted in triplicate and the resulting data were later subjected to averaging.

Table 1. Standard test microorganisms

Bacteria	*Staphylococcus aureus* ATCC 29213
	Bacillus subtilis NRRL B478
	Enterococcus faecalis ATCC 51299

Statistical Analysis

The average ± SD of three replicates for every sample was used to present the results. The DPPH techniques' IC_{50} values were determined by the use of linear regression analysis. Test findings for ABTS are reported in millimolar (mM).

RESULT AND DISCUSSION

The antioxidant capacity of two different extracts of *H. myrtifolium* obtained by DPPH and ABTS methods and total phenolic substance content are given in Table 2. The extracts showed differences in their total phenol content and antioxidant capacity depending on the solvent polarity. The effective dose for both extracts was observed to be 2.5 mg/ml. The methanol extract of *H. myrtifolium* showed higher antioxidant activity (IC_{50} 0.1207±0.0086 mg/ml) compared to the *n*-hexane extract (IC_{50} 0.256±0.0150 mg/ml). According to ABTS test results, the data closest to the TEAC standard was seen in methanol extract (1.783±0.0062 mM). Total phenol content was measured using gallic acid equivalents (GAE). The total phenolic content was 44.69±0.021 mg GAE/g for the methanol extract. On the other hand, the *n*-hexane extract contained 37.59±0.007 mg GAE/g of total phenols, indicating a lower content compared to the methanol extract. These results confirm a correlation between the total flavonoid content and antioxidant activity.

Table 2. Antioxidant activity and total phenolic contents of extracts

Extracts and standart	Total phenolic content-mg GAE/g extract	DPPH Test-IC_{50} (mg/ml)	ABTS Test-TEAC (mM)
Methanol	44.69±0.0210	0.1207±0.0086	1.783±0.0062
***n*-Hexane**	37.59±0.0072	0.2560±0.0150	1.512±0.0096
Gallic acid (GA)	-	0.0016±0.0001	2.93± 0.03

In the study conducted by Zengin et al., total phenol content, DPPH and ABTS tests were applied to the extracts of *H. myrtifolium*. Total phenolic content was found in for methanol (34.53 mg GAE/g extract), and petroleum ether (32.32 mg GAE/g extract) extracts. DPPH radical scavenging activity (mg TE/g extract) was listed as follows: methanol 84.49±2.59> water 82.42±4.53> ethyl acetate 67.45± 2.94> petroleum ether 43.77±0.83. The ABTS radical scavenging effect was found for methanol 169.77 mg TE/g extract, and petroleum ether 129.30±1.95 mg TE/g extract [23]. Total phenol content of methanol extract of *H. myrtifolium* (HM-1 and HM-2, Konya) collected from two different localities was calculated as 48.0±0.4 mg GAE/g extract for HM-1 and 56.1±0.8 mg GAE/g extract for HM-2. % inhibition values of ABTS activity were measured as 76.5% for HM1 and 71.1% for HM-2. % inhibition of DPPH activity was observed to be more than 80% for HM-1 and HM-2 [32]. It is observed that the antioxidant effect and total phenol content of extracts prepared with various solvents from plants collected in different localities vary.

Antimicrobial Activity

The MBC values for plant extracts are listed in Table 3. The MBC values of the *H. myrtifolium* methanol extracts varied between 125-2000 µg/ml and the *n*-hexane extracts showed antimicrobial activity only on *E. feacalis* with the 3000 µg/ml concentration and 750 µg/ml on *S. aureus*. According to the MBC values *n*-hexane extracts of *H. myrtifolium* exhibited poor antimicrobial activity with high concentrations. As a result, only the *S. aureus* is more sensitive than other bacteria species.

Table 3. MBC values of *Haplophyllum myrtifolium* extracts (μg/ml)

Extracts and standards	*E. feacalis*	*S. aureus*	*B. subtilis*
Methanol	2000	125	250
***n*-Hexane**	3000	750	-
Chloramphenicol	125	0.97	15.6

-: No activity

Studies on the *Rutaceae* family suggest that this family exhibits antileishmanial effects due to its alkaloid and terpenic compound content [33]. For this reason, when this effect of the endemic *H. myrtifolium* extracts and two isolated alkaloid compounds (Skimmianine and Gamma-fagarine) were investigated, it was reported that they exhibited more than 50% inhibition against promastigotes of *Leishmania tropica* at a concentration of 25 μg/ml [34]. The fact that it showed a significant effect in terms of biological activity led us to plan new studies to investigate this plant further.

In the study conducted by Ashfaq et al., when the antimicrobial activity of various extracts obtained from endemic *H. gilesii* was investigated using the agar well diffusion method, methanol extract showed significant antibacterial activity especially against *S. aureus* and *E. faecalis* [35]. The limited number of studies conducted on *H. myrtifolium*, an endemic plant, reveals the originality of our study.

In the study conducted by Al-Saeghi et al., when the antimicrobial activity of the methanol extract and its subfractions of *H. tuberculatum* was tested against various strains using the agar disk method, it was observed that the activity was similar between different solvents and showed moderate inhibition against the applied strains [36]. In our study, although the methanol extract was more active than the *n*-hexane extract, the methanol extract exhibited low antibacterial activity. It is thought that this difference may have changed due to the different preparation methods and methods used. For this reason, there is a need for a more comprehensive evaluation of the antimicrobial studies on this plant.

Conclusion

Numerous pharmacological actions were present in *Haplophyllum* species, including acetylcholinesterase inhibitory, cytotoxic, cardiovascular, insecticidal, antiprotozoal, molluscicidal, antioxidant, and antimicrobial properties [37]. This study was interested in the antioxidant and antimicrobial effect of *H. myrtifolium* to find new natural sources. It contributed to the literature due to the limited antimicrobial studies on *H. myrtifolium*. This study provided valuable scientific data to the literature and showed that methanol extracts of *H. myrtifolium* may have antibacterial properties. The antioxidant study findings show a parallel relationship between the total phenolic content and antioxidant activity of *H. myrtifolium* extracts. Detailed and comparative studies on this species are planned for future studies.

AUTHOR CONTRIBUTIONS

Concept: N.S., F.T., Z.G., P.S., Y.B.K.; Design: F.T., Y.B.K.; Control: N.S., F.T., Z.G., P.S., Y.B.K.; Sources: N.S., F.T., Z.G., P.S.; Materials: N.S., F.T., Z.G., P.S., Y.B.K.; Data Collection and/or Processing: F.T.; Analysis and/or Interpretation: N.S., F.T., Z.G., P.S.; Literature Review: F.T.; Manuscript Writing: F.T.; Critical Review: N.S., F.T., Z.G., P.S., Y.B.K.; Other: -

CONFLICT OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

The authors declare that the ethics committee approval is not required for this study.

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J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 49(3): 859-869, 2025
Doi: 10.33483/jfpau.1663961

ORIGINAL ARTICLE / ÖZGÜN MAKALE

INTERACTION BETWEEN ELTROMBOPAG AND HUMAN SERUM ALBUMIN: FLUORESCENCE AND ELECTROCHEMICAL STUDIES

ELTROMBOPAG VE İNSAN SERUM ALBÜMİNİ ARASINDAKİ ETKİLEŞİM: FLORESANS VE ELEKTROKİMYASAL ÇALIŞMALAR

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ABSTRACT

Objective: *This research investigated the interaction between Eltrombopag (EB) and Human Serum Albumin (HSA) by fluorescence spectroscopy and voltammetry while explaining the quenching mechanism.*
Material and Method: *The electrochemical studies were conducted in an acetate buffer solution (AB) at pH 4 by differential pulse voltammetry (DPV). For the fluorescence studies, EB, HSA, and a mixture of EB and HSA solutions were designed with a pH of 4.7, AB containing 20% DMSO.*
Result and Discussion: *The interaction between EB and HSA was examined by fluorescence and electrochemical titrations, which showed a quenching effect and peak shifting. Fluorescence titrations indicate that EB's HSA quenching process is static and has a hypsochromic shift. Analysis of thermodynamic parameters and higher binding constants concluded a strong and spontaneous interaction. Electrochemical titrations show the intercalation of EB into HSA.*
Keywords: *Differential pulse voltammetry, drug protein interaction, eltrombopag, fluorescence spectroscopy, human serum albumin*

ÖZ

Amaç: *Bu araştırmada Eltrombopag (EB) ile İnsan Serum Albümini (HSA) arasındaki etkileşim floresan spektroskopisi ve voltametri ile incelenerek sönümlenme mekanizması açıklanmıştır.*
Gereç ve Yöntem: *Elektrokimyasal çalışmalar, pH 4'te asetat tampon çözeltisinde (AB) diferansiyel darbe voltametrisi (DPV) ile gerçekleştirildi. Floresan çalışmaları için EB, HSA ve EB ve HSA çözeltilerinin karışımı, %20 DMSO içeren pH 4.7 AB ile tasarlandı.*
Sonuç ve Tartışma: *EB ve HSA arasındaki etkileşim floresan ve elektrokimyasal titrasyonlar ile incelendi ve sönümlenme etkisi ile pik kayması elde edildi. Floresan titrasyonları, EB'nin HSA sönümleme işleminin statik olduğunu ve hipsokromik bir kayma olduğunu gösterir. Termodinamik*

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Submitted / Gönderilme : 23.03.2025
Accepted / Kabul : 16.05.2025
Published / Yayınlanma : 19.09.2025

parametrelerin ve daha yüksek bağlanma sabitlerinin analizi güçlü ve kendiliğinden bir etkileşimin olduğunu ortaya koydu. Elektrokimyasal titrasyonlar, EB'nin HSA'ya interkalasyonunu gösterir.
Anahtar Kelimeler: *Diferansiyel darbe voltametrisi, eltrombopag, floresan spektroskopisi, ilaç protein etkileşimi, insan serum albümini*

INTRODUCTION

Eltrombopag (EB) is named 2-aminoethanol;3-[3-[[2-(3,4-dimethyl phenyl)-5-methyl-3-oxo-1H-pyrazol-4-yl]diazenyl]-2-hydroxyphenyl]benzoic acid (Figure 1). It is an oral drug used to treat thrombocytopenia, aplastic anemia, and chronic hepatitis C infection-associated thrombocytopenia [1–3]. EB is a thrombopoietin receptor (TpoR) agonist that activates megakaryocyte differentiation and proliferation and increases platelet production [4]. Studies about EB usage in chemotherapy groups concluded that patients receiving gemcitabine-based chemotherapy and eltrombopag medication experienced decreased dose delays and reductions brought on by thrombocytopenia [5].

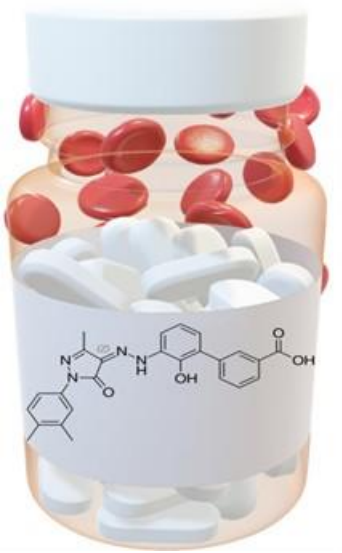


Figure 1. Chemical structure of Eltrombopag

The interactions between drugs and plasma proteins are crucial in pharmacokinetics and pharmacodynamics. These interactions give information about doses of drugs, the drug's half-life, and therapeutic effect. Plasma proteins are responsible for drug absorption, diffusion, and distribution, known as drug carriers in the bloodstream [6].

The most prevalent plasma protein in humans is human serum albumin (HSA). HSA consists of 585 amino acids and three homologous domains with a molecular weight of 66.439 Da [7]. HSA generally works as a transporter for hormones, fatty acids, ions, and drugs [8]. Each drug has a different binding site on HSA, and when the drug interacts with HSA, its conformational structures change. Drug-site interaction is crucial for investigating the function of HSA in drug delivery [9]. Therefore, many methods exist to investigate the correlation between drugs and HSA. These methods are fluorescence spectroscopy, UV-visible spectroscopy, circular dichroism (CD) spectroscopy, and molecular modeling [10–15]. Electrochemical and fluorescence studies offer easy, cheap, and fast responses for analyzing drugs and biomolecules [16–18].

To the best of our knowledge, no studies in the literature report binding a drug (EB) to a protein (HSA). Thus, this research may help to see EB’s pharmacokinetics, appropriate dosage, efficacy, and availability in future studies. The purpose of this work is to use spectroscopic and electrochemical techniques to examine EB's binding with HSA for the first time. Fluorescence titration analysis indicates that EB quenches the fluorescence effect of HSA. In the light of this analysis, the quenching constant

and binding constants were determined at three different temperature values (288.15 K, 298.15 K, 310.15 K). Thermodynamic parameters show a strong binding between EB and HSA, which continues without energy input. The interaction of EB to HSA was found to be static quenching. The quenching effect is also obtained with electrochemical studies. When the concentration ratio increases to [EB: HSA] 1:0.025, the peak voltage of EB disappears, and a positive peak potential shift is observed due to the intercalation. Therefore, the interaction between HSA and EB is proven via the quenching effect in the fluorescence and electrochemical titrations.

MATERIAL AND METHOD

Reagents and Chemicals

HSA (purity ≥ 96%), sodium chloride (NaCl), dimethyl sulfoxide (DMSO), sodium acetate trihydrate, acetic acid, hydrochloric acid (HCl), tris(hydroxymethyl)aminomethane (Tris) were obtained from Sigma-Aldrich. EB (purity > 99.5%) was taken from DEVA Holding A.S, R&D Center, Türkiye. 1.0 mM HSA and 10 mM EB stock solutions were composed of pH 7.4 Tris-HCl buffer solution and DMSO, respectively, and kept at 4°C. Using ultrapure water, all solutions were made from scratch in 0.05 M Tris buffer solution containing 0.1 M NaCl (pH 7.4). Different concentrations of EB solutions varying between 0.5 μM to 64.0 μM were made.

A supporting electrolyte, acetate buffer solution (AB) at pH 4.7, was prepared for electrochemical studies. EB, HSA, and a mixture of EB and HSA solutions were designed with a pH 4.7 AB containing 20% DMSO.

Fluorescence Spectroscopy Measurements

Fluorescence titration was performed using a Varian-Cary Eclipse model fluorescence spectrophotometer with a Peltier temperature regulation module. The fluorescence quenching data of HSA (20 μM) were measured with concentrations of EB (0.5 μM to 64 μM) at varying temperatures (288.15, 298.15, 310.15 K). Before the measurement, the HSA solution was allowed to wait 15 minutes to reach the desired temperature. The excitation of each sample is 270 nm; both the slits of excitation and emission were adjusted to 5 nm. Corrections were made in the fluorescence experiments to get rid of the inner filter effect in HSA-EB binding. The formula below was utilized to determine the corrected fluorescence intensity (Fcor) values [19].

$$F_{cor} = F_{abs} 10^{(Aexc + Aem)/2}$$

Fabs is the measured fluorescence result of HSA, Aexc is the absorbance result at the excitation wavelength, and Aem is the absorbance result at the emission wavelength. Fcor readings were utilized for the remaining computations.

Electrochemical Measurements

An Autolab potentiostat was used to record all electrochemical measurements (PGSTAT 302). The electrode system comprised a glassy carbon electrode (3 mm in diameter), a Pt plate counter electrode, and an Ag/AgCl reference electrode. 3×10^{-5} M EB and HSA solution, and different concentrations of HSA solution mixed with 3×10^{-5} M EB solution and scanned via differential pulse voltammetry in the pH 4.7 acetate buffer solution.

RESULT AND DISCUSSION

Fluorescence Spectroscopy Experiments

The binding of EB to HSA was examined at three different temperature values (288.15 K, 298.15 K, 310.15 K) to investigate binding and thermodynamic parameters that depend on temperature. The fluorescence quenching spectra and binding isotherms at three temperature values are shown in Figures 2-4, respectively, at pH 7.4. The addition of EB at concentrations changing from 0.5 μM to 50 μM caused a progressive decrease at both these temperatures. When the HSA molecule is excited at 270 nm, the emission peak of HSA is observed at 336 nm without the addition of EB. After adding 50 μM EB,

the emission peak is shifted to 306 nm in all temperature values. This hypsochromic shift indicates that the interaction of EB with HSA results in less polarity around the fluorophore and more hydrophobicity [20,21]. Association constants (Ka) were found by fitting binding isotherms.

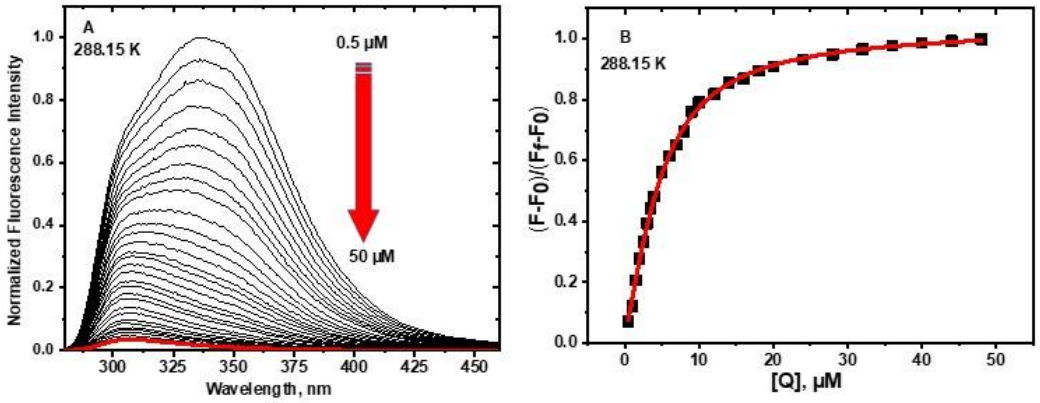


Figure 2. A) Fluorescence quenching spectrum of 20 µM HSA with the addition range between 0.5 µM to 50 µM EB at 288.15 K. B) Non-linear curve fitting of binding isotherm (Initial fluorescence intensity is denoted by F_0 and final fluorescence intensity by F_f.)

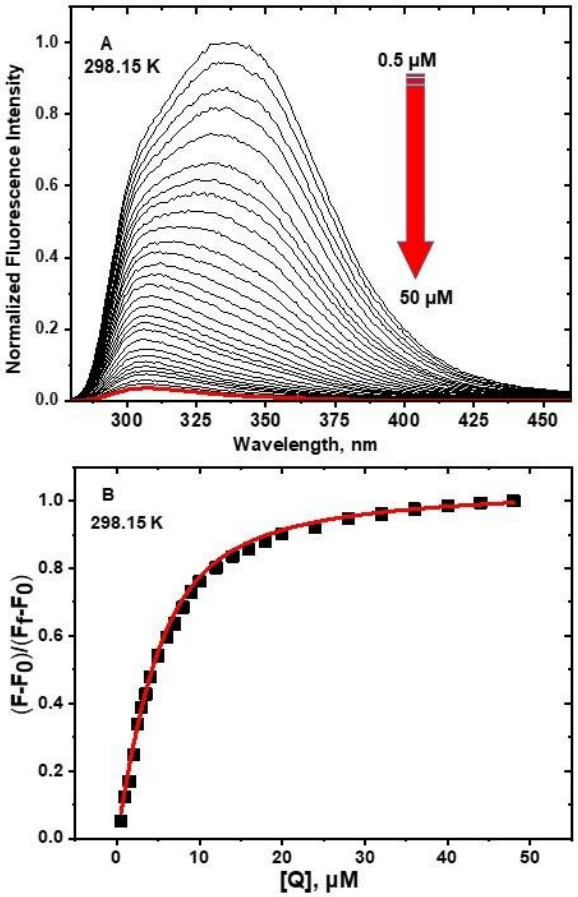


Figure 3. A) Fluorescence quenching spectrum of 20 µM HSA with the addition range between 0.5 µM to 50 µM EB at 298.15 K. B) Non-linear curve fitting of binding isotherm

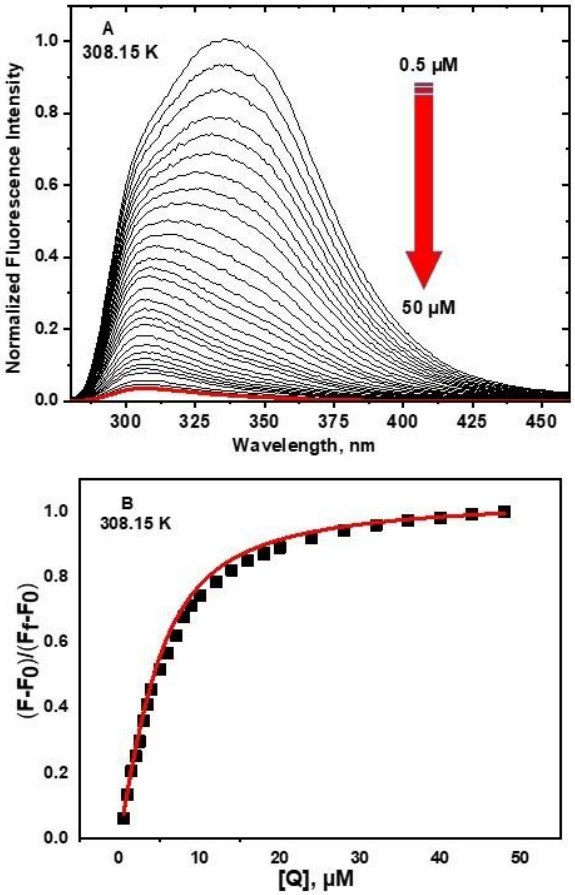


Figure 4. A) Fluorescence quenching spectrum of 20 µM HSA with the addition range between 0.5 µM to 50 µM EB at 308.15 K. B) Non-linear curve fitting of binding isotherm

Quenching Mechanism Calculation

Stern-Volmer plots were used to examine the fluorescence quenching mechanism at various temperatures (Figure 5). From this plot and the equation below, fluorescence quenching constants were calculated.

$$F0/F = 1 + kq\ \tau0\ [Q] = 1 + K_{sv}\ [Q]$$

Where F and F0 are the fluorescence values of the HSA in the presence of EB and without EB, respectively; τ0 is the half-life (8-10 s) of the HSA in the absence of EB; K_{sv} (Stern-Volmer quenching constant) and EB concentration ([Q]), whereas kq represents the quenching rate. The slopes of the F0/F versus [Q] from the Stern-Volmer plots were used to get the K_{SV} values. As seen in Figure 5, when the EB concentration ([Q]) increases, the quenching constant also increases at lower temperatures. Also, Table 1 shows that the K_{SV} values are higher when the temperature decreases. These inverse relationships can be explained via static quenching, where the quenching rate is inversely proportional to temperature [11,22,23]. When HSA and EB interact, the stable, non-fluorescent ground state complex is formed, which leads to quenching. Based on the characteristics of static quenching, raising the temperature will make the complex less stable, thus lessening the impact of static quenching [24].

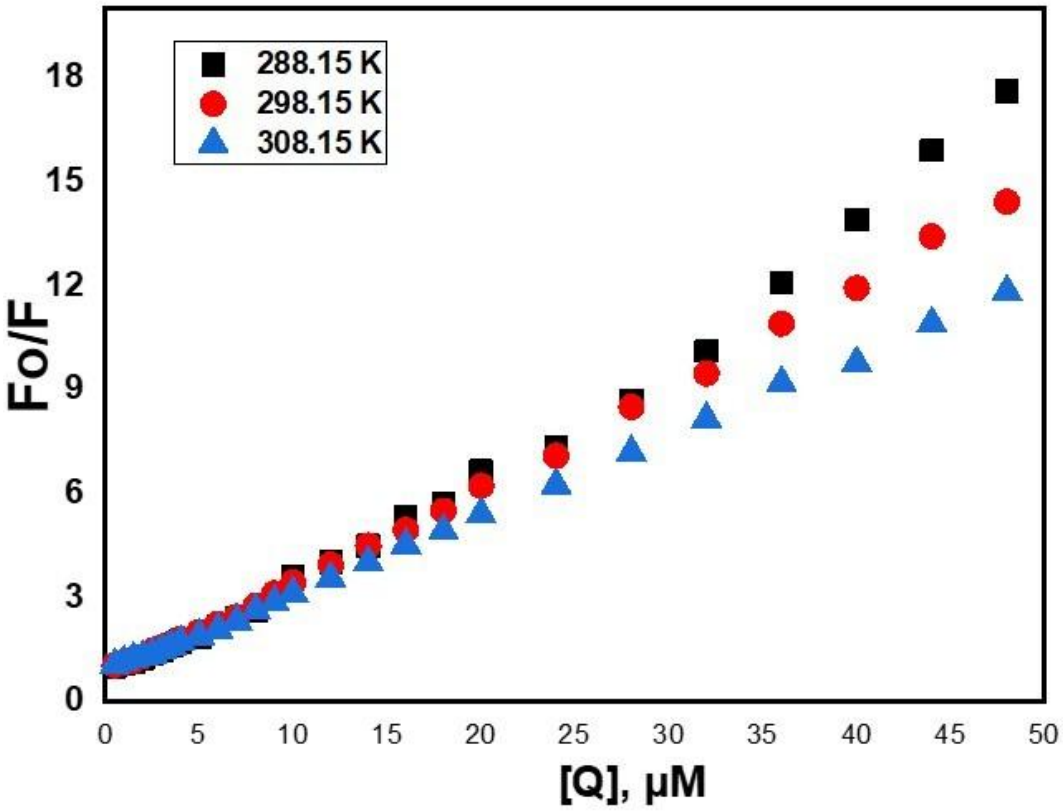


Figure 5. Stern-Volmer plots of HSA-EB interaction

Table 1. (K_{SV}) and (kq) constants and regression coefficients (R) for the binding of EB with HSA at various temperatures

T (K)	K_{sv} ($\times 10^5$ l.mol^{-1})	R	kq ($\times 10^{13}$ M^{-1}.s^{-1})
288.15	3.81	0.996	3.81
298.15	2.64	0.995	2.64
310.15	2.15	0.995	2.15

Analysis of Thermodynamic Parameters and Binding Constant

Thermodynamic variables like change in enthalpy (ΔH°), entropy (ΔS°), and Gibbs free energy (ΔG°) provide information about the interaction types of complex, the ease of complex formation, and the process of systems. The vann't Hoff equation provides these thermodynamic parameters, which are shown in the following equation. The vann't Hoff graph is shown in Figure 6. The slope of the lnKa versus 1/T plot gives ΔH°, and the intercept value gives ΔS°. Ka is denoted as the binding constant, and R is denoted as the universal gas constant.

$$\ln Ka = -\Delta H°/RT + \Delta S°/R$$

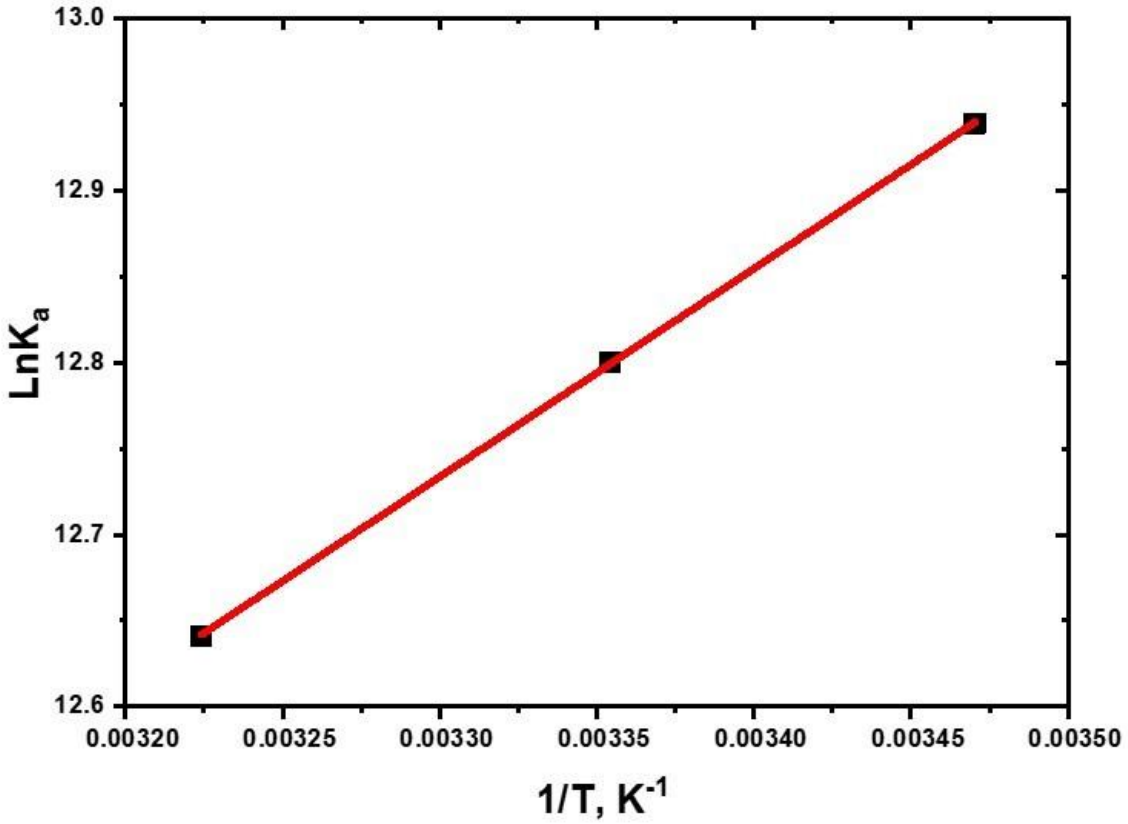


Figure 6. HSA-EB binding by Vant Hoff plot

A plot of the van't Hoff of HSA-EB binding and equation calculates and summarizes thermodynamic variables and binding constants in Table 2. When temperature increases, Ka also increases like K_{sv} value due to static quenching. The negative Gibbs free energy, enthalpy, and high binding constant demonstrate the strong interaction between EB and HSA and the spontaneous reaction. The entropy value must be positive for the spontaneous process, which is also confirmed by the positive entropy result.

A plot of the van 't Hoff of HSA-EB binding and its equation, the variables of thermodynamic and binding constants are calculated and summarized in Table 2. Ka also decreases like K_{sv} value when temperature increases due to static quenching. The negative Gibbs free energy, enthalpy, and high binding constant demonstrate the strong interaction between EB and HSA and the spontaneous reaction. The entropy value must be positive for the spontaneous process, which is also confirmed by the positive entropy result.

Table 2. The variables of thermodynamic parameters and binding constants for EB-HSA binding at various temperatures

T (K)	K_a ($\times 10^6$ l.mol^{-1})	n	R	ΔG° (kJ mol^{-1})	ΔH° (kJ mol^{-1})	ΔS° (J.mol^{-1}K^{-1})
288.15	4.162	1.049	0.995	-30.988	-10.058	72.676
298.15	3.623	1.056	0.999	-31.715		
310.15	3.090	1.068	0.999	-32.587		

* K_a: binding constants; n: number of binding sites of EB to HSA

Electrochemical Studies

Like fluorescence titration analysis, electrochemical titration is also completed at 288 K in the pH 4.7 acetate buffer solution. Firstly, 30 µM EB, 30 µM HSA, and their 1:1 mixture of differential pulse voltammogram measurements are completed (Figure 7). The quenching effect is around both 1.25 and 0.45 volts after adding HSA to the EB solution. Then, at 1.25, the voltage is examined more deeply with different concentration mixtures. As shown in Figure 8, 30 µM EB was measured first, and then 8 different molarity ratios of HSA solutions were added to the EB solution. When the ratio reaches EB: HSA (1:0.04), the peak totally disappears. In addition to the quenching effect, there is a positive shift in the peak potential after adding HSA. It is suggested by the intercalation of EB into HSA via phenyl ring interactions [25]. Thus, electrochemical studies prove the HSA and EB interaction via the quenching effect and peak potential shift.

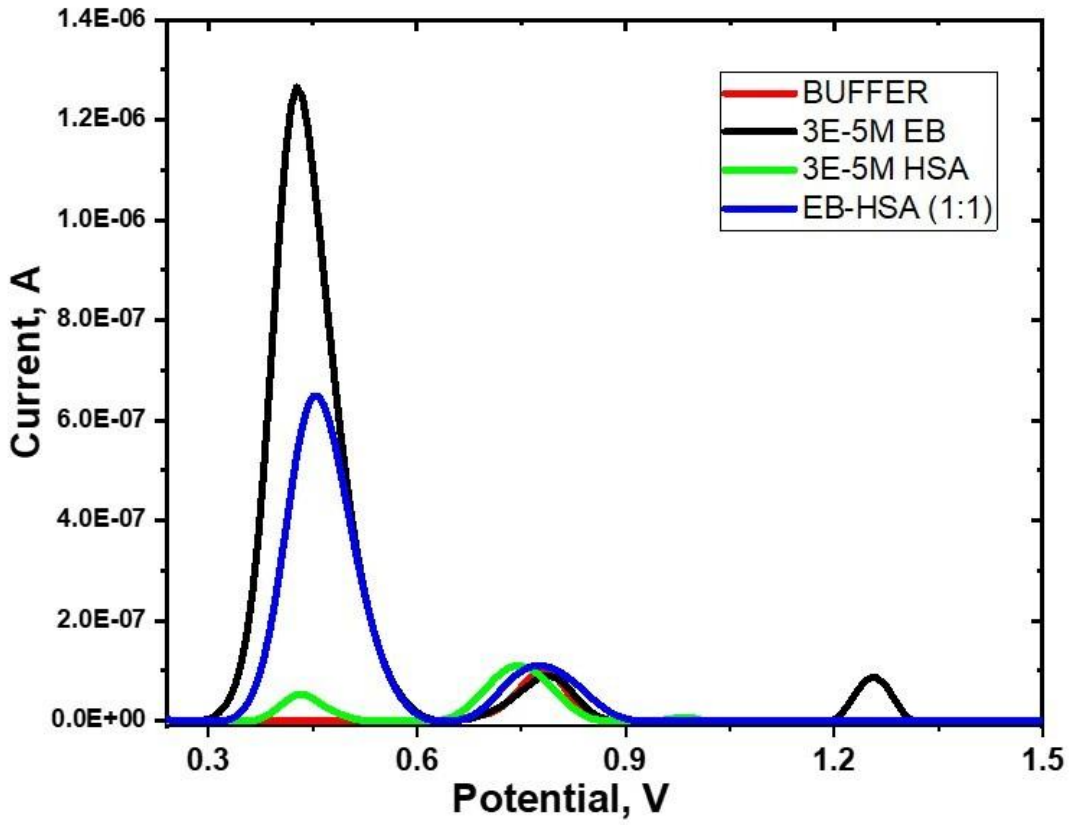


Figure 7. DPV of 3×10^{-5} M EB, 3×10^{-5} M HSA, and their mixture (1:1) in acetate buffer solution

For the first time, the interactions between EB and HSA were investigated using fluorescence spectroscopy and differential pulse voltammetry. At 288 K, 298 K, and 310 K, the fluorescence emission of HSA was quenched, and a hypsochromic shift (25 nm) was obtained with the addition of EB. The binding constant of the EB to HSA and the thermodynamic parameters were calculated using fluorescence quenching data. The K_{sv} value decreased from 3.81×10^5 to 2.15×10^5 as the temperature increased. When the temperature increases, the binding constant decreases; this situation shows static quenching. 288 K, which has the higher binding and quenching constant, are calculated as 4.16×10^5 and 3.81×10^5, respectively. Higher binding constant, Gibbs Free energy, enthalpy (-10.058), and entropy (72.676) results demonstrate EB's strong and spontaneous binding interaction to HSA. According to electrochemical results, if the concentration of HSA increases, the peak voltage of the EB decreases in

the DPV voltammograms, and a positive potential shift (0.04V) is seen, which reveals that EB is intercalated with HSA.

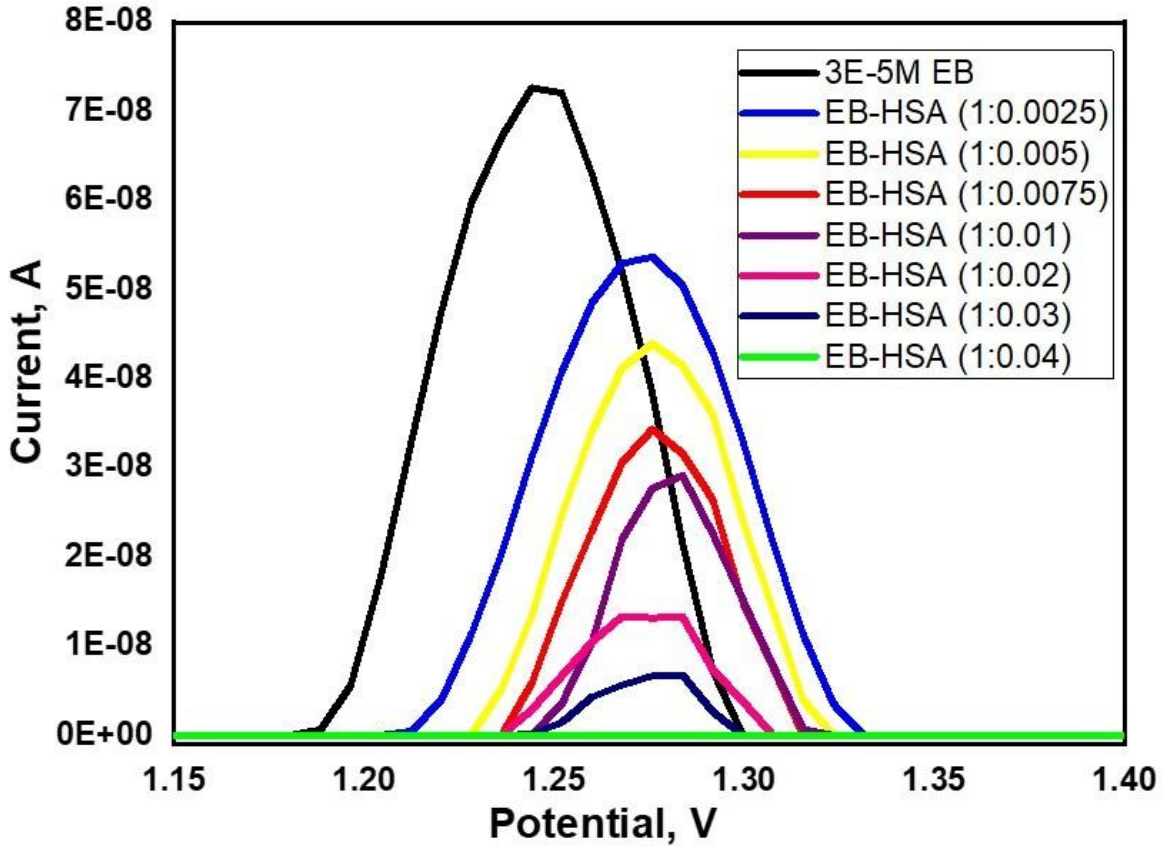


Figure 8. DPV of 3×10^{-5} M EB, different concentrations of HSA with EB in acetate buffer solution

ACKNOWLEDGEMENTS

Ahmet Cetinkaya acknowledges the funding provided by the ARDEB/1004-Center of Excellence Support Program (Project No: 20AG003) and the Scientific and Technological Research Council of Türkiye (TUBITAK) through the BIDEB/2218-National Postdoctoral Research Scholarship Program.

AUTHOR CONTRIBUTIONS

Concept: M.Y., A.C., S.A.O.; Design: M.Y., S.A.O.; Control: M.Y., A.C., E.B.A., S.A.O.; Sources: E.B.A., S.A.O.; Materials: E.B.A., S.A.O.; Data Collection and/or Processing: M.Y., A.C.; Analysis and/or Interpretation: M.Y., A.C.; Literature Review: M.Y., A.C.; Manuscript Writing: M.Y., A.C.; Critical Review: M.Y., A.C., E.B.A., S.A.O.; Other: -

CONFLICT OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest in this article.

ETHICS COMMITTEE APPROVAL

The authors declare that the ethics committee approval is not required for this study.

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J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 49(3): 870-877, 2025
Doi: 10.33483/jfpau.1652814

ORIGINAL ARTICLE / ÖZGÜN MAKALE

GREEN SYNTHESIS OF GOLD NANOPARTICLES USING *PRUNUS CERASUS L.* LEAVES EXTRACT AND STUDY OF THEIR CYTOTOXIC EFFECT ON LIVER CELL LINE

PRUNUS CERASUS L. YAPRAK EKSTRAKTINI KULLANARAK ALTIN NANOPARTİKÜLLERİNİN YEŞİL SENTEZİ VE KARACİĞER KANSERİ HÜCRE HATTI ÜZERİNDE ANTİPROLİFERATİF ETKİLERİNİN ARAŞTIRILMASI

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ABSTRACT

Objective: *Gold nanoparticles (AuNPs) possess unique chemical, physical and biological properties that make them valuable in nanotechnology applications such as drug delivery, biosensor and photothermal therapy. This study aims to synthesize AuNPs using Prunus cerasus (P. cerasus) leaves extract as a green reducing and stabilizing agent and to evaluate their structural and biological properties.*
Material and Method: *Green synthesis of AuNPs using P. cerasus leaves extract. The obtained AuNPs were characterized using UV-Vis spectroscopy (UV-Vis), Fourier transform infrared spectroscopy (FTIR), X-ray diffraction (XRD), transmission electron microscopy (TEM) and zeta potential analysis to evaluate their structural, morphological and stability properties.*
Result and Discussion: *TEM analysis confirmed that the synthesized AuNPs were predominantly spherical and had an average size of 33±13 nm. Furthermore, the synthesized AuNPs were determined to have high stability by zeta potential analysis. Biological evaluations showed that AuNPs exhibited selective cytotoxic effects, approximately three times higher antiproliferative activity against liver cancer (HepG2) cells compared to P. cerasus leaves extract. These findings emphasize the potential of P. cerasus extract-mediated AuNPs for cancer treatment, supporting their applications in biomedical nanotechnology.*
Keywords: *Antiproliferative activity, AuNPs, biosynthesis, Prunus cerasus L.*

ÖZ

Amaç: *Altın nanopartiküller (AuNP'ler), ilaç dağıtımı, biyosensör ve fototermal terapi gibi nanoteknoloji uygulamalarında değerli olmalarını sağlayan benzersiz kimyasal, fiziksel ve biyolojik özelliklere sahiptir. Bu çalışma, yeşil indirgeyici ve stabilize edici ajan olarak Prunus cerasus (P. cerasus) yaprak özütü kullanılarak AuNP'leri sentezlemeyi ve yapısal ve biyolojik özelliklerini değerlendirmeyi amaçlamaktadır.*
Gereç ve Yöntem: *AuNP'ler, çevre dostu koşullar altında P cerasus yaprak özütü kullanılarak sentezlendi. Elde edilen nanopartiküller, yapısal, morfolojik ve kararlılık özelliklerini değerlendirmek için X-ışını kırınımı (XRD), Fourier dönüşümlü kızılötesi spektroskopisi (FTIR),*

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Submitted / Gönderilme : 06.03.2025
Accepted / Kabul : 22.05.2025
Published / Yayınlanma : 19.09.2025

transmisyon elektron mikroskobu (TEM), UV-Vis spektroskopisi ve zeta potansiyel analizi kullanılarak karakterize edildi.
Sonuç ve Tartışma: *TEM analizi, sentezlenen AuNP'lerin ağırlıklı olarak küresel olduğunu ve ortalama boyutunun 33±13 nm olduğunu doğruladı. XRD sonuçları yüz merkezli kübik kafes yapıda olduğunu kanıtladı. Ayrıca sentezlenen AuNP'lerin yüksek stabiliteye sahip olduğu zeta potansiyel analizi ile belirlenmiştir. Biyolojik değerlendirmeler AuNP'lerin seçici sitotoksik etkiler sergilediğini, P. cerasus yaprak özütüne kıyasla karaciğer kanseri (HepG2) hücrelerine karşı yaklaşık üç kat daha yüksek antiproliferatif aktivite gösterdiğini gösterdi. Bu bulgular P. cerasus özütü aracılı AuNP'lerin kanser tedavisi için potansiyelini vurgulayarak biyomedikal nanoteknolojideki uygulamalarını desteklemektedir.*
Anahtar Kelimeler: *Antiproliferatif aktivite, AuNP'ler, biyosentez, Prunus cerasus L.*

INTRODUCTION

Nanotechnology has led to groundbreaking advancements in cancer treatment by introducing innovative approaches within the field of biomedicine. Despite progress in conventional treatments such as chemotherapy and radiotherapy, challenges like low specificity, systemic toxicity, and drug resistance continue to limit their effectiveness [1]. The ability of nanoscale materials to interact precisely with biological systems enables more targeted diagnostics and therapies. In this context, gold nanoparticles (AuNPs) have emerged as highly promising nanomaterials due to their chemical stability, surface functionalization potential, and distinctive optical properties. AuNPs are widely applied in targeted drug delivery systems, photothermal and photodynamic therapies, and advanced imaging techniques [2-6]. These features help overcome the limitations of traditional approaches, offering a more efficient and safer strategy for cancer diagnosis and treatment.

Traditional methods of synthesizing AuNPs, such as chemical reduction and physical deposition, often rely on hazardous reagents, require high energy inputs, and produce toxic by-products [7]. These drawbacks emphasize the need for green synthesis aproach. Green synthesis, which utilizes biological resources such as plant extracts, microorganisms, and biomolecules, has emerged as an appealing alternative for the eco-friendly production of nanoparticles [8,9]. Among these, plant-based synthesis has gained considerable attention due to its cost-effectiveness, accessibility, and compatibility with green chemistry principles. Plants are rich in bioactive compounds such as phenolic acids, flavonoids, tannins, and alkaloids, which serve as natural reducing and stabilizing agents, enabling the formation of nanoparticles.

Numerous plant species have been successfully employed for the green synthesis of AuNPs, including *Diospyros kaki*, *Magnolia kobus* [10], *Terminalia catappa* [11], *Cacumen platycladi* [12], *Gymnocladus assamicus* [13], *Musa acuminata colla* [14], *Prunus domestica* [15] and *monogyna* [16]. However, the biosynthesis of AuNPs using *P. cerasus* leaves extract has not been previously reported. This study aims to fill this gap by synthesizing AuNPs using *P. cerasus* leaves extract, which is rich in phenolic compounds, and evaluating their biological properties, including their anticancer potential.

MATERIAL AND METHOD

Preparation of Plant Extract

The leaves of *P. cerasus* were collected from the Eğirdir region of Isparta, and the plant species was taxonomically identified. To prepare the leaves extract, five grams of air-dried *P. cerasus* leaves were placed in a flask containing 100 ml of 80% ethanol. The maceration process was carried out over 24 hours to ensure efficient extraction of bioactive compounds. After the initial maceration, the extract was filtered, and the residual plant material was subjected to re-extraction using fresh ethanol. This process was repeated three times to maximize the yield of the extract [17]. The obtained filtrate was stored at +4°C for subsequent experimental use.

Synthesis of Au NPs

Sodium chloroaurate ($NaAuCl_4.2H_2O$) was supplied by Sigma Aldrich. To synthesize AuNPs, 1 mL of 0.01 M $HAuCl_4$ was added to 10 ml of the *P. cerasus* extract and stirred at room temperature in

a dark for 24 hours. The change in color of the solution from dark yellow to purple confirmed the formation of AuNPs by reduction reactions [18]. The synthesized AuNPs were centrifuged and washed with distilled water.

Cytotoxic Assay (MTT Assay) of Synthesized AuNPs

The liver cancer cell line (HepG2, ATCC® HB-8065™) and the human normal fibroblast cell line (L929) were procured from the American Type Culture Collection. Cells were cultured in Dulbecco's Modified Eagle's Medium-high-glucose (DMEM) supplemented with 10% fetal bovine serum (FBS), and %1 glutamax. The cell seeding was done at a density of $4.5x10^3$ (for L929), $5x10^3$ (for HepG2) cells/well into sterile 96-well plates. After seeding, the cells were exposed to AuNPs at varying concentrations: 5, 2.5, 1.25, 0.625, and 0.3125 µg Au /ml for L929 and 5, 2.5, 1.25, and 0.625 µg Au/ml for HepG2. The exposure duration was 48 hours. Following the exposure period, an MTT solution was added to each well to evaluate cell viability, and the plates were incubated for an additional 2 hours. Absorbance readings were obtained using an Epoch 2 ELISA plate reader at 590 nm. The half-maximal inhibitory concentration (IC_{50}) values were determined using GraphPad Prism Software 5.

Characterization of Synthesized AuNPs

The characterization of the synthesized AuNPs was carried out using UV-Vis, FTIR, XRD, TEM and zeta potential analysis. UV-Vis spectra were obtained using a JASCO V-770 UV/Vis spectrometer with a resolution of 1 nm, scanning wavelengths from 350 to 800 nm. FTIR spectroscopy was performed to identify functional groups in the plant extract and AuNPs, with spectra recorded at room temperature in the 400–4000 cm^{-1} range at a resolution of 4 cm^{-1}. XRD analysis was conducted utilizing monochromatic Cu-Kα radiation (wavelength: 0.154056 nm) to determine the crystalline structure of AuNPs, with data collected over a 2θ range of 20° to 90°. The size and morphology of the nanoparticles were examined using TEM on a JEOL JEM-1020 instrument. Zeta potential measurements were also conducted to evaluate the stability of the synthesized AuNPs.

RESULT AND DISCUSSION

AuNPs are well-known for their characteristic surface plasmon resonance (SPR) band, which is observed in the UV-Vis spectrum. This SPR band enables qualitative analysis of AuNPs, providing insight into their presence and stability. For spherical AuNPs, this band typically appears within the wavelength range of 500–550 nm, depending on factors such as particle size, shape, and the surrounding medium. The UV-Vis spectrum of the *P. cerasus* leaves extract and synthesized AuNPs by using *P. cerasus* leaves extract is shown in Figure 1. Synthesized AuNPs exhibit a characteristic SPR band at 545 nm in the UV-Vis spectrum. The obtained UV-Vis spectrum is consistent with findings reported in the literature [19-21].

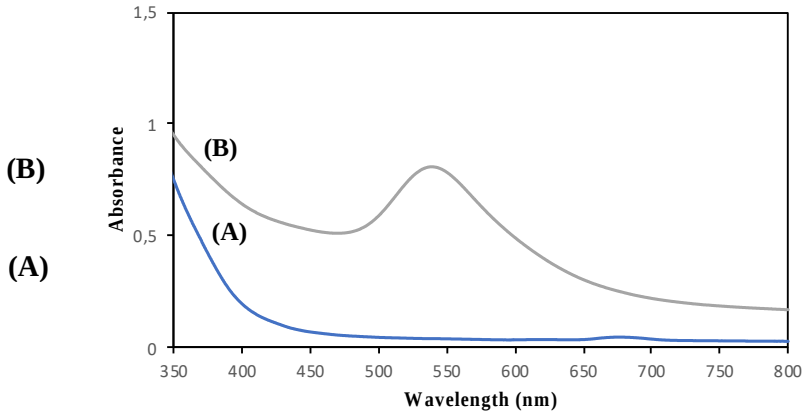


Figure 1. UV-Vis spectrum of (A) *P. cerasus* leaves extract, (B) synthesized AuNPs in the *P. cerasus* leaves extract

FTIR analysis was performed to identify the bioactive components in *P. cerasus* leaves extract that contribute to the synthesis and stabilization of AuNPs. The FTIR spectra, as shown in Figure 2, revealed prominent absorption bands at 3291, 2923, 1600, 1375, and 1029 cm^{-1} for both the leaves extract and synthesized AuNPs using the extract. The broad peak at 3291 cm^{-1} corresponds to O–H and N–H stretching vibrations, indicative of the presence of phenolic compounds and proteins. The peak at 2923 cm^{-1} is attributed to C–H stretching vibrations. The band observed at 1600 cm^{-1} represents C=O stretching vibrations, suggesting the presence of key flavonoids and terpenoids. The band at 1375 cm^{-1} corresponds to C=C stretching vibrations, associated with alkenes and aromatic compounds. Lastly, the peak at 1029 cm^{-1} is linked to C–N stretching vibrations, which are characteristic of aliphatic amines, alcohols, or polyphenols [22]. Notably, reductions in intensity or slight shifts in these peaks were observed in the spectra of AuNPs compared to the extract alone, as shown in **Fig. 2.** These changes imply that functional groups in the *P. cerasus* leaves extract, such as phenolic and amine groups, play an active role as reducing and stabilizing agents during the biosynthesis of AuNPs.

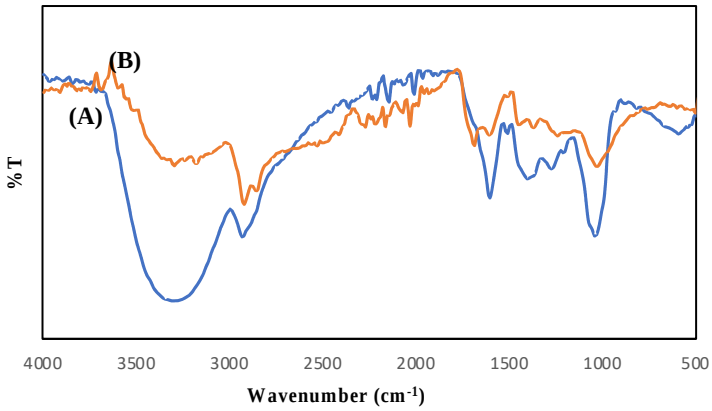


Figure 2. FTIR spectrum of (A) *P. cerasus* leaves extract (B) synthesized AuNPs

The XRD analysis of the AuNPs synthesized using *P. cerasus* leaves extract, shown in Figure 3, was conducted over a 2θ range of 20° to 90°. The diffraction pattern revealed five prominent peaks at 38.1°, 44.4°, 64.6°, 77.8°, and 81.8°, corresponding to the (111), (200), (220), (311), and (222) crystalline planes, respectively. These results align with the Powder Diffraction Standards Joint Committee data (card number 04-0784). The intense diffraction peak at 38.1° is particularly notable, confirming the presence of pure crystalline gold [23]. Additionally, the XRD analysis clearly indicates that the AuNPs formed by the reduction of $AuCl_4^-$ ions by the bioactive components present in the using *P. cerasus* leaves extract possess a face-centered cubic (fcc) lattice structure [23]. This analysis confirms the crystalline nature of the nanoparticles and highlights the role of the plant extract in their synthesis.

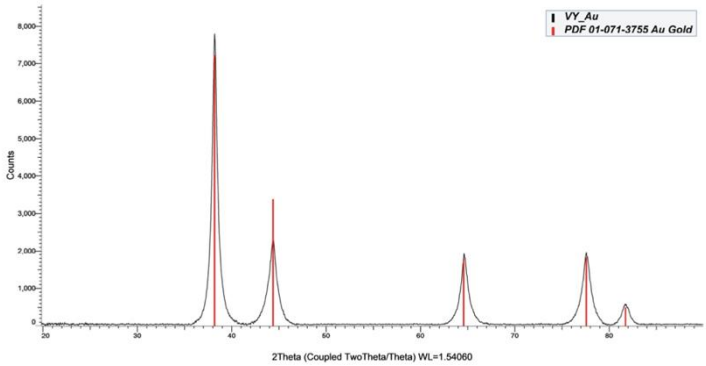


Figure 3. XRD spectrum of synthesized AuNPs

The TEM analysis provides information on the morphology and size of the synthesized AuNPs. As shown in Figure 4, the TEM results indicate that the synthesized AuNPs are approximately spherical, with an average diameter of 33±13 nm.

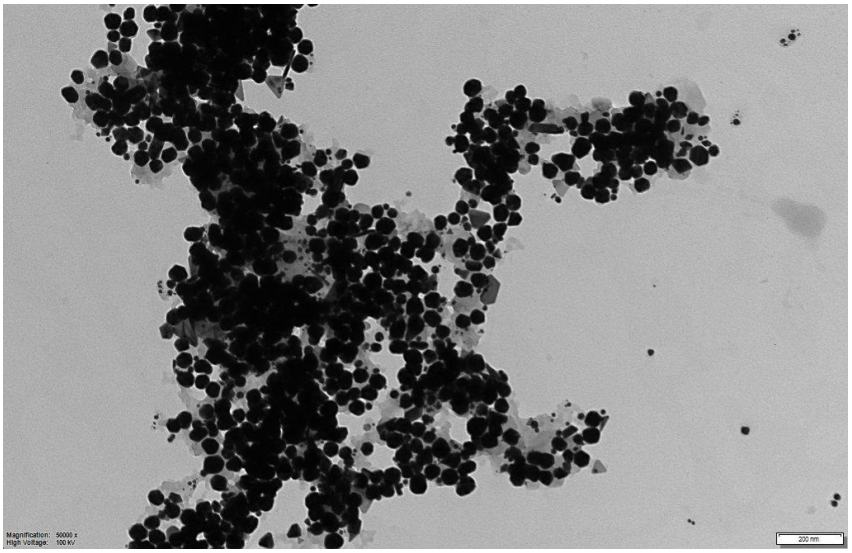


Figure 4. TEM images of synthesized AuNPs (Scale bar: 200 nm)

The size distribution of the synthesized AuNPs was determined by analyzing TEM images using Adobe Photoshop 7. Measurements were taken for 100 individual nanoparticles, and the resulting size distribution is illustrated in Figure 5. According to the analysis, the nanoparticles ranged in size from 10 nm to 59 nm.

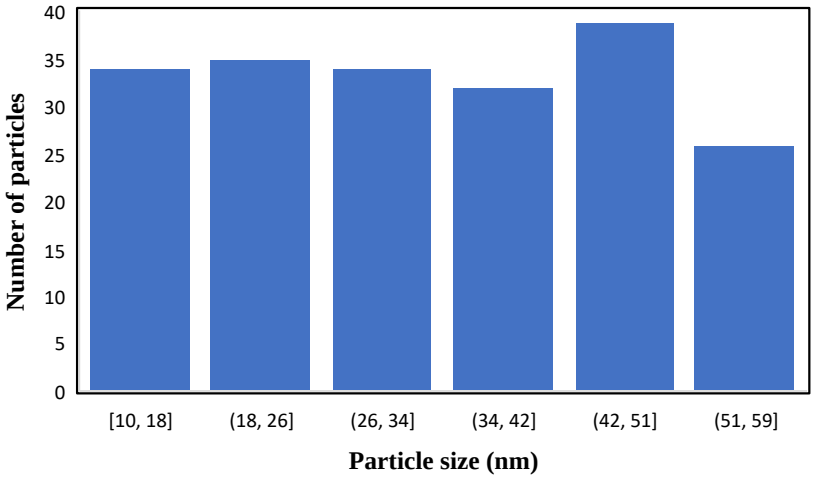


Figure 5. Particles size distribution of synthesized AuNPs

Zeta potential is a widely used parameter for characterizing the surface charge properties of nanoparticles. It provides insights into the electrical potential at the particle surface and is influenced by both the composition of the nanoparticles and the characteristics of the dispersing medium. Nanoparticles with a zeta potential above ±30 mV are considered stable in suspension due to the surface charge preventing particle aggregation [24]. The zeta potential of synthesized AuNPs using *P. cerasus* extract was found to be –54.4 mV (Figure 6), indicating that the synthesized AuNPs are stable.

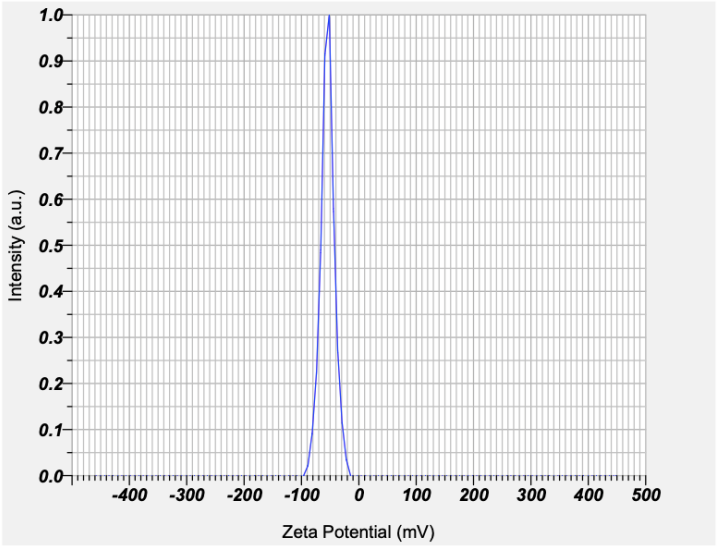


Figure 5. Zeta potential of synthesized AuNPs

The anticancer potential of the synthesized AuNPs was assessed against liver cancer cell lines *in vitro*. Both human liver cancer cells and normal human cell lines were used for the evaluation, with tests performed in duplicate, and the results averaged. As presented in Table 1, the AuNPs demonstrated approximately threefold greater cytotoxicity in inhibiting liver cancer cell growth compared to the plant extract alone. Moreover, the AuNPs exhibited higher cytotoxic activity against human liver cancer cells (HepG2) compared to normal human cell lines (L929), clearly demonstrating their selective activity.

Table 1. IC_{50} results for AuNPs against liver cancer and the human normal fibroblast cell line

Compounds	**IC_{50} (µl/ml)**	
	HepG2	**L929**
P. cerasus extract	11.09	19.42
Synthesized AuNPs	4.133	9.451

Several studies have similarly explored the cytotoxic properties of AuNPs synthesized from various plant extracts against different human cancer cell lines [25-28]. In one study, AuNPs synthesized using *Marsdenia tenacissima* extract with an approximate size of 50 nm were evaluated for their cytotoxic effects on lung cancer A549 cell lines. The findings indicated that the cytotoxicity of the nanoparticles was dependent on both their size and concentration [29]. For example, Al-Khedhair and wahap studied the cytotoxic effects of AuNPs of different sizes (10-15 nm, 20-30 nm, 45 nm) against Liver Cancer Cells. They found that among three different sizes of AuNPs, small-sized nanoparticles were the most effective in controlling cancer cells. They also reported that small size facilitates easy entry into cells and reaction with cell organelles [30]. In another study, AuNPs synthesized using *Curcumae kwangsiensis folium* leaves extract with an average diameter of 16.6 nm demonstrated significant activity against ovarian cancer cell lines. It was also reported that the cytotoxic effects of the synthesized AuNPs increased in a dose-dependent manner [31].

Conclusion

This study highlights the significant potential of green synthesis methods in medical nanotechnology by demonstrating the successful synthesis of AuNPs using *P. cerasus* leaves extract and evaluating their structural properties and anticancer activity. The characterization techniques, including UV-Vis, FTIR, XRD, TEM and zeta potential analyses, confirmed the formation of stable,

crystalline, and morphologically desirable AuNPs. Biological assays further revealed that these nanoparticles exhibit approximately threefold higher antiproliferative activity against HepG2 liver cancer cells compared to the leaf extract alone, while also showing selective cytotoxicity over normal cells. These findings underscore the unique physicochemical and therapeutic potential of green-synthesized AuNPs, positioning them as strong candidates for targeted cancer therapy. Moreover, the use of P. cerasus leaves as a natural and sustainable reducing agent reinforces the feasibility of eco-friendly approaches in nanoparticle production. Overall, this research provides valuable insights into sustainable nanomaterial development and opens new avenues for future applications of green nanotechnology in biomedical sciences.

CONFLICT OF INTEREST

The author declares that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

The author declares that the ethics committee approval is not required for this study.

AUTHOR CONTRIBUTION

Concept: F.M.S.; Design: F.M.S.; Control: F.M.S.; Sources: F.M.S.; Materials: F.M.S.; Data Collection and/or Processing: F.M.S.; Analysis and/or Interpretation: F.M.S.; Literature Review: F.M.S.; Manuscript Writing: F.M.S.; Critical Review: F.M.S.; Other: -

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Ankara Ecz. Fak. Derg. / J. Fac. Pharm. Ankara, 49(3): 878-887, 2025
Doi: 10.33483/jfpau.1650925

ÖZGÜN MAKALE / ORIGINAL ARTICLE

İNCİR ÇEKİRDEĞİ YAĞININ KOLOREKTAL KANSER HÜCRE HATLARI ÜZERİNDEKİ SİTOTOKSİK VE ANTİ-İNVAZİV ETKİLERİ

EFFECTS OF FIG SEED OIL ON THE PROLIFERATION AND INVASION OF COLORECTAL CANCER CELL LINES

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ÖZ

Amaç: *Kolorektal kanser (KRC), dünya genelinde yüksek morbidite ve mortaliteye sahip yaygın bir malignitedir. Cerrahi ve kemoterapi gibi geleneksel tedavilerin yanında, doğal kaynaklı biyoaktif bileşiklerin destekleyici terapötik potansiyelleri üzerine çalışmalar giderek artmaktadır. Bu çalışma, incir (Ficus carica) çekirdeği yağının HT-29 ve DLD-1 kolorektal kanser hücre hatlarında proliferasyon ve invazyon üzerindeki etkilerinin in vitro koşullarda değerlendirilmesini amaçlamaktadır.*
Gereç ve Yöntem: *İncir çekirdeği yağının kimyasal bileşimi GC-FID ve HPLC analizleri ile karakterize edilmiştir. Sitotoksik etkiler Alamar Blue testi ile değerlendirilmiş ve IC_{50} değerleri hesaplanmıştır; invazyon kapasitesi ise Matrigel transwell invazyon analizi ile belirlenmiştir.*
Sonuç ve Tartışma: *Analiz sonuçlarına göre, incir çekirdeği yağı yüksek düzeyde α-linolenik asit (%39.97), linoleik asit (%33.24), oleik asit (%16.71) ve γ-tokoferol (4800 mg/kg) içermektedir. Yağ, HT-29 ve DLD-1 hücrelerinde doza bağlı antiproliferatif etki göstermiş, sağlıklı PNT1A hücrelerinde ise daha düşük toksisite sergilemiştir. Ayrıca, her iki kanser hücre hattında anlamlı düzeyde invazyon inhibisyonu saptanmıştır. Bulgular, incir çekirdeği yağının antikanser potansiyel taşıyan doğal bir ajan olabileceğini göstermektedir.*
Anahtar Kelimeler: *İncir çekirdeği yağı, invazyon, kolorektal kanser, sitotoksisite, γ-tokoferol*

ABSTRACT

Objective: *Colorectal cancer (CRC) is a common malignancy associated with high morbidity and mortality worldwide. In addition to conventional treatments such as surgery and chemotherapy, there is a growing interest in the therapeutic potential of naturally derived bioactive compounds. This study aims to evaluate the in vitro effects of fig (Ficus carica) seed oil on the proliferation and invasion of HT-29 and DLD-1 colorectal cancer cell lines.*
Material and Method: *The chemical composition of fig seed oil was characterized by GC-FID and HPLC analyses. Cytotoxic effects were assessed using the Alamar Blue assay, and IC_{50} values were calculated. Invasion capacity was determined through a Matrigel-transwell invasion assay.*

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Gönderilme / Submitted : 04.03.2025
Kabul / Accepted : 23.05.2025
Yayınlanma / Published : 19.09.2025

Result and Discussion: *According to the analysis results, fig seed oil was found to be rich in α-linolenic acid (39.97%), linoleic acid (33.24%), oleic acid (16.71%), and γ-tocopherol (4800 mg/kg). The oil exhibited dose-dependent antiproliferative activity on HT-29 and DLD-1 cells, while demonstrating lower toxicity on healthy PNT1A cells. Furthermore, a significant inhibition of invasion was observed in both cancer cell lines. These findings suggest that fig seed oil may serve as a natural agent with anticancer potential for use in CRC management.*
Keywords: *Colorectal cancer, cytotoxicity, fig seed oil, invasion, γ-tocopherol*

GİRİŞ

Kolorektal kanser (KRC), hem dünya genelinde hem de Türkiye'de yaygın görülen ve ciddi sağlık yükü oluşturan maligniteler arasında yer almaktadır. Uluslararası Kanser Araştırma Ajansı'nın 2020 tarihli Dünya Kanser Raporu'na göre, kanser, 30–69 yaş aralığındaki bireylerde 134 ülkede birinci veya ikinci sırada ölüm nedenidir [1]. Türkiye verilerine göre ise KRC insidansı erkeklerde yüz binde 24.1, kadınlarda yüz binde 13.3 olup, 2020 yılında toplam 18.825 yeni vaka bildirilmiştir [2]. Bu veriler, KRC'nin küresel ve ulusal düzeyde artan önemini ortaya koymakta ve yeni tedavi yaklaşımlarına duyulan gereksinimi vurgulamaktadır.

Dünya genelinde kolorektal kansere yakalanma olasılığı %4-5 arasında değişmekte olup, bireysel faktörler ve yaşam tarzı alışkanlıkları bu riski önemli ölçüde etkileyebilmektedir. Yaş, genetik yatkınlık ve inflamatuvar bağırsak hastalıkları gibi değiştirilemez risk faktörlerinin yanı sıra; sedanter yaşam tarzı, obezite, sağlıksız beslenme, sigara ve alkol kullanımı gibi değiştirilebilir risk faktörleri de hastalığın gelişimine katkıda bulunmaktadır [2,3].

Kolorektal kanser tedavisinde cerrahi müdahale ve kemoterapi temel yaklaşımlar olmakla birlikte, metastatik vakalarda prognoz genellikle zayıf olup, hedefe yönelik tedavi, immünoterapi ve gen tedavisi gibi yenilikçi stratejiler geliştirilmiştir [4]. Ayrıca, kemoterapi ile doğal bileşenler kombine edilerek ilaç direncini azaltabilmekte ve kolorektal kanser tedavisinde alternatif yaklaşımlar sunabilmektedir [5]. Doğal kaynaklar, birçok ciddi hastalığın tedavisinde birincil seçenek olarak kullanılan ve terapötik açıdan önemli bileşenler içeren zengin bir potansiyele sahiptir. Yapılan tahminlere göre, dünya nüfusunun yaklaşık %75–80'i, sağlık hizmetlerine erişimdeki kısıtlılıklar ve sentetik ilaçların güvenilirliği ile etkinliğine yönelik endişeler nedeniyle ilk tedavi yöntemi olarak geleneksel tıp uygulamalarına yönelmektedir [6,7].

Geleneksel tedavi uygulamalarında yaygın olarak kullanılan doğal ürünlerin önemli bir bölümünü bitkisel yağlar oluşturmaktadır. Bu yağlar, içeriklerinde bulunan linoleik, linolenik ve oleik asit gibi doymamış yağ asitlerinin yanı sıra fitosteroller, tokoferoller ve fenolik bileşikler gibi biyoaktif komponentler sayesinde antioksidan, anti-inflamatuvar ve anti-kanser etkiler göstermektedir [8-11]. Bu kapsamda yapılan çeşitli araştırmalar, farklı bitkisel yağların yağ asidi ve tokoferol içeriklerini ortaya koymuştur. Çörek otu (Nigella sativa) yağı da sağlık açısından değerli yağlar arasında yer almakta olup, linoleik asit (%55.6), oleik asit (%8.1) ve linolenik asit (%2.45) içermektedir [12]. Ayrıca çörek otu yağının tokoferol içeriği 91.5–279.2 mg/kg arasında değişmektedir [13]. Keten tohumu yağı (Linum usitatissimum), özellikle yüksek linolenik asit (%45.72–48.59) içeriğiyle dikkat çekerken, linoleik asit içeriği %13.83–14.61 ve oleik asit içeriği %25.66–27.31 aralığında raporlanmıştır [14]. Keten tohumu yağının tokoferol içeriği ise 271–788 mg/kg düzeyindedir [15]. Susam yağı (Sesamum indicum) da önemli bir bitkisel yağ olarak öne çıkmakta olup, oleik asit içeriği %37.8–47.5, linoleik asit içeriği %36.4–46.2 ve linolenik asit oranı ise %0.2–0.7 aralığında değişmektedir [16]. Ayrıca susam yağı, 161.7–647.0 mg/kg aralığında tokoferol içeriği göstermektedir [17].

İncir çekirdeği yağı (*Ficus carica* seed oil) üzerine yapılan çalışmalarda, bu yağın yüksek düzeyde linolenik asit (%40.00–41.27), linoleik asit (%30.06–31.97) ve oleik asit (%17.06–18.10) içerdiği bildirilmiştir [18-20]. Ayrıca, özellikle γ-tokoferol açısından zengin olduğu, içeriğinin 3888.21–4132.09 mg/kg aralığında olduğu ve bu özelliğiyle oksidatif stresle ilişkili hücresel hasarı önleyerek potansiyel bir anti-kanser ajanı olabileceği belirtilmiştir [20]. İncir çekirdeği yağının içerdiği fenolik bileşikler sayesinde yüksek serbest radikal temizleme kapasitesine sahip olup; bu yönüyle antikarsinojenik, antioksidan, antimutajenik, serbest radikal giderici ve lipid peroksidasyonunu inhibe edici etkiler gösterebildiği bildirilmiştir [21]. Bu bulgular genel olarak değerlendirildiğinde, incir çekirdeği yağının

hem çoklu doymamış yağ asitleri (PUFA) hem de tokoferoller bakımından zengin içeriğiyle dikkat çektiği ve diğer yaygın bitkisel yağlarla karşılaştırıldığında potansiyel biyolojik aktiviteleri destekleyebilecek nitelikte olduğu görülmektedir. İncir çekirdeği yağı, yapılan çalışmalarda yüksek düzeyde PUFA—özellikle linolenik asit (%40.00–41.27), linoleik asit (%30.06–31.97) ve oleik asit (%17.06–18.10)—içeriğiyle dikkat çekmektedir [18–20]. Ayrıca, bu yağın γ-tokoferol açısından zengin olduğu ve içeriğinde 3888.21–4132.09 mg/kg düzeyinde γ-tokoferol bulunduğu bildirilmiş; bu özelliğiyle oksidatif stresle ilişkili hücresel hasarı önlemede etkili olabileceği ve potansiyel bir anti-kanser ajanı olarak değerlendirilebileceği ifade edilmiştir [20]. Bununla birlikte, incir çekirdeği yağında bulunan fenolik bileşiklerin de yüksek serbest radikal temizleme kapasitesi sağladığı ve bu sayede yağın antioksidan, antimutajenik ve lipid peroksidasyonunu inhibe edici etkiler gösterebildiği çeşitli çalışmalarla ortaya konmuştur [21]. Genel olarak değerlendirildiğinde, incir çekirdeği yağı hem yağ asidi profili hem de tokoferol ve fenolik bileşik içeriği açısından zengin bileşimiyle, diğer yaygın bitkisel yağlarla karşılaştırıldığında biyolojik aktivite potansiyeli yüksek bir fonksiyonel bileşen olarak öne çıkmaktadır.

Bu çalışma, ilk kez incir çekirdeği yağının HT-29 ve DLD-1 kolon kanseri hücre hatları üzerindeki proliferatif ve invazif etkilerini değerlendirmeyi amaçlamaktadır. Değerlendirme kapsamında, incir çekirdeği yağında bulunan biyoaktif bileşenlerin kanser hücrelerinin büyümesini baskılama ve invazyon kapasitesini azaltma potansiyeli araştırılmıştır. Elde edilen bulgular doğrultusunda, incir çekirdeği yağının potansiyel terapötik önemi tartışılmıştır.

GEREÇ VE YÖNTEM

Kullanılan Kimyasal Maddeler

HT-29 (HTB-38) ve DLD-1(CCL-221) hücre hatları American Tissue Culture Company (ATCC, Manassas, VA, ABD) firmasından; PNT1A hücre hattı ise Sigma-Aldrich (CB_95012614, St. Louis, MO, ABD) firmasından temin edilmiştir. Bu çalışmada kullanılan bovine serum albumin (BSA; A7511) Sigma-Aldrich firmasından; Alamar Blue ise Invitrogen Life Technologies firmasından sağlanmıştır. Modifiye McCoy's 5a besiyeri (ATCC-30-2007), RPMI 1640 besiyeri (ATCC-30-2001) ve fetal bovin serumu (FBS; ATCC-30-2020) da ATCC firmasından temin edilmiştir. Bu çalışmada kullanılan tüm kimyasal maddeler ve çözücüler, analitik saflık derecesine sahip olup, ticari olarak mevcut en yüksek saflık seviyesinde temin edilmiştir.

İncir Çekirdeği Yağının Tedariki ve Kimyasal Analizleri

Bu çalışmada kullanılan incir çekirdeği yağı, ticari olarak Zade Vital firmasından temin edilmiştir. Soğuk sıkım yöntemiyle üretilmiş olması ve GMP (Good Manufacturing Practices) sertifikasına sahip bulunması nedeniyle bu araştırmada tercih edilmiştir.

İncir çekirdeği yağının yağ asidi kompozisyonu, Zade Vital firması bünyesindeki laboratuvarlarda Shimadzu GC-FID 2010 cihazı kullanılarak analiz edilmiştir. E vitamini içeriği de yine aynı firma tarafından Shimadzu HPLC Floresan dedektör sistemi kullanılarak analiz edilmiştir.

Hücrenin İncir Çekirdeği Yağı ile Muamele Edilmesi

İncir çekirdeği yağı, öncelikle DMSO ile çözdürülmüştür. Hücre kültür ortamında toksisiteyi önlemek amacıyla DMSO oranı %0.2'nin altında tutulmuş, ardından çözelti hücre besiyeri ile seyreltilerek vortekslenmiş ve homojen hale getirilmiştir. Hücreler farklı doz aralıklarında incir çekirdeği yağına maruz bırakılmak üzere 6-60 mg/ml konsantrasyonlarında çözeltiler hazırlanmıştır. Önceden ekilmiş hücrelerin besiyerleri aspire edilerek uzaklaştırılmış ve hazırlanan farklı konsantrasyonlardaki incir çekirdeği yağı solüsyonları hücrelerin üzerine ilave edilmiştir. Son olarak, hücreler 48 saat boyunca inkübasyona bırakılmıştır.

Hücre Kültür Koşulları

Bu çalışmada, kolorektal adenokarsinom kökenli HT-29 ve DLD-1 hücre hatları ile sağlıklı kontrol grubu olarak PNT1A hücre hattı kullanılmıştır. PNT1A, normal insan prostat epitelinden türetilmiş, immortalize bir hücre hattı olup, sağlıklı epitel hücre modelini temsil etmesi amacıyla

karşılaştırmalı sitotoksisite analizlerinde tercih edilmiştir. Çalışma kapsamında kolorektal kökenli normal epitel hücre hattı temin edilemediğinden, alternatif olarak PNT1A referans grup olarak değerlendirilmiştir. Literatürde de PNT1A hücre hattının, farklı doku kökenli tümör hücre hatlarıyla yapılan in vitro toksisite çalışmalarında kontrol grubu olarak kullanıldığı görülmektedir [22].

HT-29, DLD-1 ve PNT1A hücre hatları, %10 fetal bovin serumu, 2 mM L-glutamin ve %1 penisilin/streptomisin içeren McCoy's 5a ve RPMI-1640 besiyerlerinde kültüre edilmiştir. Hücre hatları, %5 CO_2 atmosferinde, %95 nem oranında ve 37 °C sıcaklıkta muhafaza edilmiştir.

Hücre Canlılık Testi

İncir çekirdeği yağının HT-29 ve DLD-1 hücre hatları üzerindeki proliferasyon etkisini ve IC50 değerlerini belirlemek amacıyla Alamar Blue testi uygulanmıştır [23]. Deney kapsamında, toplam 1×10^4 hücre, 96 kuyucuklu plaklara ekilmiş ve 37°C'de, %5 CO_2 ortamında 24 saat boyunca inkübe edilmiştir. Ardından, hücreler 6-60 mg/ml aralığındaki farklı konsantrasyonlardaki incir çekirdeği yağına maruz bırakılmıştır. Hücre canlılığı, 570 nm ve 610 nm dalga boylarında absorbans ölçümü yapılarak Multiskan Go (Thermo Scientific Co., Waltham, MA, ABD) marka spektrofotometre ile belirlenmiştir. IC50 değerleri, inhibisyon oranı (%) ile incir çekirdeği yağı konsantrasyonunun logaritması arasındaki sigmoidal eğri üzerinden hesaplanmıştır.

Hücre İnvazyon Analizi

HT-29 ve DLD-1 hücre hatlarının invazyon kapasitesi, BioCoat Matrigel İnvazyon odacıkları (BD Biosciences, Bedford, MA, ABD) kullanılarak değerlendirilmiştir. Matrigel, 37°C'de ve %5 CO_2 ortamında büyüme besiyeri ile 2 saat rehidre edilmiştir. Hücreler, sitotoksisite analizi sonucunda belirlenen IC_{50} konsantrasyonlarında incir çekirdeği yağı ile 48 saat süreyle muamele edildikten sonra, %1 FBS içeren ortamda 1×10^4 hücre olacak şekilde süspanse edilerek üst bölmeye eklenmiştir. Hücrelerin alt bölmeye göç etmesi için %20 FBS içeren ortam kullanılarak 37 °C'de, %5 CO_2 ortamında 24 saat inkübe edilmiştir. Üst bölmede kalan hücreler uzaklaştırılmıştır, ardından membranlar %3.7 formaldehit ile sabitlenmiş ve %100 metanol ile geçirgen hale getirilmiştir. Hücreler, %1 toluidin ile boyanmış ve mikroskop altında görüntülenerek sayılmıştır.

İstatistiksel Analiz

Elde edilen verilerin istatistiksel analizleri, Windows GraphPad Prism (Sürüm 5.0) istatistiksel yazılım paketi (GraphPad Software, San Diego, CA, ABD) kullanılarak gerçekleştirilmiştir. İkili grup karşılaştırmaları, Student t-testi ile analiz edilmiştir. İstatistiksel anlamlılık eşiği $p < 0.05$ (*) olarak ifade edilmiştir.

SONUÇ VE TARTIŞMA

İncir Çekirdeği Yağının Kimyasal İçeriğinin Analizi

Ticari olarak temin edilen incir çekirdeği yağının yağ asidi profili, Shimadzu GC-FID 2010 cihazı kullanılarak belirlenmiştir (Tablo 1). Bunun yanı sıra, incir çekirdeği yağının tokoferol (E vitamini) içeriği, Shimadzu HPLC Floresans dedektörü ile analiz edilmiştir (Tablo 2).

İncir çekirdeği yağı, yüksek oranda doymamış yağ asitleri içermesi nedeniyle besinsel açıdan değerli bir yağ kaynağıdır. Yapılan analizler sonucunda, incir çekirdeği yağının başlıca yağ asitleri α-linolenik asit (%39.97), linoleik asit (%33.24) ve oleik asit (%16.71) olarak belirlenmiştir. Bu oranlar, Tarlacı (2021) tarafından bildirilen α-linolenik asit (%40.2), linoleik asit (%31.2) ve oleik asit (%17.0) değerleriyle büyük ölçüde uyumludur [24]. Benzer şekilde, literatürde yapılan çalışmalarda da incir çekirdeği yağının linolenik asit (%40-41.27), linoleik asit (%30.06-31.97) ve oleik asit (%17.06-18.10) içerdiği rapor edilmiştir [18–20]. Çalışmamızda hesaplanan doymamış yağ asidi/doymuş yağ asidi oranı (UFA/SFA) 7.62 olup, bu değer Soltana ve ark. (2016) tarafından bildirilen oranla büyük ölçüde örtüşmektedir [25]. Çalışma bulgularına göre, incir çekirdeği yağı %88.39 oranında doymamış yağ asitleri (UFA) içermekte olup, bu oran soya ve yer fıstığı gibi önemli bitkisel yağlarla kıyaslandığında daha yüksektir. PUFA oranının %73.21 olarak belirlenmesi, incir çekirdeği yağının diğer yağ tohumlarına [26,27] kıyasla daha yüksek doymamış yağ asidi içeriğine sahip olduğunu göstermektedir.

Tablo 1. İncir çekirdeği yağının yağ asidi kompozisyonu (%)

Yağ Asidi	Konsantrasyon (%)
Palmitik asit (C16:0)	6.72
Stearik asit (C18:0)	2.69
Oleik asit (Omega-9) (C18:1)	16.71
Linoleik asit (Omega-6) (C18:2)	33.24
α-Linolenik asit (Omega-3) (C18:3)	39.97
Araşidik asit (C20:0)	0.18
Eikosenoik asit (Omega-9) (C20:1)	0.23
Behenik asit (C22:0)	0.07
Toplam doymuş yağ asitleri (SFA)	9.66
Toplam doymamış yağ asitleri (UFA)	90.15
Toplam çoklu doymamış yağ asitleri (PUFA)	73.21
PUFA/SFA	7.58
UFA/SFA	9.33

SFA: doymuş yağ asitleri; UFA: doymamış yağ asitleri; PUFA: çoklu doymamış yağ asitleri

Bu çalışmada, incir çekirdeği yağında γ-tokoferol düzeyi 4800 mg/kg, *α*-tokoferol 120 mg/kg, *δ*-tokoferol 80 mg/kg olarak belirlenmiş; β-tokoferol ise tespit edilmemiştir. Bu değerler, literatürde fig çekirdeği yağına ilişkin bildirilen bazı verilerle benzerlik gösterirken, bazı çalışmalarla anlamlı farklılıklar sergilemektedir. Örneğin, bazı araştırmalarda *γ*-tokoferol içeriğinin 3914–4016 mg/kg aralığında olduğu bildirilmiş olup [18-20], bu değerler çalışmamızla oldukça uyumludur. Benzer şekilde, Tarlacı (2021) tarafından bildirilen 391 mg/100 g (yaklaşık 3910 mg/kg) *γ*-tokoferol içeriği de çalışmamızda elde edilen değerlerle paralellik göstermektedir [24]. Öte yandan, bazı çalışmalarda *γ*-tokoferol içeriği yalnızca 634 mg/kg olarak bildirilmiş olup [19], bu farklılığın, kullanılan ekstraksiyon yöntemine, bitkinin yetiştirildiği coğrafi bölgeye, olgunluk evresine ve işleme koşullarına bağlı olarak değişebileceği düşünülmektedir [28-30]. Özellikle soğuk pres gibi termal bozulmanın en aza indirildiği ekstraksiyon tekniklerinin tokoferol stabilitesini koruyarak ölçülen değeri artırabileceği bilinmektedir [30].

Elde edilen bulgular yalnızca incir çekirdeği yağına yönelik önceki araştırmalarla değil, aynı zamanda farklı bitkisel yağların raporlanan tokoferol içerikleriyle karşılaştırıldığında da dikkat çekici bir profil ortaya koymaktadır. Nitekim literatürde, çörek otu (*Nigella sativa*) yağı için toplam tokoferol içeriği 91.5–279.2 mg/kg [13], keten tohumu (*Linum usitatissimum*) yağı için 271–788 mg/kg [15] ve susam (*Sesamum indicum*) yağı için 161.7–647.0 mg/kg [17] aralığında değerler bildirilmektedir. Bu kapsamda değerlendirildiğinde, incir çekirdeği yağı, özellikle *γ*-tokoferol içeriği bakımından dikkat çekici bir profilde olup, bu özelliği sayesinde antioksidan kapasitesi yüksek bitkisel yağlar arasında potansiyel bir aday olarak değerlendirilebilir.

Tokoferoller, yenilebilir bitkisel yağlarda doğal olarak bulunan antioksidan bileşiklerdir ve E vitamini bileşenleri olarak insan sağlığı açısından önemli işlevler üstlenmektedir [20]. Tokoferoller, yağın antioksidan kapasitesine katkıda bulunarak oksidatif stabilitesini artırmaktadır. *γ*-tokoferol, güçlü antioksidan aktivitesi sayesinde zararlı reaktif oksijen türleri ve reaktif azot türlerini nötralize edebilir ve oksidatif stresin neden olduğu hücresel hasarı azaltabilir [31]. Bu nedenle, *γ*-tokoferolün oksidatif stres kaynaklı hastalıkların önlenmesi ve tedavisinde önemli bir potansiyele sahip olduğu düşünülmektedir. Linoleik asit (LA) açısından zengin olan incir çekirdeği yağı, özellikle vitamin E gibi antioksidanlarla birlikte serbest radikal temizleme kapasitesini artırabildiği ve sinerjistik etkiler gösterebildiği bildirilmektedir [32]. Bu bağlamda, incir çekirdeği yağında hem PUFA düzeyinin hem de *γ*-tokoferol içeriğinin yüksek olması, bu yağın oksidatif stabilite ve biyoaktif potansiyel açısından dikkate değer bir profil sunduğunu göstermektedir.

Bu çalışmada incelenen incir çekirdeği yağının kimyasal kompozisyonu, özellikle linoleik asit (%33.24), alfa-linolenik asit (%39.97) ve *γ*-tokoferol (4800 mg/kg) düzeylerinin yüksekliği ile dikkat çekmektedir. Literatürde, linoleik asit içeren yağların, kolon kanseri hücre hatlarına karşı belirli bir antikanser aktivite sergilediği bildirilmiştir [33]. Benzer şekilde, *γ*-tokoferolün insan HCT116 kolon kanseri hücrelerinin büyümesini inhibe ettiği rapor edilmiştir [34]. Alfa-linolenik asit (ALA), vücutta uzun zincirli omega-3 yağ asitlerine dönüşerek antioksidan savunmayı artırabilir ve bu sayede oksidatif

stresle mücadelede etkili olabilir [35,36]. ALA açısından zengin diyetlerin, meme kanseri gibi bazı kanser türlerinin büyümesini ve metastazını engelleyebileceği öne sürülmüştür [37,38]. Bu veriler ışığında değerlendirildiğinde, incir çekirdeği yağının içerdiği yüksek düzeyde çoklu doymamış yağ asitleri ve γ-tokoferol, bu yağın da antikanser potansiyele sahip fonksiyonel bir bileşen olabileceğini düşündürmektedir.

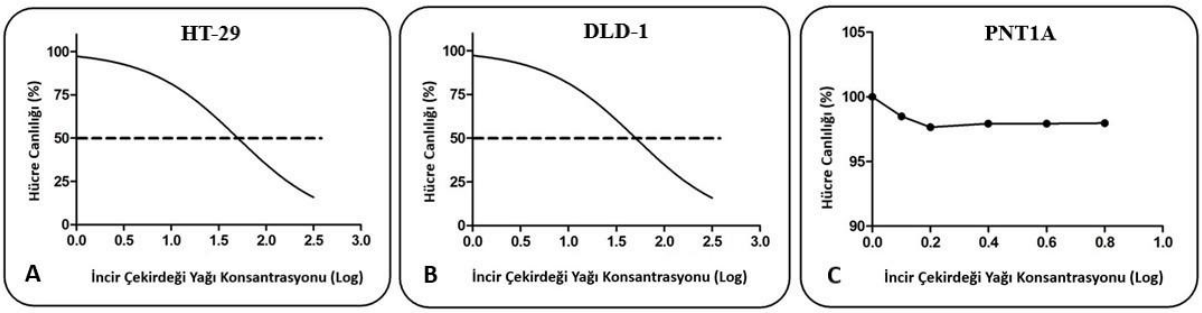
Tablo 2. İncir çekirdeği yağında bulunan tokoferol bileşenleri

Tokoferol Bileşenleri	Konsantrasyon (mg/kg)
α- Tokoferol	120
β- Tokoferol	0
γ- Tokoferol	4800
δ- Tokoferol	80

İncir Çekirdeği Yağının Sitotoksisite Analizi

Hücre canlılık analizleri, incir çekirdeği yağının HT-29, DLD-1 ve PNT1A hücre hatları üzerindeki sitotoksik etkilerini değerlendirmek amacıyla gerçekleştirilmiştir. Alamar Blue testleri sonucunda, incir çekirdeği yağının kolorektal kanser hücrelerinin proliferasyonunu baskıladığı ve IC50 değerlerinin hücre tipine bağlı olarak farklılık gösterdiği belirlenmiştir. HT-29 hücreleri için IC50 değeri 57 mg/ml, DLD-1 hücreleri için ise 32.16 mg/ml olarak hesaplanmıştır (Şekil 1). Şekil 1'de gösterildiği üzere, incir çekirdeği yağının DLD-1 hücrelerine karşı daha güçlü sitotoksik etki gösterdiğini ortaya koymaktadır. Buna karşılık, PNT1A hücre hattında IC50 değeri 96.6 mg/ml olarak hesaplanmış (Şekil 1), bu durum incir çekirdeği yağının sağlıklı hücreler üzerindeki toksisitesinin kanser hücrelerine kıyasla daha düşük olduğunu göstermektedir. Bu bulgu, incir çekirdeği yağının seçici sitotoksisite gösterebileceğini düşündürmektedir.

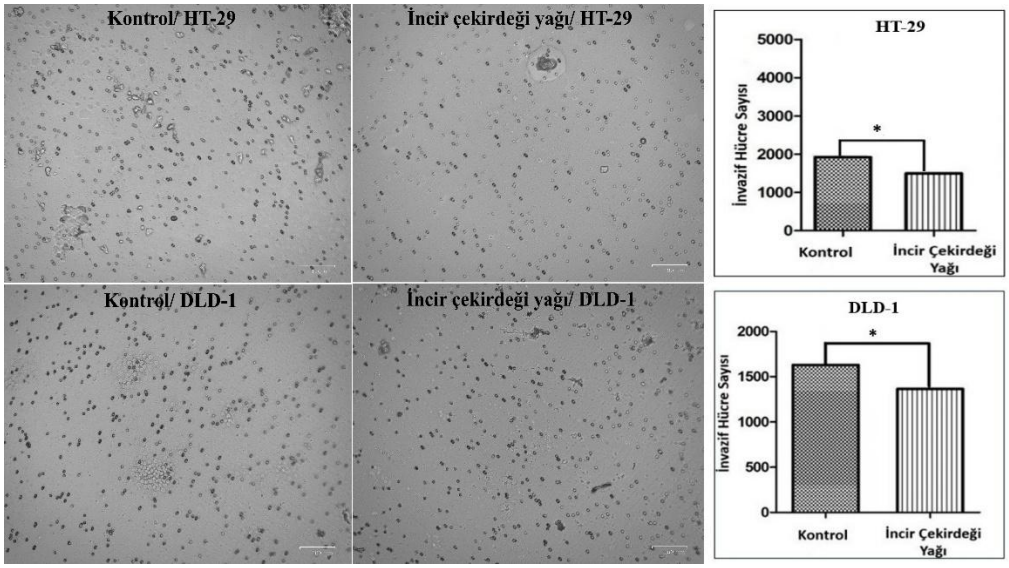
Literatürde incir çekirdeği yağının doğrudan kanseri tedavi edici veya önleyici etkisine dair kesin bulgular bulunmamaktadır. Farklı bitkisel yağların kolorektal kanser hücre hatlarına yönelik sitotoksik etkileri değerlendirildiğinde, çörek otu (*Nigella sativa*) tohum yağı için HCT116 hücre hattında bildirilen IC_{50} değeri 43.56±1.95 µM olarak raporlanmıştır [39]. Gözyaşı otu (*Coix lachryma*-jobi) tohum yağı için ise HT-29 hücre hattında yaklaşık 5.30 mg/ml'lik bir IC_{50} değeri bildirilmiştir [40]. Öte yandan, keten tohumu yağına ait IC_{50} değeri LoVo kolorektal kanser hücre hattında 4.30±0.60 mg/ml olarak belirlenmiştir [41]. Bu çalışma kapsamında incelenen incir çekirdeği yağı, HT-29 hücreleri için 57 mg/ml, DLD-1 hücreleri için ise 32.16 mg/ml IC_{50} değerleri ile ölçülmüş olup, özellikle DLD-1 hücre hattında daha belirgin bir sitotoksik etki göstermiştir. Bu değerlerle karşılaştırıldığında, incir çekirdeği yağı daha yüksek IC_{50} değerleri göstermiştir. Bu fark, kullanılan ekstraksiyon yöntemi, yağların kimyasal bileşimi, test süresi, hücre hattı özellikleri gibi parametrelerle ilişkilendirilebilir.



Şekil 1. İncir çekirdeği yağının farklı konsantrasyonlarının HT-29, DLD-1 ve PNT1A hücre hatları üzerindeki sitotoksik etkisinin değerlendirilmesi. (A) HT-29 hücre hattında, (B) DLD-1 hücre hattında ve (C) PNT1A hücre hattında belirlenen IC50 değerleri gösterilmektedir

İncir Çekirdeği Yağının HT-29 ve DLD-1 Hücre Hatlarının İnvazyonu Üzerine Etkisi

İncir çekirdeği yağının HT-29 ve DLD-1 hücre hatları üzerindeki anti-invaziv etkisi, sitotoksisite analizi sonucunda belirlenen IC_{50} konsantrasyonları kullanılarak değerlendirilmiştir. Yapılan analizler sonucunda, incir çekirdeği yağı ile 48 saat süreyle muamele edilen gruplarda (n=3), HT-29 hücrelerinin invazyon potansiyelinin %22 oranında anlamlı şekilde azaldığı tespit edilmiştir (p=0.028). Benzer şekilde, DLD-1 hücre hattında invazyon kapasitesinde %16 oranında istatistiksel olarak anlamlı bir azalma gözlemlenmiştir (p=0.041) (Şekil 2). Mevcut literatür incelendiğinde, özellikle tohum yağlarının kolorektal kanser hücre hatlarında invazyon üzerine etkilerini araştıran çalışmaların sınırlı olduğu dikkat çekmektedir. Gill ve ark. (2005) tarafından yürütülen bir çalışmada, HT115 kolorektal kanser hücre hattında, farklı konsantrasyonlardaki sızma zeytinyağı fenolleri (25, 50, 75 ve 100 µg/ml) ile 24 saatlik inkübasyon sonrası yapılan Matrigel invazyon testlerinde, invazyonun doza bağlı olarak anlamlı şekilde azaldığı ve 25 µg/ml üzerindeki konsantrasyonlarda yaklaşık %60'a varan inhibisyon elde edildiği bildirilmiştir [42]. Zeytinyağı gibi fenolik bileşen içeriği yüksek bitkisel yağların invazyonu baskılayıcı etkileri daha önce raporlanmış olmakla birlikte [42] incir çekirdeği yağına ilişkin veriler bu alanda ilk örneklerden biri olup, tohum kökenli bir yağın kolorektal kanser hücre hatlarında invazyonu anlamlı düzeyde baskılayabileceğini göstermesi bakımından önem taşımaktadır. Bu sonuçlar, incir çekirdeği yağının potansiyel anti-metastatik özellikleri olabileceğini düşündürmektedir.



Şekil 2. Kontrol ve incir çekirdeği yağı ile muamele edilen HT-29 ve DLD-1 hücrelerine ait mikroskobik görüntüler ve invazif hücre sayılarının kantitatif analizi (n=3)

Elde edilen bulgular, incir çekirdeği yağının kolorektal kanser hücrelerinde proliferasyonu baskılayıcı ve invazyonu azaltıcı etkiler gösterdiğini ortaya koymaktadır. Bu etkilerin, yağın içerdiği yüksek düzeyde PUFA ve γ-tokoferol gibi biyoaktif bileşenlerle ilişkili olabileceği düşünülmektedir. Literatürde, linoleik asit ve α-linolenik asit gibi PUFA'ların, NF-κB gibi inflamatuvar transkripsiyon faktörlerinin aktivitesini baskılayarak tümör hücre proliferasyonunu azalttığı, apoptozu indükleyebildiği ve invazyon/metastaz ile ilişkili MMP (matrix metalloproteinaz) ekspresyonunu düşürdüğü rapor edilmiştir [32, 38, 43-45]. Benzer şekilde, γ-tokoferol'ün, reaktif oksijen türlerini ve reaktif azot türlerini etkili biçimde nötralize ederek oksidatif stres kaynaklı DNA hasarını ve hücre proliferasyonunu önleyebildiği, ayrıca COX-2 ve iNOS gibi inflamatuvar belirteçlerin ekspresyonunu inhibe ettiği çeşitli çalışmalarda bildirilmiştir [31,46,47]. Bu bağlamda, çalışmamızda doğrudan moleküler analizler gerçekleştirilmemiş olsa da, gözlemlenen antiproliferatif ve anti-invaziv etkilerin, incir çekirdeği yağında bulunan PUFA ve γ-tokoferol gibi biyoaktif bileşenlerin inflamatuvar ve oksidatif stresle ilişkili hücresel yolakları dolaylı olarak modüle etme potansiyeliyle ilişkili olabileceği değerlendirilmektedir.

Bu çalışma, incir çekirdeği yağının kimyasal bileşimi ile kolorektal kanser hücre hatları üzerindeki antiproliferatif ve anti-invaziv etkilerini in vitro düzeyde ortaya koymaktadır. Yağın yüksek oranda PUFA ve γ-tokoferol içermesi, gözlemlenen antikanser etkilerin potansiyel olarak antioksidan ve anti-inflamatuar mekanizmalarla ilişkili olabileceğini düşündürmektedir. Hücre kültürü deneyleri, incir çekirdeği yağının tümör hücrelerinin proliferasyonunu ve invazyonunu anlamlı düzeyde baskıladığını; buna karşın, sağlıklı hücreler üzerinde daha düşük toksik etki gösterdiğini ortaya koymuştur. Ancak çalışmada apoptotik, inflamatuvar veya oksidatif stresle ilişkili yolaklara dair moleküler düzeyde mekanistik analizler gerçekleştirilmemiştir. Bu nedenle, elde edilen bulguların translasyonel anlamda daha güçlü biçimde desteklenebilmesi için, ileride gerçekleştirilecek moleküler mekanizma çalışmalarına ve in vivo doğrulamalara ihtiyaç duyulmaktadır.

TEŞEKKÜR

Bu araştırma Selçuk Üniversitesi Bilimsel Araştırma Projeleri Koordinatörlüğü tarafından 16202041 proje numarası ile desteklenmiştir.

YAZAR KATKILARI

Kavram: Z.D.; Tasarım: Z.D.; Denetim: Z.D., M.A., H.V., S.K.; Kaynaklar: Z.D., M.A., H.V., S.K.; Malzemeler: Z.D., M.A., H.V., S.K.; Veri Toplama ve/veya İşleme: Z.D., S.K.; Analiz ve/veya Yorumlama: Z.D., S.K.; Literatür Taraması: Z.D.; Makalenin Yazılması: Z.D.; Kritik İnceleme: Z.D.; Diğer; -

ÇIKAR ÇATIŞMASI BEYANI

Yazarlar bu makale için gerçek, potansiyel veya algılanan çıkar çatışması olmadığını beyan ederler.

ETİK KURUL ONAYI

Yazarlar bu çalışma için etik kurul onayının zorunlu olmadığını beyan etmektedir.

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Ankara Ecz. Fak. Derg. / J. Fac. Pharm. Ankara, 49(3): 888-899, 2025
Doi: 10.33483/jfpau.1621281

ÖZGÜN MAKALE / ORIGINAL ARTICLE

ECZACILIK FAKÜLTESİ ÖĞRENCİLERİNİN NİKOTİN İÇERİKLİ ÜRÜNLERİ TÜKETİMİNİN ARAŞTIRILMASI

INVESTIGATION OF NICOTINE CONTAINING PRODUCTS CONSUMPTION BY PHARMACY STUDENTS

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ÖZ

Amaç: *Piyasada, nikotin içerikli (sigara, nargile, e-sigara, puro, sarma tütün ve tek dolumlu e-sigara) çok fazla ürün bulunmaktadır ve bu ürünlerin sayısı ve çeşidi her geçen gün artmaktadır. Son zamanlarda nikotin içerikli ürünlere başlama yaşının giderek düşmesi ve özellikle gençlerin büyük bir kısmını oluşturan üniversite öğrencilerinin bu ürünleri tüketmesi, gençlere yönelik tedbirler oluşturulmasının ve piyasada yer alan nikotin içerikli ürünlere dikkat çekilmesi gerektiğini göstermektedir.*

Gereç ve Yöntem: *Çalışmamızda Mersin Üniversitesi Eczacılık Fakültesi öğrencilerinin nikotin içerikli hangi ürünleri tükettikleri ve bu ürünleri tüketim alışkanlıkları araştırılmıştır. Çalışmamız kapsamında 05.02.2024-24.05.2024 tarihleri arasında Mersin Üniversitesi Eczacılık Fakültesi öğrencilerinden 246 kişiye Google Formlar üzerinden çevrimiçi bir anket uygulanılmıştır. Ankete katılan Mersin Üniversitesi Eczacılık Fakültesi öğrencilerine nikotin içerikli hangi ürünü ne kadar sıklıkla tükettikleri sorulmuştur.*

Sonuç ve Tartışma: *Ankete katılan 246 öğrencinin 116'sı (%47.2) nikotin içerikli ürünlerden birini en az bir kez kullandığını ifade ederken, 130'u (%52.8) nikotin içerikli ürünlerden birini hiç kullanmadığını ifade etmiştir. Nikotin içerikli ürünlerin tercih edilme sırasında sigara (n=69) en yüksek orana sahip iken diğer ürünlerin nargile (n=27), sarma tütün (n=20), tek dolumlu e-sigara ürünleri (n=18) ve e-sigara (n=11) olduğu görülmektedir. Çalışmamızda değişkenler arasında arasında anlamlı bir ilişki olduğu tespit edilmiştir ($X^2(1)=15.694$, $p\leq0.05$). Ayrıca çalışmamızda nikotin içerikli ürünleri tüketen öğrenci sayısının tüketmeyene oranla daha az olduğu görülmektedir. Nikotin içerikli ürünlerinin üniversite öğrencileri tarafından hala tüketiliyor olmasının nedenleri arasında stres, tadını sevme, alışkanlık, arkadaş ortamı gibi sebepler sayılmaktadır. Çalışmamızın üniversite öğrencilerinin nikotin içerikli ürünleri tüketim alışkanlıklarına bir kaynak oluşturabileceği düşünülmektedir.*

Anahtar Kelimeler: *Eczacılık öğrencileri, e-sigara, nargile, nikotin tüketim, sigara*

ABSTRACT

Objective: *There are many nicotine containing products on the market (cigarette, hookah, cigar, e-cigarette, rolled tobacco, and disposable e-cigarette), and the number and variety of these products are increasing day by day. The fact that the age of starting nicotine containing products has been decreasing recently and that university students, who constitute a large proportion of young people, consume these products, shows that measures should be taken for young people and attention should be drawn to nicotine containing products in the market.*

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Gönderilme / Submitted : 16.01.2025
Kabul / Accepted : 25.05.2025
Yayınlanma / Published : 19.09.2025

Material and Method: *Our study examined which nicotine containing products are consumed by the students of the Mersin University's Faculty of Pharmacy and their consumption habits of these products were investigated. Within the scope of our study, an online survey was applied to 246 students of Mersin University's Faculty of Pharmacy between 05.02.2024-24.05.2024 via Google Forms. The students of the Mersin University's Faculty of Pharmacy who took part in the survey were asked which nicotine containing product they used and how often they used it.*

Result and Discussion: *Out of the 246 students who participated in the survey, 116 (47.2%) reported having used at least one nicotine containing product, while 130 (52.8%) stated that they had never used any nicotine containing products. The preferred nicotine containing products were reported as follows: cigarettes (n=69), hookah (n=27), rolling tobacco (n=20), disposable e-cigarettes (n=18), and e-cigarettes (n=11). In our study, a significant correlation was found between the variables ($X^2(1)=15.694$, $p\leq0.05$). According to our study, students who use nicotine products are less likely than those who do not. The reasons why nicotine containing products are still consumed by university students include stress, liking the taste, habitual friend environment. It is thought that our study may be a source for the consumption habits of nicotine containing products by university students.*

Keywords: *Cigarette, e-cigarette, hookah, nicotine consumption, pharmacy students*

GİRİŞ

Nikotin, patlıcangiller (*solanaceae*) familyasından *"nicotiana"* cinsi bir bitki türüdür. *"Nicotiana Tabacum" ve "Nicotiana Rustica", "Nicotiana rustica"* isimli bitkiler endüstriyel yöntemler kullanılarak sigara, puro, pipo gibi ürünler için tütün yapımında kullanılmaktadır. Nikotin yıllar boyunca insanlar tarafından tüketilen ve psikoaktif maddeler arasından bağımlılık oluşturma özelliği kuvvetli olan bir madde olarak bilinmektedir [1]. Nikotin, lipolifik ve küçük molekül yapısına sahip bir kimyasal olmasından dolayı cilt ve mukozalardan kolayca, hızla emilmektedir [1-3]. Nikotin içerikli ürünlerden sigara, sarma tütün, puro, pipo yaygın kullanıma sahip ürünler arasında olsa da son zamanlarda bu ürünlere ilaveten nargile, elektronik sigara (e-sigara) ve tek dolumlu e-sigara gibi nikotin içerikli yeni nesil ürünler de karşımıza çıkmaktadır. Dünyada ve ülkemizde nikotin içerikli ürünlerden en çok ve en sık tüketilen ürün sigara olduğu bilinmektedir [4]. Sigara tütünün içerisinde yaklaşık %0.5-8 oranında nikotin bulunmaktadır. Dünya Sağlık Örgütü'ne (DSÖ) göre 15 yaş üstü yetişkinlerde sigara kullanma yaygınlığı dünya genelinde %26.0, Avrupa Bölgesi'nde %35.3 olarak bulunurken, Türkiye'de ise 2012 yılı Küresel Yetişkin Tütün Araştırması (KYTA) sonuçlarına göre sigara kullanma yaygınlığı erkeklerde %41.4, kadınlarda %15.2 oranında bulunmuştur [4,5]. Küresel Gençlik Tütün Araştırması'nın (KGTA), 2009-2012-2017 yıllarında yaptığı araştırmada ise Türkiye'de 13-15 yaş arası sigara kullanan gençlerin oranı en yüksek 2012 yılında kızlarda %12 oranında bulunmuştur. Ayrıca KGTA'nın 2017 yılında yaptığı araştırmada en az 1 defa tütün ürünü deneyenlerin sayısı %40 olarak bulunmuştur [6]. Türkiye'de üniversite öğrencileri arasında sigara içme sıklığı ise %20 ile %48 arasında değişmektedir ve üniversitede okunan yıl arttıkça, sigara içme oranı da giderek artmaktadır [7-9]. Sigara tüketimine bağlı iskemik kalp hastalıkları, kanser türleri, inme, solunum yolu hastalıkları, kardiyovasküler rahatsızlıklar ve diş eti hastalıkları gibi pek çok kronik hastalık bulunmaktadır [10]. Sigara kullanımının, bağışıklık sistemine zarar verdiği ve enfeksiyonlara karşı vücudun direncini azalttığı belirtilmektedir. Akciğer kanserinin %80'i sigara kullanımına bağlı olduğu bilinmektedir [11]. Sigara kullanımının akciğer kanserinin yanısıra ağız, yemek borusu, farinks, larinks (gırtlak), ağız boşluğu (dil, dudak, yutak, yanak), pankreas, mesane, renal pelvis (böbrek pelvisi), kolorektal (kalın bağırsak ve rektum), sinonazal (burun ve sinüs), adrenal, mide, uterus (rahim), serviks (rahim ağzı) ve karaciğer gibi çeşitli kanser türlerine yol açtığı bilinmektedir. Ayrıca, sigara kullanımının miyeloid lösemi gibi kan kanseri türlerine de katkıda bulunduğu bilinmektedir [12]. Sigara tüketime ek olarak düzenli puro içen bireylerde de akciğer, yemek borusu, gırtlak ve ağız boşluğu kanseri oranının yüksek olduğu görülmüştür [13]. Sarma tütün ve nargile gibi tütün ürünleri üzerine yapılan sağlık araştırmaları sınırlı kalmaktadır. Sigara kullanımı, nargile ve sarma tütün kullanımını teşvik eden bir risk faktörü olarak öne çıkarken, erkek cinsiyetin sarma tütün tercihinde etkili olduğu görülmektedir [14]. Sarma tütünün, genellikle daha düşük maliyetli olması nedeniyle, kullanımı giderek artış göstermektedir. Ekonomik nedenlerin bu tercih üzerinde belirleyici bir rol oynadığı gözlemlenmektedir [15]. Örneğin, 1930'larda ABD'de hazır sigara

tüketiminde azalma yaşanırken, sarma tütün kullanımında belirgin bir artış kaydedilmiştir [16]. Son on yılda tütün ve sigara endüstrisi, hükümetlerin sigaraya karşı aldığı önlemlere karşı piyasaya yeni ürünler sunma çabası içerisinde olmuş ve bu yeni ürünler arasında da en popüler ürün e-sigara olmuştur [17]. E-sigaralar literatürde Elektronik Nikotin İletim Sistemi (Electronic Nicotine Delivery System) olarak adlandırılmaktadır [18]. E-sigaralar, tipik olarak bir pil, ısıtma elemanı ve nikotin içeren sıvı için bir bölme içerir. Sıvı ısıtılır ve solunabilen bir aerosol üretir. E-sigara olarak adlandırılan ürünlerin çeşitliliği her geçen gün artmakta ve farklı ürünler bu yelpazeye dahil olmaktadır. E-sigara türleri farklı mekanizmalarla (ateşlemeli, ısıtmalı vb.) 4 nesilde, nikotin bağımlılarına piyasada sunulmaktadır [19]. Yeni e-sigara türlerinin çoğunda yakma yerine ısıtma kullanılmakta, bazılarında nikotin sıvıları kullanılmakta, bazılarında ise sigaraya benzer şekilde özel olarak hazırlanmış tütünün kendisi kullanılmaktadır. E-sigara türlerinin en son versiyonu olan 4. nesil e-sigara türü tek dolumlu e-sigaralar, FDA (Food and Drug Agency) düzenlemelerinden kaçarak son yıllarda popülerliğini artırmış ve birçok farklı isim ile piyasada yer almaktadır [19,20]. E-sigaralar başlangıçta yetişkin tüketiciler için tasarlanmış bir cihaz olsa da özellikle tek dolumlu e-sigaralar gençler arasında oldukça yaygınlaşmıştır. Tek dolumlu e-sigaraların ambalajlarının ve tatlarının çekici olması, sosyal medya etkisi, COVID-19 salgını, tek dolumlu e-sigaralar popülaritesinin artmasına büyük ölçüde katkıda bulunan faktörlerdir [21]. Tek dolumlu e-sigaraların popülerliği göz önünde bulundurulduğunda, nikotin içermesi, içeriklerinin bilinmezliği ve denetlenmeyen bir ürün olması nedeniyle ergen gelişimi ve sağlığı için bir tehdit oluşturmaktadır [22]. Keyif verici ve bağımlılık yapıcı nikotin içerikli ürünleri, özellikle gençlerin büyük bir kısmını oluşturan üniversite öğrencilerinin tükettiği görülmektedir [23]. Bu ürünleri kullanma nedenleri arasında aile ortamından uzaklaşma, yeni girdiği ortamda kendini kabul ettirme isteği, arkadaş ortamında oluşan teşvik, taklit, merak duygusu, gelecek kaygısı veya korkusu yer almaktadır [23]. Bu kapsamda nikotin içerikli ürünlerinin tüketilmesi açısından önemli bir risk grubu olarak üniversite öğrencileri karşımıza çıkmaktadır. Ayrıca nikotin içerikli ürünlerin piyasa formlarının sürekli değişmesi ve bu ürünlerin bilinirliğinin analiz edilebilmesi açısından da üniversite öğrencileri önemli bir gösterge olacaktır. Bu yüzden, çalışmamızda Eczacılık Fakültesi öğrencilerinin nikotin içerikli ürünleri tüketim alışkanlıkları ve kullandıkları ürün formlarının ortaya konulması amaçlanmıştır.

GEREÇ ve YÖNTEM

Çalışmamız kapsamında Mersin Üniversitesi Eczacılık Fakültesi öğrencilerine 05.02.2024-24.05.2024 tarihleri arasında bir anket uygulanmıştır. Anket formunda 40 adet soru (bulunmaktaydı) belirlenmiştir. Anket formunda çalışmaya katılan Eczacılık Fakültesi öğrencilerinin demografik özeliklerinin belirlenmesine (yaş, cinsiyet, kaçıncı sınıfta olduğu, üniversite hayatındaki konaklama şekli ve aile iletişimi); nikotin içerikli ürünlerin (sigara, nargile, e-sigara, puro, sarma tütün ve tek dolumlu e-sigara) tüketim akışkanlıklarının belirlenmesine (tüketim durumu ve hangi sıklıkta tükettiği) ve nikotin içerikli ürünler hakkında bilgi sahip olup olmadıklarının belirlenmesine yönelik sorular yer almaktadır. Çalışmamız kesitsel bir çalışma olup, evreni bilinen örneklem büyüklüğü hesabına göre %95 güç ve %5 hata marjini ile en az 239 öğrencinin çalışmaya alınması planlanmış olup 246 öğrenci katılmıştır. Örneklem büyüklüğü hesaplaması https://www.openepi.com sitesinde yapılmıştır. Anket uygulaması için Helsinki Deklarasyonu prensiplerine uygun olarak etik kurul onayı Mersin Üniversitesi Tıp Fakültesi Girişimsel Olmayan Etik Kurulundan (13/12/2023 tarihli ve 2023/860 sayılı) alınmış olup ankete katılan bütün katılımcılardan bilgilendirilmiş onamları alınmıştır. Ayrıca veri toplama aracı olarak Google Formlar üzerinden çevrimiçi bir anket uygulanılmıştır. Gönüllülerin çalışmaya dahil edilme kriterleri; katılımcıların 18-28 yaş arasında yer alan Mersin Üniversitesi Eczacılık Fakültesi öğrencileri olması, 05 Şubat 2024 ile 14 Haziran 2024 tarihleri arasında bilgilendirilmiş gönüllü olur formu'nu imzalaması ile anket formu dolduran gönüllüler ile sınırlandırılmıştır. Dahil edilmeme kriterleri ise gönüllünün Mersin Üniversitesi Eczacılık Fakültesi öğrencisi olmaması, çalışmaya katılmak istememesi, bilgilendirilmiş gönüllü olur formunu imzalamaması olarak belirlenmiştir. İstatistiksel yöntemde kullandığımız kategorik değişkenler (sürekli olmayan değişkenler) sayı ve yüzde ile devamlı değişkenler ortalama ± standart sapma ile elde edilmiştir. Kategorik değişkenler arasındaki ilişki ki kare testi kullanılarak değerlendirilmiştir. Hesaplamalar SPSS (versiyon 10.0) istatistik paket programı ile yapılmıştır.

SONUÇ VE TARTIŞMA

Çalışmamızda Mersin Üniversitesi Eczacılık Fakültesinde öğrenim gören 246 öğrencinin anket formları değerlendirmeye alınmıştır. Çalışmaya katılan 246 öğrencinin sosyo-demografik özelliklerinin verildiği Tablo 1'de verilmiştir. Tüm katılımcılarımızın yaş ortalaması 22.19 ± 2.7 yıl olarak görülürken, erkeklerde 22.48 ± 2.02 yıl, kadınlarda 22.37 ± 2.36 yıl olarak görülmüştür. Ankete katılan öğrencilerin gözlemlenen en yaygın yaş grubu 22 yaş (%22.8) olduğu görülmüştür.

Katılımcıların sosyo-demografik özelliklerine bakıldığında, yaş dağılımı açısından %22.3'ünün 18–20 yaş aralığında, %67.9'unun 20–25 yaş aralığında ve %9.8'inin ise 25 yaş ve üzeri olduğu belirlenmiştir. Cinsiyet bakımından katılımcıların %64.2'si kadın, %35.8'i erkektir. Sınıf düzeyine göre incelendiğinde; %12.2'si 1. sınıf, %18.3'ü 2. sınıf, %14.2'si 3. sınıf, %26.4'ü 4. sınıf ve %28.9'u 5. sınıf öğrencisidir. İkamet yeri olarak katılımcıların %56.5'inin devlet yurdunda, %20.7'sinin aile yanında ve %22.8'inin ise tek başına evde kaldığı saptanmıştır. Aile içi iletişim düzeyleri değerlendirildiğinde ise %72.8'inin iletişiminin iyi, %24.4'ünün orta düzeyde ve %2.8'inin kötü olduğu görülmüştür.

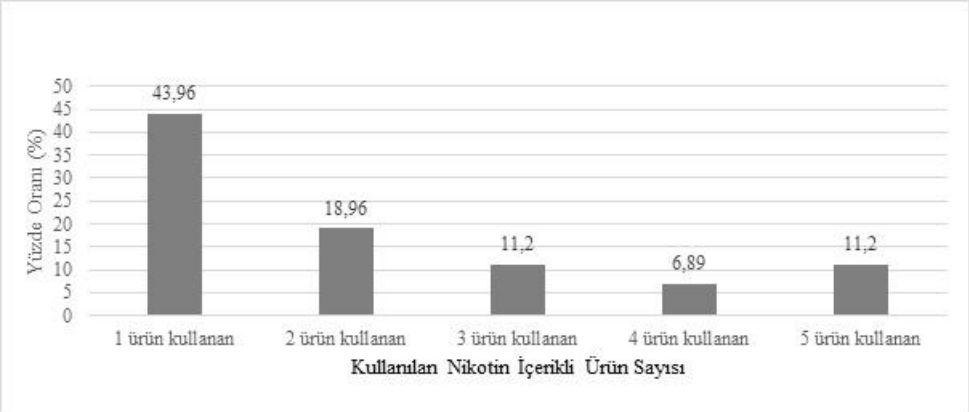
Ankete katılan 246 öğrencinin 116'sı (%47.2) nikotin içerikli ürünlerden birini *en az 1 kez* tükettiğini ifade ederken, 130'u (%52.8) nikotin içerikli ürünlerden birini *hiç tüketmediğini* ifade etti. Ankete katılan 116 kişinin hayatı boyunca nikotin içerikli hangi ürün/ürünleri tercih ettiği görülmektedir. Bu bireyler hayatlarının bir döneminde bu ürünleri *en az 1 kez* tüketmişlerdir. Öğrencilerin en çok tükettiği ürünün sigara (%97.2) olduğu görülmekte olup ikinci sırayı nargilenin (%52) aldığı görülmektedir. E-sigaralar (%24.7) ve tek dolumlu e-sigaralar (%25.1) yeni bir nikotin tüketim aracı olmasına rağmen büyük bir orana sahip olduğu görülmektedir. Sarma tütün eskiden beri tercih edilen bir ürün olup (%27) hala e-sigara türlerine göre daha fazla tüketildiği görülmektedir. Ayrıca ankete katılan öğrencilerin %31'i hayatının bir döneminde puro denediği görülmektedir.

Tablo 1. Kullanılan nikotin içerikli ürün ile cinsiyet arasındaki ki-kare analiz sonuçları

Cinsiyet					X^2	*SD*	*p*
	Erkek		Kadın				
Kullanılan ürün	**Evet %**	**Hayır %**	**Evet %**	**Hayır %**			
Sigara	61.9	38.1	35.3	64.7	15.694	1	0.000
Nargile	41.7	58.3	10.3	89.7	32.190	1	0.000
E-sigara	20.2	79.8	4.5	95.5	15.051	1	0.000
Tek dolumlu e-sigara ürünleri	15.5	84.5	9.6	90.4	1.820	1	0.177
Sarma tütün	23.8	76.2	3.2	96.8	24.840	1	0.000

SD: serbestlik derecesi, $p \leq 0.05$

Tablo 1'ye göre cinsiyet ile nikotin kullanma durumu arasında anlamlı bir ilişki olup olmadığını incelemek için ki-kare bağımsızlık testi yapılmıştır. Değişkenler arasında anlamlı bir ilişki olduğu tespit edilmiştir. $X^2(1)=15.694$, $p \leq 0.05$. Çalışmamıza katılan bireylerin cinsiyeti ile nikotin içerikli ürünleri tercih etmeleri arasında bir ilişki olduğu belirlenmiştir. Tablo 1'ye göre nikotin içerikli ürünlerden tek dolumlu e-sigaranın dışındaki bütün diğer nikotin içerikli ürünler ile cinsiyet faktörü anlamlı bulunmaktadır. Kullanılan ürünlere baktığımız zaman sigara her iki cinsiyette de en yüksek yüzdeye sahip nikotin içerikli ürün olarak karşımıza çıkmaktadır. Ankete katılan öğrencilerin %43.96'sı sadece 1 tane nikotin içerikli ürünü tüketirken, %11'i 5 farklı nikotin ürününü tükettiğini dile getirmiştir (Şekil 1). Öğrencilerin nikotin içerikli ürünleri tüketme yüzdeleri Şekil 1'de gösterilmiştir.



Şekil 1. Nikotin içerikli ürünleri kullanan katılımcıların kullandıkları nikotin içerikli ürünlerin dağılımı

Tablo 2'e göre ankete katılan öğrencilerin nikotin içerikli ürün tüketen katılımcı bilinç düzeyine baktığımız zaman tüketilen ürün içerisindeki nikotin miktarı hakkında bilgi sahibi olmadıkları, tükettikleri her ürünü kendi içerisinde değerlendirdiğimizde ise ürünün oluşturabileceği sağlık riskleri hakkında bilgi sahibi oldukları görülmektedir. 2018 yılında yapılan bir çalışmada, Eylül 2014 - Haziran 2015 tarihleri arasında, bir üniversitenin sağlık bilimleri ve sosyal bilimlerinde öğrenim gören ve araştırmaya katılmayı kabul eden 602 öğrenci ile öğrencilerin sigara içme durumu ve sigaranın neden olduğu hastalıkları bilme durumu anket formu ile incelenmiştir. Çalışmaya katılan öğrencilerin %20.6'sı sigara içmekte olup, öğrencilerin %97.8'i sigaranın kardiyovasküler hastalıklara, %97.0'sı akciğer kanserine, %95.3'ü gırtlak kanserine, %94.9'u serebrovasküler hastalıklara, %92.7'si kronik obstrüktif akciğer hastalığına sebep olduğunu bildiğini belirttiği görülmüştür [24].

Tablo 2. Nikotin içerikli ürün tüketen katılımcıların bilinç düzeyi

	Ürünlerin nikotin miktarı hakkında bilgi sahibi olma N (%)		**Tüketiminin insan sağlığında oluşturabileceği riskler hakkında bilgi sahibi olma N (%)**	
	Evet	**Hayır**	**Evet**	**Hayır**
Sigara	8 (11.6)	61 (88.4)	69 (100)	0 (0)
Nargile	0 (0)	27 (100)	26 (96.2)	1 (3.8)
E-sigara	0 (0)	11 (100)	9 (81.8)	2 (18.2)
Tek dolumlu e-sigara ürünleri	0 (0)	18 (100)	16 (88.9)	2 (11.1)
Sarma tütün	0 (0)	20 (100)	18 (90)	2 (10)

Nikotin içerikli ürünler geniş bir ticari yelpazeye sahiptir ve başta gençler olmak üzere geniş halk kitlelerine ulaştığı için nikotin tipi bağımlılık tüm toplumlarda ilk sırada yer alan bağımlılık türü olarak karşımıza çıkmaktadır. Bu bağımlılığın insan sağlığı için önemli olmasının sebebi başta akciğer kanseri olmak üzere pek çok kanser türü, solunum yolu hastalıkları, kalp damar hastalıkları, üreme sağlığı problemleri gibi pek çok önemli hastalığa sebep olmaktadır [25]. Bu hastalıkların gelişmesinde ise, başta sigara olmak üzere birçok nikotin içerikli ürün içerisinde yer alan on bine yakın farklı kimyasal etkin olmaktadır [25]. DSÖ'nün raporuna göre 15 yaş ve üstü kişiler arasında mevcut tütün içme oranları incelendiğinde yaklaşık %29.3 belirtilmektedir [26]. Türkiye İstatistik Kurumu (TUİK)'in açıkladığı 2019 yılı "Türkiye'de Sağlık Araştırması" verilerine bakıldığında 15 yaş ve üzeri tütün kullanan bireylerin oranı %28 olarak belirtilmiş olup oranın %41.3'ü erkek, kadınlar ise %14.9 olarak belirtilmektedir [27]. Tablo 3'e göre nikotin içerikli ürün kullanan bireylerin tüketim sıklıkları kullanıcılara göre farklılık göstermektedir. Sigaraya başlama yaşı olarak yaklaşık her 3 kişiden birinin (%31.9) 18 yaş ve daha küçük yaşta başlaması ve nerdeyse tamamının 24 yaş ve daha küçük yaşta

(%94.8) olması dikkat çekmektedir. Sigara kullanan bireylerin %75.4'ünün ailesinde sigara kullanımı görülmez iken nargile kullanan bireylerin %88.9'unun ailesi nargile kullanmadığı görülmektedir. Ayrıca nargile kullanan bireylerin %96.3'nün nargile kafede ortak nargile kullanarak nargile tükettiği görülmektedir. Ankete verilen cevaplar doğrultusunda, nargile ve sigara tüketiminde çevresel faktörlerin etkilediği görülmektedir. Ankete katılan bireylerin tüketim sebeplerine baktığımız zaman sigarada stres ve alışkanlık (%38), nargilede tadını sevme (%66.7) ön plana çıkarken e-sigarada ise tadını sevme (%72.7) parametreleri en üst sırada yer almaktadır. Çalışmamız verilerine göre ankete katılan üniversite öğrencilerinin aile iletişimi yüksek olsa da nikotin içerikli ürünleri tüketme alışkanlıklarını belirleyen en önemli faktörler arasında stres yer almaktadır. E-sigara ve tek dolumlu e-sigara ürünleri tüketiciler için yeni ve ilgi çekici olsa da hala en çok tüketilen nikotin içerikli ürünün sigara olduğu görülmektedir. E-sigara'da ise en çok likit ürünler kullanılmakta olup bu ürünlerin tüketimi, yeni nesil e-sigara türü olan tek dolumlu e-sigara ürünlerinin tüketimine ortam sağlamaktadır. Sigara tüketimi ile ilgili yapılan çalışmalara baktığımızda, 2013-2014 eğitim-öğretim döneminde aktif öğrenci olan toplam 332 öğrenciden 197'sinin (%59.33) katıldığı bir anket çalışmasında, üniversite öğrencilerinin sorumluluklarla baş etme, kendini mutlu hissetme, boş zamanlarını doldurma, üniversiteye verilen ücretin sorumluluğu gibi sebeplerden dolayı sigara içtikleri sonucuna ulaşılmıştır. Yapılan anket çalışması sonuçlarına göre; sigara kullanımında ve alışkanlık haline gelmesinde etken olarak stres ve arkadaş ortamı ön planda olduğu sonucuna varılmıştır [28]. 2017 yılında 300 üniversite öğrencisi ile yapılan bir çalışmada elde edilen verilere uygulanan binary lojistik regresyon analizi sonucuna göre üniversite öğrencilerinin sigara tüketimine cinsiyet, yaş, sınıf, yakın arkadaş çevresi, ailedeki bireylerin sigara içmesi, prestij, köken, sosyal olduğunu düşünme ve stres faktörlerinin etkili olduğu görülmüştür [29]. Bozkurt vd. tarafından 2022 yılında yapılan bir çalışmaya katılan 191 üniversite gençliğinde ciddi düzeyde nikotin bağımlılığı ve pasif sigara maruziyeti saptanmış olup öğrencilerde saptanan öksürük, nefes darlığı şikâyetlerinin, bu durumdan olumsuz etkilendiklerini göstermektedir [30]. 2019 yılında yapılan çalışmaya katılan üniversite öğrencilerinin günde 15 (10-20) dal sigara içmekte olduğu ve %54.3'ünün babası ve %62.3'ünün yakın arkadaşının sigara içicisi olduğu görülmektedir. Yapılan nikotin bağımlılık testine göre %31.4'ü orta ve %13.4'ü ise yüksek düzeyde bağımlı olduğu görülmüştür. Yüksek bağımlı olan üniversite öğrencilerinin babası ve yakın arkadaşının sigara içiciliğinin daha yüksek olduğu görülmüştür (p<0.05) [7]. 2022 yılında, bir vakıf üniversitesindeki öğrencilerin sigara kullanımı, nikotin bağımlılık düzeyi ve ilişkili faktörlerin incelenmesi için 100 öğrencinin katıldığı ve %77.3'ü (n=85) sağlık bilimleri yüksekokulu, %22.7'si (n=25) mühendislik-mimarlık fakültesi öğrencisi olduğu bir çalışmada sigara kullanım sıklığının %35.5 (n=39), fagerström nikotin bağımlılığı testi (FBNT) puan ortalamasının 3.54±2.70 olduğu bulunmuştur. Sağlık bilimleri yüksekokulu erkek öğrencilerinin ve ailede sigara kullanımı olanların sigara kullanım durumlarının daha yüksek olduğu sonucuna ulaşılmıştır [31]. 2021 yılında üniversite öğrencilerinde tütün kullanım alışkanlıklarının belirleyicileri ve algılar üzerine yapılan sistematik bir derleme çalışmasında tütün alışkanlıkları ile ilgili 102 adet çalışma tespit edilmiş olup 56 çalışma değerlendirmeye alınmıştır. Bu çalışmalarda üniversite öğrencilerinin nikotin kullanım alışkanlıkları çoğunlukla sigara üzerinden değerlendirilmiş olup ikinci sırayı 15 çalışma ile üniversite öğrencilerinin nargile tüketim alışkanlıklarının araştırılması almıştır. Ayrıca aynı çalışmada yer alan verilere göre, öğrencilerin sigara kullanım oranları 5 yıllık hareketli ortalamalar ile değerlendirildiğinde, sigara kullanım oranında genel bir düşüş eğilimi olduğu görülmektedir [32]. 2024 yılında yapılan bir çalışmada, 2022-2023 akademik güz döneminde Türkiye'nin güney bölgesinde yer alan bir vakıf üniversitesinin sağlık bilimleri fakültesi ve meslek yüksekokuluna kayıtlı 802 öğrenciye uygulanan veri toplama işlemi ve FBNT sonucunda araştırmaya katılan öğrencilerin %20.8'inin sigara kullanıcısı olduğu ve %71.9'unun sigara kullanımına 16 yaşından sonra başladığı tespit edilmiştir. Sigara kullanıcılarının %34.1'i çok hafif derecede bağımlı, %35.9'u hafif-orta derecede bağımlı, %30.0'ı ise yüksek ve çok yüksek derecede bağımlı olarak sınıflandırılmıştır. Bu çalışmada yapılan ikili lojistik regresyon analizinden elde edilen sonuçlar göz önüne alındığında, erkek olmak, parçalanmış bir aileye sahip olmak ve orta veya düşük akademik başarıya sahip olmak öğrencilerin sigara kullanımını etkileyen faktörler olarak belirlenmiştir [33].

Tablo 3. Nikotin kullanan katılımcıların tükettikleri nikotin içerikli ürünlerin dağılımı

SİGARA	**(N=69)** **N (%)**	**NARGİLE**	**(N=27)** **N (%)**
Tüketim sıklığı		**Tüketim sıklığı**	
Günde 1 paketten fazla	5 (7.2)	Haftada 2 kez	5 (18.5)
Günde 1 paket	21 (30.4)	Ayda 2 kez	4 (14.8)
Günde yarım paket	8 (11.4)	Ayda 1 kez	4 (14.8)
Günde 1-3 tane	21 (30.4)	Yılda birkaç kez	12(44.5)
Nadiren	14 (20.6)	Yılda 1 kez	2 (7.4)
Tüketim sebebi*		**Tüketim sebebi***	
Stres	38 (55.1)	Stres	2 (7.4)
Alışkanlık	38 (55.1)	Tadını sevme	18 (66.7)
Tadını sevme	16 (23.2)	Keyif verici	3 (11.1)
Diğer	15 (21.5)	Arkadaş ortamı	4 (14.8)
Kullanmaya başlama yaşı		**Tüketim yeri***	
18 yaş altı	37 (31.9)	Evde	5 (18.5)
18-20	49 (42.2)	Nargile kafede	26 (96.3)
21-24	24 (20.7)		
24 yaş üstü	6 (5.2)		
		Nargile takımı	
Ailede sigara kullanımı		Kendime ait	4 (14.8)
Evet	17 (24.6)	Ortak	23 (85.2)
Hayır	52 (75.4)	**Ailede nargile kullanımı**	
		Evet	3 (11.1)
		Hayır	24 (88.9)
E-SİGARA	**(N=11)** **N (%)**	**TEK DOLUMLU E-SİGARA ÜRÜNLERİ**	**(N=18)** **N (%)**
Tüketim sıklığı		**Tüketim sıklığı**	
Günde3-5 saat	1 (9.09)	Günde 20 nefesten fazla	3 (16.6)
Haftada 3-5 saat	4 (36.6)	Günde 10-15 nefes	4 (22.2)
Nadiren	6 (54.4)	Haftada 10-15 nefes	1 (5.6)
		Ayda 10-15 nefes	8 (44.5)
		Nadiren	2 (11.1)
Tüketim sebebi*		**Satın alınma yeri**	
Stres	3 (27.7)	Tekel bayii	6 (33.3)
Alışkanlık	2 (18.1)	Dükkan/Market	6 (33.3)
Tadını sevme	8 (72.7)	İnternet	2 (11.1)
		Yurtdışı	3 (16.7)
		Arkadaş	1 (5.6)
Kullanılan Tür*			
Likit e-sigaralar	9 (81.8)		
İçten yanmalı	2 (18.2)		
SARMA TÜTÜN	**(N=20)** **N (%)**		
Tüketim sıklığı			
Günde 5-15 Adet	4 (20.0)		
Haftada 10-15	6 (30.0)		
Ayda 10-15	10 (50.0)		
Satın alınma yeri			
Tekel bayii	6 (30.0)		
Dükkan/market	12 (60.0)		
Yurt dışı	2 (10.0)		

*Katılımcılar birden fazla şık işaretleme seçeneği bulunmaktadır

Nikotin içerikli diğer ürünlerde sigara kadar dikkat çekmeye başladığı görülmektedir. Yapılan bir çalışmada sigara tüketmeyen ve alternatif nikotin/tütün ürünü tüketenlerin dağılımına bakıldığında 89 kişi nargile, 17 kişi e-sigara ve 1 kişi Puro kullanmaktadır [34]. Sigaraya ek olarak en çok denenen ve tüketilen nikotin içerikli ürün nargile olduğu tespit edilmiştir. Tütün ürünü içerikli alternatif ürünlerin sigaraya göre zarar düşüncesine ilişkin verilerde dikkat çeken bir husus, toplumun ısıtılmış tütün ürünleri konusunda bilgi düzeyinin çok düşük düzeyde olduğu bilinmektedir. [34]. Yapılan çalışmada içten yanmalı tütün ürünlerinin sigaraya göre zararlı olup olmadığı konusunda 890 kişi (%63.6) "bilgim yok" cevabı verilmiştir. Bu durumun zarar algısından ziyade içten yanmalı tütün ürünleri hakkında bilgi eksikliğinden kaynaklandığı düşünülmektedir [34]. Bizim çalışmamızda da nikotin içerikli ürünleri tüketen katılımcıların bilinç düzeyine bakıldığında, sadece sigara içerisindeki nikotin miktarı hakkında 8 kişi bilgi sahibi iken diğer ürünler hakkında bilgi sahibi değildir. Ayrıca çalışmamıza katılan üniversite öğrencilerinin kullandıkları ürünün oluşturduğu sağlık riski hakkında bilgi sahip olmama yüzdelerinin daha yüksek olduğu görülmüştür.

Nargile, Amerikan ve Avrupa pipolarının aksine kolayca taşınabilir olmayan ve kahvehanelerde kullanıldığı için ortak bir sosyal deneyim sağlayan nikotin içerikli bir üründür. Jackson ve Aveyard (n=937 öğrenci) tarafından yapılan bir çalışmada, 355 (%37.9) öğrencinin nargile denediği, 75'inin (%8.0) düzenli sigara kullanıcısı olduğu görülmüştür. Düzenli nargile içen 21 kişiden 7'si (%33.3) nargile tüketimine karşı bir arzulama/bağımlılık yaşadığını belirtmiştir. Düzenli nargile kullanıcılarının neredeyse tümü nargile içmenin, sigara içmekten daha az zararlı olduğunu düşünmektedir [35]. Jaffri ve arkadaşlarının 2012 yılında Pakistan Karaçi'de Kolej ve Üniversitelerde Nisan-Ekim 2009 ayları arasında kesitsel anket çalışmasına 422 üniversite öğrencisi katılmış olup, nargile kullanma oranının %45.2 olduğu bulunmuştur [36]. Poyrazoğlu ve arkadaşlarının 2010 yılında 645 Tıp ve Mühendislik Fakültesi öğrencisi ile yaptıkları araştırmada nargile kullanma oranı %32.7 olarak bulunmuştur. Bu oranın %20.2'sini kız, %41.6 ise erkekler oluşturmaktadır [37]. Nargile tüketimi ile ilgili bir devlet üniversitesinin tıp ve güzel sanatlar fakültesi öğrencisi olan toplam 156 öğrencinin katıldığı bir çalışmada ise erkeklerin (%48.8) kadınlara (%45.9) oranla daha yüksek oranda nargile içtiği görülmekte olup, nargile tüketenlerin çoğunluğunun Güzel Sanatlar Fakültesi öğrencisi (%67.7) olduğu görülmüştür (p <0.001). Nargile içen bireylerin %76.3'ü aynı zamanda sigara da içtiği görülmüştür [38]. Ayrıca, nargile tüketimi incelendiğinde son 20 yılda nargile tüketiminin arttığı ve nargile tüketiminin sigara tüketimini de arrtırdığı görülmüştür. Çalışmalarda nargile içme oranlarının yıllar bazında artmasında; aromalı tütün ürünlerinin piyasaya sürülmesinin, artan nargile kafeler, gençlerin nargilenin sağlık üzerindeki potansiyel zararları konusunda yeterince bilgiye sahip olmamasının, sosyal ortama uyum çabası ve nargile içimini özendirici reklamlar gibi etkenlerin etkili olduğu belirlenmiştir [38]. Bizim çalışmamıza katılan 246 Eczacılık Fakültesi öğrencisinden ise 27 öğrenci nargile tüketmekte olup, katılımcıların %66.7'sinin sevdiği için nargile tükettiği görülmüştür. Nargile tüketen öğrencilerin, %41.7'sinin erkek %10.3'ün ise kız öğrencilerden oluştuğu görülmektedir. Ayrıca nargile tüketen katılımcıların tamamı kullandığı ürünün içerdiği nikotin miktarı hakkında bilgi sahibi değilken, %96.2'si İnsan sağlığında oluşturabileceği riskler hakkında bilgisi bulunmaktadır. Bu sebeple hem bizim hem de diğer çalışmaların da gösterdiği üzere nargile tüketim oranları azımsanmayacak düzeyde olup nargile, halk sağlığı için potansiyel bir tehdit olduğu göz ardı edilmemelidir. E-sigaralar, sigaraya oranla daha sağlıklı alternatif iddiası ile sigarayı bırakmak ve sigara tüketimini azaltmak için piyasaya sürülmüş olup bunun yanı sıra sigara yasaklarına takılmadan "her yerde içilebilir" olması iddiası ile de kullanıcıyı cezbetmiştir. Ancak halk sağlığı üzerindeki etkisi hala kanıtlanabilmiş değilken pazara girişi hızlı olmuştur.

Yapılan çalışmalarda e-sigaraların %7'ye kadar nikotin içerdiği görülmektedir [39]. E-sigaranın içerisinde sıvı nikotin konsantrasyonları yüksek olması nedeni ile akut toksisite riski oluşmaktadır. Ayrıca bu ürünlerin tüketimlerinin bağımlılık oluşturma potansiyeli de önemli bir konu olarak karşımıza çıkmaktadır. E-sigaraların solüsyonlarında ve aerosollerinde nikotine ek olarak çeşitli bileşikler tespit edilmiştir. Bunlar arasında diğer tütün alkaloidleri, tütüne özgü nitrozaminler, formaldehit, asetaldehit, akrolein, metaller, polisiklik aromatik hidrokarbonlar (PAH) ve propilen glikol veya gliserin bulunmaktadır [40]. Bu kimyasalların çoğu geleneksel sigara dumanında da bulunmuştur ve bazıları kanser başlangıcıyla ilişkili olabilecek DNA hasarına neden olmaktadır. DNA hasarı, sigara dumanının neden olduğu karsinogenez mekanizmasının merkezinde yer almaktadır. Sigara dumanındaki

kimyasallar doğrudan DNA ile reaksiyona girebilir veya metabolizma sonrasında DNA bazları ve fosfatları ile ek ürünler oluşturabilir [41]. E-Sigaralarda sigara dumanına kıyasla çok daha az kanserojen madde ve önemli ölçüde daha düşük miktarda kanserojen madde bulunur, ancak aynı temel DNA hasarı mekanizması geçerli olabilmektedir. E-sigaralarda bulunan ve inhale edilen çeşitli kimyasalların aerosol ile ilk teması ağız boşluğunda gerçekleşmektedir [42]. Bu sebeple e-sigaralar oral hücreler için toksiktir ve oral kanseri indükleme potansiyeline sahip olabilmektedirler. E-sigaraya maruziyet sonucunda, disbiyoz, inflamasyon, periodontal hastalıklar, diş ve diş eti sağlığının bozulması ve oral mikrobiyomda değişiklikler dahil olmak üzere çeşitli ağız sağlığı etkilerine neden olabilmektedir. E-sigaraya maruz kalmanın ayrıca baş-boyun ve ağız hücreleri üzerinde anormal morfoloji, sitotoksisite, oksidatif stres, azalmış canlılık ve genotoksisite açısından olumsuz etkileri olduğu tespit edilmiştir [42]. 2008'den 2012'ye kadar birçok ülkede e-sigara denemesi incelendiğinde yetişkinler ve ergenler arasında hızlı bir şekilde arttığı görülmüştür. Kore'de gençlerin e-sigara tüketimi 2008'de %0.5 iken, 2011'de %9.4'e; Amerika Birleşik Devletleri'nde 2011'de %3.3 iken 2012'de %6.8'e yükselmiştir. Diğer taraftan hiç geleneksel sigara denemeyen gençlerin de e-sigaraya yöneldiği ve denediği bildirilmiştir [37]. Erinoso ve arkadaşlarının 2021 yılında Nijerya'nın Lagos kentindeki 15-35 yaş arası ergen ve genç yetişkin katılımcılar ile gerçekleştirdikleri kesitsel bir çalışmada, e-sigara tüketiminin yaygınlığı %7.9 olarak bulunmuş ve e-sigaranın arkadaş tüketiminin ile istatistiksel olarak anlamlı olduğu bulunmuştur [43]. Avrupa'da ise, 15 yaş ve üzeri kişiler arasında e- sigara tüketiminin yaygınlığı 2012 yılında %7.2 iken 2014 yılında %11.6 olarak bildirilmiştir. Bununla birlikte e-sigaraya ilişkin zarar algısı ise 2012'de %27.1 iken 2014'te %51.6'ya yükseldiği bildirilmiştir. E-sigara tüketim yaygınlığı ile zarar algısının aynı oranda arttığı görülmektedir. İstanbul ilinde Sağlık Hizmetleri Meslek Yüksekokulunda eğitim gören 307 öğrencinin 41'i (21 kız %9.5, 20 erkek %27) e-sigaralara geleneksel sigaradan daha az zararlıdır cevabı verdiği, 165'inin (124 kız (%56.4), 31 erkek (%41.9) geleneksel sigara ile aynı zarara sahiptir cevabını aldıklarını bildirmiştir [44]. 2020 yılında 1241 lisans öğrencisinin katılımı ile gerçekleştirilen bir çalışmada e-sigara kullanan 9 kişi olduğu görülmüştür [45]. Bizim çalışmamızda ankete katılan öğrencilerden 11 tanesi e-sigara kullandığını belirtirken 18 tanesi ise tek dolumlu e-sigara ürünleri kullandığını belirtti. E-sigara kullanan öğrencilerden 9 tanesi likit e-sigara türünü kullandığını belirtmiştir. E sigara kullanan öğrencilerin % 20.2'si erkek % 4.5'i kız öğrencilerden oluşurken tek dolumlu e-sigara kullanan öğrencilerin ise %15.5'i erkek %9.6'sı kız öğrencilerden oluşmaktadır.

Nikotin, insanlar tarafından yıllar boyunca tüketilen ve psikoaktif maddeler arasında bağımlılık oluşturma özelliği kuvvetli olan bir madde olarak bilinmektedir [1]. Bu kapsamda önemli bir risk grubu olan üniversite öğrencilerinin nikotin içerikli ürünleri tüketim alışkanlıklarının araştırılması ve mevcut durumun ortaya konulmasına ihtiyaç vardır. Günümüze kadar üniversite öğrencileri ile nikotin içerikli ürünlerin tüketiminin araştırılması ile ilgili yapılan çalışmalar incelendiğinde genellikle sigara tüketimi ve az da olsa nargile tüketimi ile ilgili sonuçlar göze çarpmaktadır. Bizim çalışmamızda nikotin içerikli ürünlerden sigara ve nargileye ek olarak diğer ürünlerin de tüketim alışkanlıkları (e-sigara, tek kullanımlık e-sigara ve sarma tütün) incelenmiştir. Özellikle popülerliği giderek artan e-sigaralara dikkat çekilmesi ve üniversite öğrencilerinin farkındalığının ölçülmesi açısından bu çalışma önemli önemli olacaktır. Bunun yanısıra çalışmamızın örneklem büyüklüğünün sadece Mersin Üniversitesi Eczacılık Fakültesi öğrencisi olan 246 kişiyi içeriyor olması ise çalışmamızın sınırlılıkları olarak karşımıza çıkmaktadır. Piyasada, nikotin içerikli (sigara, nargile, e-sigara, puro, sarma tütün ve tek dolumlu e-sigara ürünleri) çok fazla ürün bulunmaktadır ve bu ürünlerin sayısı ve çeşidi her geçen gün artmaktadır [19]. Günümüzde, tüketimi, sınırlamaları ve sağlık etkileri açısından en çok değerlendirilen, yetişkinler ve gençler arasında en popüler olan nikotin içerikli ürün sigaradır. Fakat son zamanlarda nikotin içerikli ürünler sürekli farklı şekillerde piyasaya sürülmekte ve gençlerin daha kolay temin edeceği ürünlere dönüştürülmektedir. Kolay taşınabilir olması ve sigara içilemeyen ortamlarda da tüketilebilmesi açısından e-sigara ve tek dolumlu e-sigara çeşitlerine özellikle risk grubu olan gençler tarafından tüketilmesine dikkat çekilmelidir. Gençlerin bu yeni nesil nikotin içerikli ürünlerin içeriği hakkında bilgi sahibi olmaması özellikle bağımlılık oluşturmasından dolayı toplum sağlığı açısından konuyu daha önemli hale getirmektedir. Son zamanlarda nikotin içerikli ürünlere başlama yaşının giderek düşmesi ve özellikle gençlerin büyük bir kısmını oluşturan üniversite öğrencilerinin bu ürünleri tüketmesi, gençlere yönelik tedbirler oluşturulmasının ve piyasada yer alan nikotin içerikli ürünlere dikkat çekilmesi gerektiğini göstermektedir. Üniversite dönemi gençlerin gelecekteki hayatlarını

planlama açısından önemli bir dönem olmasından dolayı bu çağdaki gençlerde nikotin içerikli ürünlerin tamamının kullanım prevalansını düşürmek amacıyla okullarda ve aynı zamanda toplum genelinde nikotin içerikli ürünlerin sağlık ilişkisine yer verilmeli ve zararlarının toplumsal boyutunu vurgulayan eğitimler düzenlenmelidir. Nikotin içerikli ürünlerin gençler tarafından daha net anlaşılması ve gençlerin konuyla ilgili bilgisizliklerinin giderilmesi ve tutumlarının değiştirilmesi için eğitim/müdahale programları oluşturulmalıdır. Bizim çalışmamızın da nikotin içerikli ürünlerinin tüketilmesinin azaltılmasına yönelik yapılması planlanan bütüncül çalışmalara önemli katkılar sunacağı düşünülmektedir.

YAZAR KATKILARI

Kavram: A.A.Ş.; Tasarım A.A.Ş.; Denetim: A.A.Ş.; Kaynaklar: A.A.Ş.; Malzemeler: - Veri Toplama ve/veya İşleme: A.A.Ş., B.A.; Analiz ve/veya Yorumlama: A.A.Ş., B.A.; Literatür Taraması: A.A.Ş., B.A.; Makalenin Yazılması: A.A.Ş., B.A.; Kritik İnceleme: A.A.Ş.; Diğer: -

ÇIKAR ÇATIŞMASI BEYANI

Yazarlar bu makale için gerçek, potansiyel veya algılanan çıkar çatışması olmadığını beyan ederler.

ETİK KURUL ONAYI

Bu çalışma için Mersin Üniversitesi Tıp Fakültesi Girişimsel Olmayan Etik Kurulundan (13/12/2023 tarihli ve 2023/860 sayılı) onay alınmıştır.

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J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 49(3): 900-914, 2025
Doi: 10.33483/jfpau.1651886

ORIGINAL ARTICLE / ÖZGÜN MAKALE

OPTIMIZATION FORMULATION OF INSULIN-LOADED POLY-CAPROLACTONE NANOPARTICLES FOR CHRONIC WOUND HEALING USING BOX-BEHNKEN DESIGN

KRONİK YARA İYİLEŞMESİ İÇİN İNSÜLİN YÜKLÜ POLİ-KAPROLAKTON NANOPARTİKÜLLERİNİN BOX-BEHNKEN DENEYSEL TASARIMI KULLANILARAK OPTİMİZASYONU

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ABSTRACT

Objective: *The aim of this study is to optimize the formulation of insulin-loaded poly-caprolactone nanoparticles (INS-PCL-NP) for use in chronic wound healing using Box-Behnken experimental design.*

Material and Method: *The response of independent variables (poly-caprolactone concentration, polyvinyl alcohol concentration and sonication time) on dependent variables (particle size, PDI and encapsulation efficiency) were evaluated. Nanoparticle formulations were produced by the double emulsion (w/o/w)-solvent evaporation method. The resulting formulations were characterized in terms of particle size, zeta potential, and encapsulation efficiency to determine the optimum formulation. Further analyses, including morphological analyses, Fourier-transform infrared spectroscopy (FTIR), differential scanning calorimetry (DSC), and in vitro drug release studies were conducted on the optimized formulation.*

Result and Discussion: *According to the results of the characterization studies, it was observed that the optimized nanoparticles had a particle size of 618.5±11.2 nm, a PDI of 0.194±0.021, a zeta potential of -8.21±1.1 mV and an encapsulation efficiency of 73.1±4.2%. In morphological analysis, it was observed that the nanoparticles had a spherical structure. The optimized nanoparticle formulation showed a rapid release of 49.1±2.1% within the first 24 hours, followed by a controlled release for 168 hours.*

Keywords: *Box-Behnken design, insulin, nanoparticle, polycaprolactone*

ÖZ

Amaç: *Bu çalışmanın amacı kronik yara iyileşmesinde kullanılmak üzere insülin yüklü poli-kaprolakton nanopartiküllerinin (INS-PCL-NP) formülasyonunu Box-Behnken deneysel tasarımı kullanarak optimize etmektir.*

Gereç ve Yöntem: *Bağımsız değişkenlerin (poli-kaprolakton konsantrasyonu, polivinil alkol konsantrasyonu ve sonikasyon süresi) bağımlı değişkenlere (partikül boyutu, PDI ve kapsülleme verimliliği) tepkisi değerlendirilmiştir. Nanopartikül formülasyonları çift emülsiyon (s/y/s)-çözücü buharlaştırma yöntemi ile üretilmiştir. Optimum formülasyonu belirlemek için nanopartiküller partikül boyutu, zeta potansiyeli ve enkapsülasyon etkinliği açısından karakterize edilmiştir.*

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Submitted / Gönderilme : 05.03.2025
Accepted / Kabul : 25.05.2025
Published / Yayınlanma : 19.09.2025

Deneysel tasarıma uygun olarak belirlenen optimum nanopartikül formülasyonu ile morfolojik analizler, FTIR, DSC analizleri ve in vitro ilaç salım çalışmaları yapılmıştır.
Sonuç ve Tartışma: *Karakterizasyon çalışmalarının sonuçlarına göre, optimize edilmiş nanopartiküllerin 618.5±11.2 nm partikül boyutuna, 0.194±0.021 PDI'ye, -8.21±1.1 mV zeta potansiyeline ve %73.1±4.2 enkapsülasyon etkinliğine sahip olduğu görülmüştür. Morfolojik analizde, nanopartiküllerin küresel bir yapıya sahip olduğu görülmüştür. Optimize edilmiş nanopartikül formülasyonu, ilk 24 saat içinde %49.1±2.1'lik ilk salım, ardından 168 saat boyunca kontrollü salım göstermiştir.*
Anahtar Kelimeler: *Box-Behnken tasarımı, insülin, nanopartikül, polikaprolakton*

INTRODUCTION

Type 2 diabetes mellitus is prevalent worldwide and accounts for more than $760 billion annually, accounting for 10% of total healthcare expenditures [1]. Chronic foot wounds occur in patients with nerve damage due to diabetes, and the healing of these wounds is a challenging and complex process [2]. Hemostasis, inflammation, proliferation and remodelling are the stages of wound healing [3,4]. Impairments in the healing mechanisms in chronic diabetic wounds lead to a prolonged inflammatory phase, thereby complicating the wound healing process [5].

Growth factors such as beta transforming growth factor (TGF-β) and epidermal growth factor (EGF) have been shown to promote wound healing by accelerating cell migration and proliferation [4,6]. However, the limited stability and high cost of these growth factors constrain their clinical application [7]. Insulin (INS) is a hydrophilic peptide hormone and growth factor primarily responsible for regulating blood glucose levels. However, in recent years, it has also been recognized for its effectiveness in promoting wound healing. Insulin contributes to tissue regeneration by facilitating cellular reconstruction in the wound area. Its pure crystalline form and compatibility with polymers in wound dressings and drug delivery systems enhance its applicability. However, insulin has a short half-life when administered locally, and it has been reported that effective healing in the wound area has been achieved with the controlled release of insulin [8]. Since insulin is a peptide-based protein, it is prone to rapid degradation; therefore, to mitigate this issue, it must be encapsulated within a suitable carrier system to enhance its stability and bioavailability [9]. Insulin encapsulated polymeric nanoparticles was found to be effective and stable in wound healing in diabetic rat [8,10].

Polymeric nanoparticles are drug delivery systems that can be produced from synthetic/natural polymers and typically range in size from 10-1000 nm. Insulin encapsulation in biocompatible and biodegradable polymers such as poly-caprolactone (PCL), poly-lactide-co-glycolide (PLGA), poly-lactic acid (PLA), is a common strategy [11,12]. PCL is a commonly used polymer in nanoparticle production, owing to its advantageous properties, including its biocompatibility, biodegradability, and the absence of inflammatory responses during degradation [13].

Many methods have been developed for the production of polymeric nanoparticles. Various methods have been used in the production of PCL nanoparticles, such as solvent evaporation-emulsification, nanoprecipitation and solvent displacement [14]. The double emulsion-solvent evaporation method is widely applied in the encapsulation of water-soluble proteins, and this method provides long-term drug release and high encapsulation efficiency [9].

In our study, the optimum PCL nanoparticle formulation for insulin encapsulation was prepared by the double emulsion-solvent evaporation method. We aimed to develop insulin-loaded PCL nanoparticle (INS-PCL-NP) through a design of experiments approach. A Quality by Design (QbD) approach was performed to understand the interactions on the desired properties in insulin-loaded PCL nanoparticle formulations and to obtain optimum nanoparticle. For this purpose, Box-Behnken experimental design was used to optimize formulation parameters and investigate the effect of independent variables (PCL concentration, polyvinyl alcohol (PVA) concentration and sonication time) on dependent variables (particle size, polydispersity index (PDI) and encapsulation efficiency).

MATERIALS AND METHODS

Materials

Poly-caprolactone (MW: 45.000 Da), polyvinyl alcohol (MW: 25.000 Da), dichloromethane (DCM), sorbitan monooleate 80 (Span 80) and recombinant human insulin were supplied by Sigma Aldrich. Sodium sulfate was supplied by Zag Chemistry. HPLC grade acetonitrile was supplied by Merck. Water was produced using reverse osmosis (Milli-Q, Milipore). All other reagents and solvents were of analytical grade.

Preparation of Nanoparticles

The water-oil-water (w/o/w) double emulsion solvent evaporation method was used to prepare INS-PCL-NPs. During the process, 10 ml of DCM solution containing 250 mg of Span 80 and PCL at varying ratios (1-2.5% w/v) was mixed for the preparation of the organic phase. Then, 1 ml of insulin solution (1 mg/ml) was added dropwise into the organic phase and emulsified by sonication for 60 sec using an ultrasound probe (Vibra cell, BioBlock Scientific, Strasbourg, France) at 60 amplitude and 50 W output amplitude to form the primary emulsion (w/o). The primary emulsion was then added dropwise into 40 ml of an aqueous PVA solution (0.1-0.3%) and emulsified by sonication under the same conditions for varying times (30-90 sec) to form the double emulsion (w/o/w). After the organic phase was removed by rotary evaporator, the nanoparticles were centrifuged at 15000 rpm for 20 min. The precipitated nanoparticles were re-dispersed in 3 ml of water and frozen at -80^{o}C before lyophilization.

Box-Behnken Experimental Design

The Box-Behnken design was used to obtain the optimized INS-PCL-NP formulation using 13 trials created on 3 independent and 3 dependent variables with Design Expert (Version 13.0.5.0, Stat-Ease Inc., Minneapolis, Minnesota). Response surface plots and second-order polynomial models with three factors at three levels were generated for the design [15,16]. In the preliminary formulation studies, the effects of PCL concentration (%) (X_1), PVA concentration (X_2) and sonication time (s) (X_3) were evaluated assigning low, medium, and high levels to each parameter. The objective was to formulate nanoparticles with an average particle size of approximately 600 nm, which would enable high encapsulation efficiency and prolonged controlled release. The dependent and independent variables are shown in Table 1. The polynomial equation derived from the experimental data is presented as follows:

$$Y = A_0 + A_1X_1 + A_2X_2 + A_3X_3 + A_4X_1X_2 + A_5X_1X_3 + A_6X_2X_3 + A_7X_1^2 + A_8X_2^2 A_9X_3^2$$

In the equation here, Y is the response obtained from the dependent variables and A_0 is the intersection point of this response. The regression coefficients of Y are coded between A_1-A_9 and the values are obtained as a result of experimental studies. The independent variables are expressed as X_1, X_2 and X_3. X_f^2 (f = 1, 2, 3) shows the quadratic values and X_y X_z (y, z = 1, 2, 3) shows the interaction values.

Table 1. Box-Behnken design: Dependent and independent variable levels

	Levels		
Independent Variables	-1	0	1
X_1 = PCL concentration (w/v %)	1	1.75	2.5
X_2 = PVA concentration (w/v %)	0.1	0.2	0.3
X_3 = Sonication time (sec)	30	60	90
Dependent Variables	**Restrictions**		
Y_1 = Average particle size (nm)	600		
Y_2 = PDI	Minimize		
Y_3 = Encapsulation efficiency (%)	Maximize		

Method for Quantification of Insulin

To quantify insulin in the nanoparticle formulations, the Ultra Performance Liquid Chromatography-UV/Visible Spectrophotometry (UPLC-UV) reversed phase method was used. The isocratic system (Waters, Vienna, Austria) was studied and validated using the method performed according to the USP monograph [17]. InertSustain C18 column (150 mm*4.6 mm*2µm) (GL Sciences, Tokyo, Japan) was used as the stationary phase and 0.02 M pH 3.1 Na_2SO_4 buffer: acetonitrile (65:35) was used as the mobile phase, and the column temperature was kept constant at 40 °C throughout the analysis. The mobile phase flow rate was kept constant at 0.5 ml/min throughout the analysis and the injection volume was set at 20 µl. The system consists of a solvent chamber, a processor-controlled high-pressure pump that provides dispersion of the mobile phase and a UV detector with a λ max of 214 nm.

Drug Encapsulation Efficiency

The indirect method was used to calculate the encapsulation efficiency of nanoparticles. After the centrifugation of the nanoparticles at 15000 rpm for 20 minutes (Beckman Coulter, CA, USA), they were washed 3 times, and the supernatants were analyzed with UPLC. After determining the amount of non-capsulated insulin remaining in the supernatant, the encapsulation efficiency [18] was calculated according to the following equation:

$$\text{Encapsulation efficiency (\%)} = \left(\frac{(Total\ amount\ of\ drug) - (Amount\ of\ free\ drug)}{Total\ drug}\right) \times 100$$

Particle Size, Polydispersity Index and Zeta Potential Measurement

The zeta potential, polydispersity index and particle size properties of the nanoparticles were evaluated using the Zetasizer Nano-ZS90 (Malvern, Worcestershire, England) device, which uses the photon correlation spectroscopic method. All analysis was carried out in three replicates.

Morphological Analysis of Optimized Nanoparticles

Scanning electron microscope (QUANTA 400F Field Emission) was used for morphological analysis of nanoparticles. After the optimized nanoparticle formulation was produced, the suspended samples were dropped onto an aluminum surface and waited for drying. Images were taken at 24000x and 50000x magnification of different parts of the formulations coated with gold/palladium.

Fourier Transform Infrared Spectroscopy (FTIR) Analysis

FTIR spectroscopy with an Attenuated Total Reflectance (ATR) probe was used to analyse insulin, PCL and insulin-loaded PCL nanoparticles (Spectrum 400, Perkin Elmer, USA). All analysis were carried out in the wavelength range of 4000-400 cm^{-1}.

Differential Scanning Calorimetry (DSC) Analysis

DSC analysis (DSC 60, Shimadzu, Japan) of insulin, PCL and insulin-loaded PCL nanoparticles were performed to order to assess possible interactions and structural changes. 2 mg samples were prepared in aluminum pans and heated from 25 °C to 350 °C in nitrogen atmosphere at a rate of 10 °C/min.

***In Vitro* Drug Release Studies**

Release studies of insulin from INS-PCL-NPs were performed using Franz diffusion cells and, in this study, diffusive area was 1 cm^2 [19]. Dialysis membrane (SERVA, molecular weight cutoff: 12000–14000) was placed between the donor and receptor compartments of the Franz diffusion cell. For the preparation of the simulated wound fluid (SWF) (pH:6.2), 8.298 g of sodium chloride and 0.368 g of calcium chloride were dissolved in 1 l of distilled water [20]. The re-dispersed nanoparticle suspension (50 mg/ml) was placed in the donor compartment and the receptor compartment containing simulated wound fluid thermostated at 37°C. The samples were taken from the receptor phase at the predetermined

time intervals and fresh SWF added the receptor phase at the same volume. The samples were analyzed using UPLC-UV to calculate the drug release. Using in vitro release data, the release kinetics of insulin from INS-PCL-NPs were evaluated for compatibility with the zero order, first order, Higuchi, Weilbull, Hixson-Crowell and Korsmeyer-Peppas kinetic models using DDSolver® software.

RESULT AND DISCUSSION

Experimental Analysis

In order to optimize nanoparticle formulation, 13 confirmatory trials with 1 center point including 3 independent and 3 dependent variables were performed according to Box-Behnken design for nanoparticle production. The independent variables used for nanoparticle production are shown in Table 2. Nanoparticle formulations were characterized in terms of the particle size, polydispersity index, encapsulation efficiency and zeta potential and the results are given in Table 2. Response-surface graphs were created by the Design-Expert software to evaluate the effects of independent variables on dependent variables.

Table 2. The results of the Box-Behnken design

Formulation	Independent variables			Dependent variables			
	PCL concentration (%)	PVA concentration (%)	Sonication time (sec)	Particle size (nm)	PDI	Encapsulation efficiency (%)	Zeta potential (mV)
1	1	0.1	60	438.4 ± 12.2	0.283 ± 0.034	42.1 ± 7.4	-1.3 ± 1.1
2	1	0.2	30	276.4 ± 4.2	0.212 ± 0.031	46.9 ± 11.3	-10.3 ± 3.3
3	1	0.2	90	286.2 ± 4.5	0.179 ± 0.014	77.8 ± 4.3	-10.9 ± 2.8
4	1	0.3	60	244.6 ± 6.7	0.156 ± 0.017	79.8 ± 5.9	-7.7 ± 2.6
5	1.75	0.1	30	478.1 ± 6.4	0.141 ± 0.011	42.2 ± 4.9	-9.8 ± 4.1
6	1.75	0.1	90	618.5 ± 9.1	0.194 ± 0.011	53.3 ± 7.2	-2.6 ± 8.1
7	1.75	0.2	60	309.6 ± 4.9	0.189 ± 0.089	78.6 ± 8.1	-8.5 ± 2.6
8	1.75	0.3	30	309.8 ± 3.5	0.171 ± 0.021	39.2 ± 6.1	-8.3 ± 4.9
9	1.75	0.3	90	262.4 ± 2.1	0.188 ± 0.014	44.7 ± 92	-0.8 ± 0.1
10	2.5	0.1	60	498.8 ± 6.4	0.119 ± 0.052	71.3 ± 3.1	-1.2 ± 0.9
11	2.5	0.2	30	645.9 ± 7.3	0.376 ± 0.091	57.4 ± 10.2	-11.6 ± 7.1
12	2.5	0.2	90	288.8 ±3.9	0.174 ± 0.011	66.6 ± 7.7	-11.6 ± 8.1
13	2.5	0.3	60	294.3 ± 3.1	0.213 ± 0.016	73.1 ± 4.4	-0.9 ± 0.3

Zeta Potential Measurements

Zeta potential is a value that allows the electrostatic charges on the surface to estimate the repulsive forces between the particles and provides information about the stability of the nanoparticles [18]. As a result of the zeta potential measurements, the main effect on the surface charge was considered to be PCL and it provided the nanoparticles to be anionic owing to its negative structure [21]. The surface charges of nanoparticles were found to be negative and between -0.8±0.1 and -11.6±8.1 mV. The physical stability of nanoparticles is strongly influenced by their zeta potential. At high absolute zeta potential values, particles repel each other, reducing the likelihood of aggregation and indicating good stability. Conversely, at low absolute zeta potential values, repulsive forces are insufficient to prevent aggregation, leading to potential instability during long-term storage. Therefore, the relatively low zeta potential values observed suggest that the nanoparticles may exhibit limited physical stability over extended storage periods.The data obtained from the optimized nanoparticles are shown in Figure 1. In addition, the zeta potential was not included in the dependent variables since the pre-formulation studies showed that no significant changes in the zeta potential occurred with changes in the independent parameters.

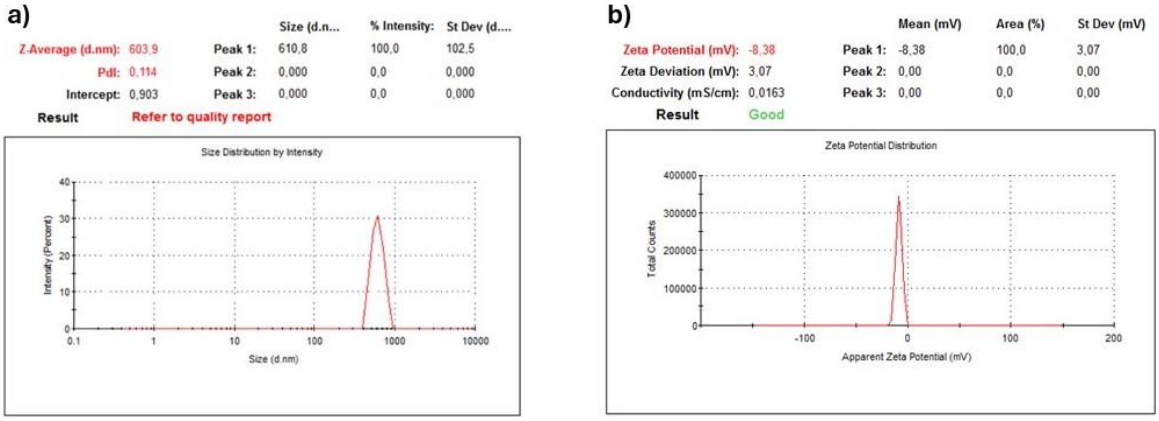


Figure 1. a) Size distribution, b) Zeta potential of optimum drug-loaded PCL nanoparticle

Effect of Independent Variables on Particle Size

The particle size of the nanoparticles was found to be between 262.4±2.1 - 618.5±9.1 nm (Table 2).

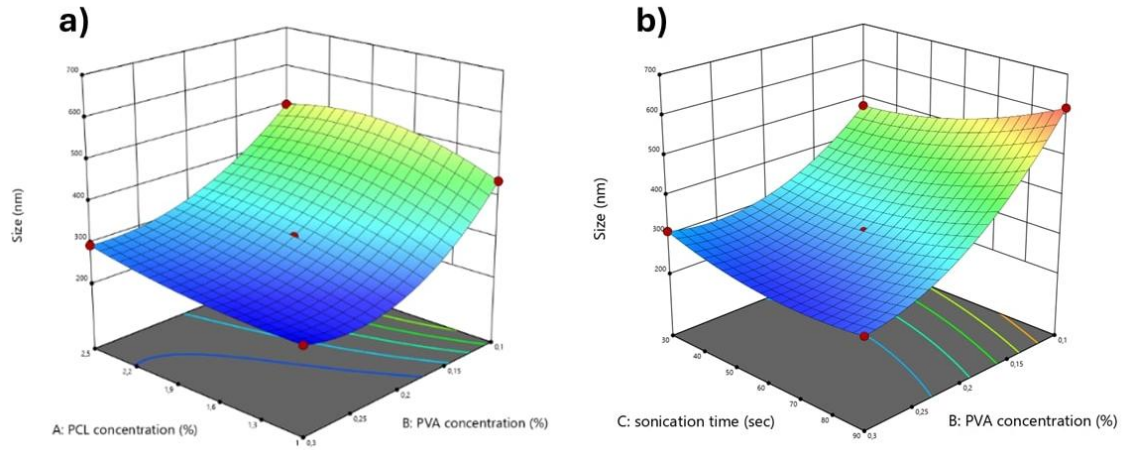


Figure 2. Surface plots presenting the effect of a) PCL and PVA concentration and b) sonication time and PVA concentration on particle size

The second-degree polynomial equation showing the effects on particle size (Y_1) is given below:

$$Y_1 = 309.6 + 35.33X_1 - 115.39X_2 - 6.84X_3 - 2.89X_1X_2 - 41.75X_1X_3 - 47.075X_2X_3 - 16.7125X_1^2 + 76.0125X_2^2 + 31.4625X_3^2$$

The negative coefficients in the obtained polynomial equation indicate antagonistic effects, whereas the positive coefficients indicate synergistic effects. A quadratic model was proposed in the design, and the R^2 and adjusted R^2 values were determined as 0.7270 and 0.6227, respectively. Since R^2 values above 0.5 are generally considered acceptable, these results suggest that the model provides a reasonable fit and the design is reliable [21]. The signal-to-noise ratio was found to be 7.059, which exceeds the threshold of 4, indicating suitability for navigation in the design area [18]. Polymer concentration influences the particle size and the size affects the drug release behavior along with the matrix behavior. The generated polynomial equation clearly indicates that the PCL concentration (X_1) has a positive effect on particle size, meaning that an increase in polymer concentration results in larger

particles (Figure 2a). This can be attributed to the increase in the viscosity of the organic phase during sonication as the polymer concentration increases, leading to the formation of larger particles. The sonication time (X_3) applied to the secondary emulsion was found to influence the size of the nanoparticles. Longer sonication times resulted in a reduction in nanoparticle size, suggesting that the increase in energy transferred during the sonication process facilitated the formation of smaller particles. This effect can be attributed to the enhanced shear forces exerted during prolonged sonication, which promote the fragmentation of larger aggregates into smaller, more uniform particles [22]. It was observed that the PVA concentration (X_2) had an inverse relationship with particle size, meaning that an increase from 0.1% to 0.3% resulted in a decrease in particle size (Figure 2b). This suggests that the PVA concentration plays a crucial role in controlling nanoparticle size. However, both excessively high and low stabilizer concentrations can lead to aggregation and clustering, highlighting the importance of using the optimal PVA concentration.

Effect of Independent Variables on PDI

Due to the low coefficients of the independent variables obtained from the polynomial equation, it was interpreted that the independent variables had a low effect on the PDI.

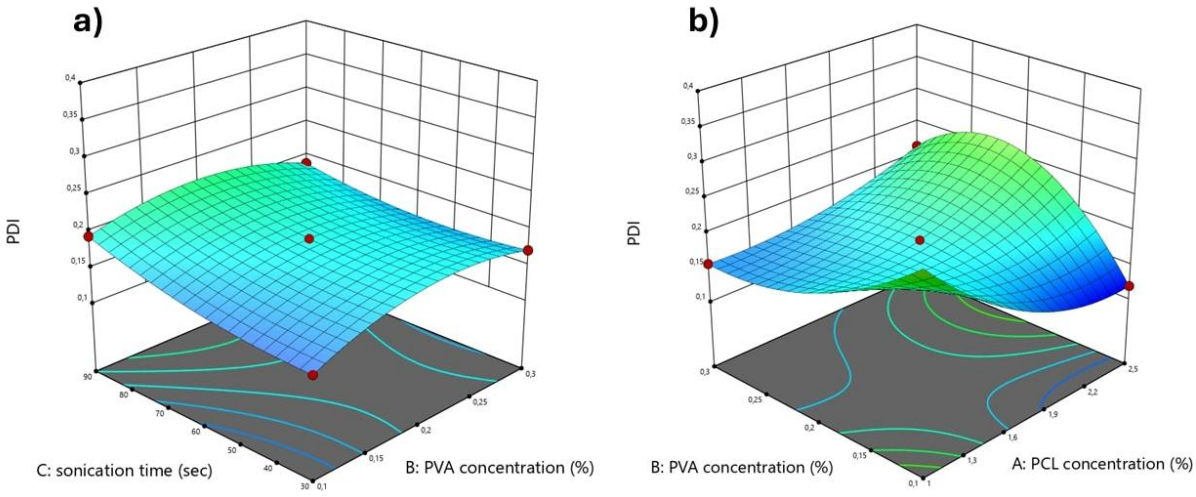


Figure 3. Surface plots presenting the effect of a) sonication time and PVA concentration and b) PCL and PVA concentration on PDI

The second order polynomial equation showing the effects on PDI (Y_2) is given below.

$$Y_2 = 0.189 + 0.03975X_1 + 0.00625X_2 + 0.01775X_3 + 0.05525X_1X_2 - 0.04225X_1X_3 - 0.00925X_2X_3 + 0.032875X_1^2 - 0.029125X_2^2 + 0.013375X_3^2$$

The R^2 value obtained from the polynomial equation was found to be 0.6008. It was found that there was a direct correlation between PCL concentration (X_1) and PDI, where a slight increase in PDI was observed with an increase in PCL concentration (Figure 3b). It was observed that the sonication time (X_2) applied to the secondary emulsion had a low positive effect on PDI though this effect was not remarkable. Additionally, the PVA concentration (X_3) showed a low positive effect on PDI, suggesting that higher stabilizer concentrations may lead to aggregation (Figure 3a). Additionally, the PDI values of the produced nanoparticles were generally found to be less than 0.3. Low PDI values indicate that the produced particles have high stability and are not prone to aggregation [23].

Effect of Independent Variables on Encapsulation Efficiency

Independent variables were found to have low effects on encapsulation efficiency.

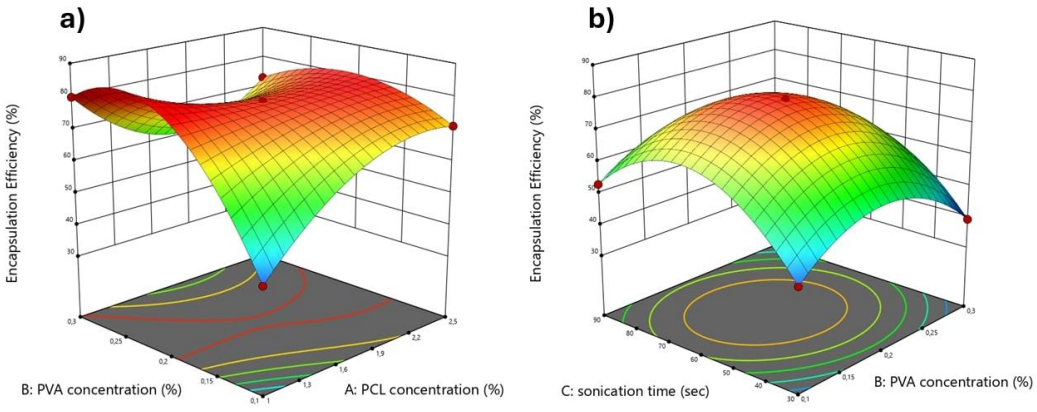


Figure 4. Surface plots presenting the effect of a) PCL and PVA concentration and b) sonication time and PVA concentration on encapsulation efficiency

$Y_3 = 78.6 - 0.3X_1 - 2.75X_2 + 4.15X_3 - 8.95X_1X_2 - 5.3X_1X_3 - 1.25X_2X_3 + 2.55X_1^2 - 14.7X_2^2 - 19.05X_3^2$

The R^2 value obtained from the polynomial equation was found to be 0.6444. A slight negative effect is observed on encapsulation efficiency with increasing PCL concentration (Figure 4a). Similarly, a negative effect on encapsulation was noted with an increase in PVA concentration. Conversely, a slight positive effect on encapsulation was observed with increasing sonication time (Figure 4b). Overall, no significant effect of the independent variables on encapsulation efficiency was detected.

UPLC-UV Method for Quantification of Insulin

The calibration curve was created for nine different concentrations between 5-45 µg/ml. The regression equation and correlation coefficient were calculated using the graph between peak area and concentration. The linear regression equation for insulin was found as y = 153211x + 91246. The coefficient of determination defined as r^2 was used to evaluate the linearity of the calibration line. The linearity of the graph increases as the r^2 value approaches 1. The correlation coefficient was found as r^2 = 0.9966 for INS and a correlation was observed between the peak areas and drug concentrations. The calibration line showed that UPLC is a sensitive method for insulin quantification and an effective analysis technique to accurately determine the active substance content in nanoparticles. The calibration curve for insulin is shown in Figure 5.

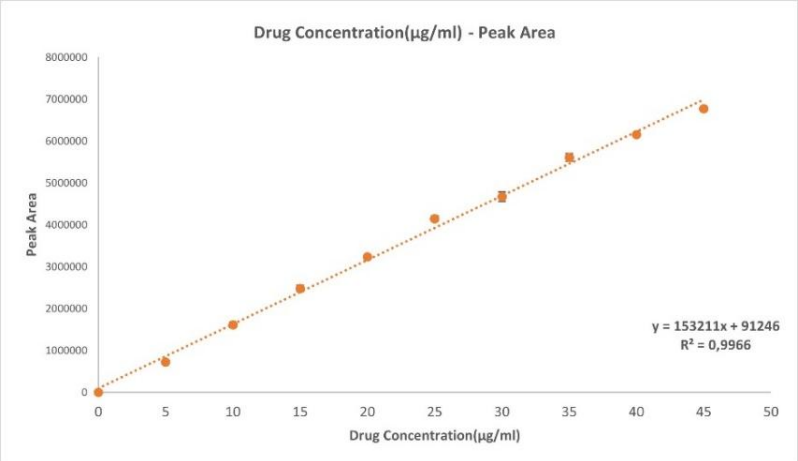


Figure 5. Calibration curve of insulin

Production of Optimized INS-PCL-NPs

The parameters required for optimum INS-PCL-NP production were determined by applying the constraints in Table 1 to the dependent factors. Point estimation was used in Design Expert software and the desirability value close to 1 is accepted in other studies [18]. According to the predictions of the software, the optimum nanoparticle formulations with a desirability value of 0.741 will be obtained with the parameters X_1 = 2.5%, X_2 = 0.15% and X_3 = 50 s and the following responses will be obtained: Y_1 = 588.7 nm, Y_2 = 0.2 and Y_3 = 79.8%. Optimized INS-PCL-NP formulations were generated and the role of the polynomial equation generated by the software in predicting the responses was investigated. Experimental results confirmed that the optimized formulations were in good agreement with the model (Table 3), giving values of $Y_1 = 618.5 \pm 11.2$ nm, $Y_2 = 0.194 \pm 0.021$ and $Y_3 = 73.1 \pm 4.2$% [24]. The zeta potential of the optimized nanoparticles was found to be -8.21 ± 1.1 mV. In a study conducted by Socha et al., insulin-containing PCL nanoparticles were produced using the w/o/w method, and 519.9 ± 34.5 nm size and 95.4 ± 1.5% encapsulation efficiency were obtained using 2.5% PCL concentration, 60 seconds sonication time and 0.1% PVA concentration in nanoparticle production. In addition, in the same study, PCL nanoparticles were found to have a negative zeta potential value (−9.5±4.9 mV) as in our study [12]. In another study insulin loaded nanoparticles were produced using the w/o/w method, Pluronic F68 was used as a stabilizer, and according to the characterization studies nanoparticles had a particle size of 796±10.5 nm, a PDI of 0.49±0.06, an encapsulation efficiency of 90.6 ± 1.6%, and a zeta potential of 29.4±2.7 mV. A thorough examination of these studies reveals that the nanoparticles obtained exhibit greater uniformity in size, attributable to their low PDI value. This observation lends credence to the efficacy of the applied factorial design.

Table 3. Optimum parameters of INS-PCL-NP formulation produced with the independent variables suggested by the Box-Behnken design

PCL concentration (%)	PVA concentration (%)	Sonication time (sec)	Particle size (nm)	PDI	Encapsulation efficiency (%)
2.5	0.15	50	618.5 ± 11.2	0.194 ± 0.021	73.1 ± 4.2

Scanning Electron Microscope (SEM) Analysis

The SEM image of the optimized INS-PCL-NP formulation is shown in Figure 6. It was observed that the nanoparticles had a near-spherical shape, with some particles connected at specific points by surface bridges. This is likely due to the adhesive properties of PVA, which can promote particle interaction and aggregation. These surface bridges imply that PVA not only functions as a stabilizer for the nanoparticles but also influences their morphological characteristics by promoting interparticle interactions [25]. In a study by Iqbal et al., PCL nanoparticles were prepared by the w/o/w method and PVA was used as a stabilizer. In the morphological analysis in the study, it was observed that PVA had an adhesive effect on the particles, similar to our study [26].

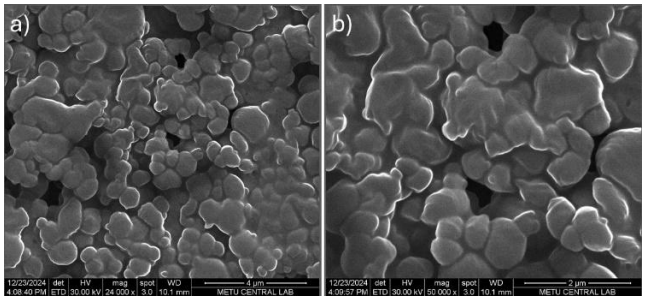


Figure 6. Scanning electron microscope image of optimized INS-PCL-NPs at a)24000x and b)50000x magnifications

Fourier Transform Infrared Spectroscopy (FTIR) Analysis

The FTIR spectra of insulin, PCL and INS-PCL-NP are shown in Figure 7. In the spectrum of insulin in Figure 7a, Amide I, Amide II and Amide III bands are seen. The Amide I band between 1700-1600 cm^{-1} is assigned to the stretching of the carbonyl groups in the protein backbone (C=O). The Amide II band, observed between 1580-1500 cm^{-1}, arises from the combination of NH and CN vibrations. The amide III band, which occurs within the range of 1350-1200 cm^{-1}, has been identified as being associated with the coupling of CN and NH vibrations. These bands have been identified as characteristic of insulin and have been found to be in accordance with the existing literature [27]. In the FTIR spectrum of PCL in Figure 7b, the symmetric aliphatic stretching is seen at 2868 cm^{-1}, the asymmetric aliphatic stretching is seen at 2944 cm^{-1}, the characteristic carbonyl (C = O) stretching band is seen at 1724 cm^{-1}, the C–O and C–C stretching vibration bands are seen at 1292 cm^{-1} and the symmetric C-O-C and asymmetric C-O-C vibration bands are seen at 1164 and 1236 cm^{-1}, respectively. In a study, indomethacin-loaded PCL nanoparticles were produced. According to the results of the FTIR analysis, it was seen that the characteristic bands of PCL were similar to our FTIR data [28]. In the spectrum of insulin-loaded nanoparticles, the characteristic amide bands of insulin almost completely disappeared, and no distinct peaks were observed in this spectrum (Figure 7c). This indicates that insulin was successfully encapsulated in PCL nanoparticles.

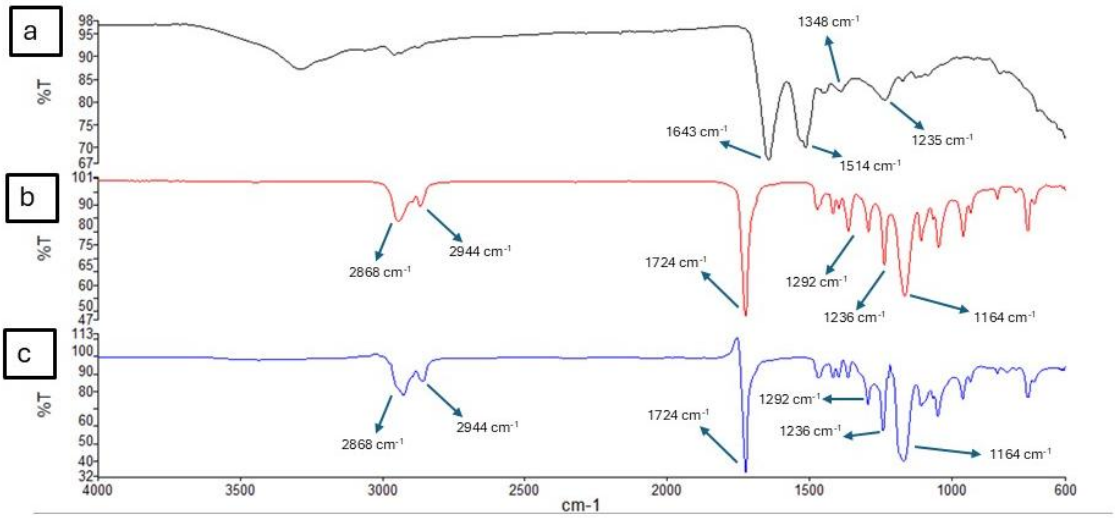


Figure 7. FTIR spectra of a) Insulin, b) PCL, c) INS-PCL-NP

Differential Scanning Calorimetry Analysis

DSC thermograms of insulin, PCL and INS-PCL-NP are shown in Figure 8. As demonstrated in Figure 8a, the DSC thermograms revealed that PCL demonstrated an endothermic peak at 61 °C. This occurrence was ascribed to the presence of its crystalline structure. Furthermore, this peak was found to be in accordance with the findings reported by Badri et al. [28]. As demonstrated in Figure 8b, insulin exhibited a small endothermic peak at 214 °C. The resulting thermogram was found to be in accordance with the thermogram obtained by Zhao et al [29]. In the thermogram of INS-PCL-NP, the endothermic peak corresponding to PCL was observed; however, the peak associated with insulin was not detected (Figure 8c). This absence is thought to be due to a decrease in the amount of insulin in the nanoparticle, possibly as a result of encapsulation within the particles.

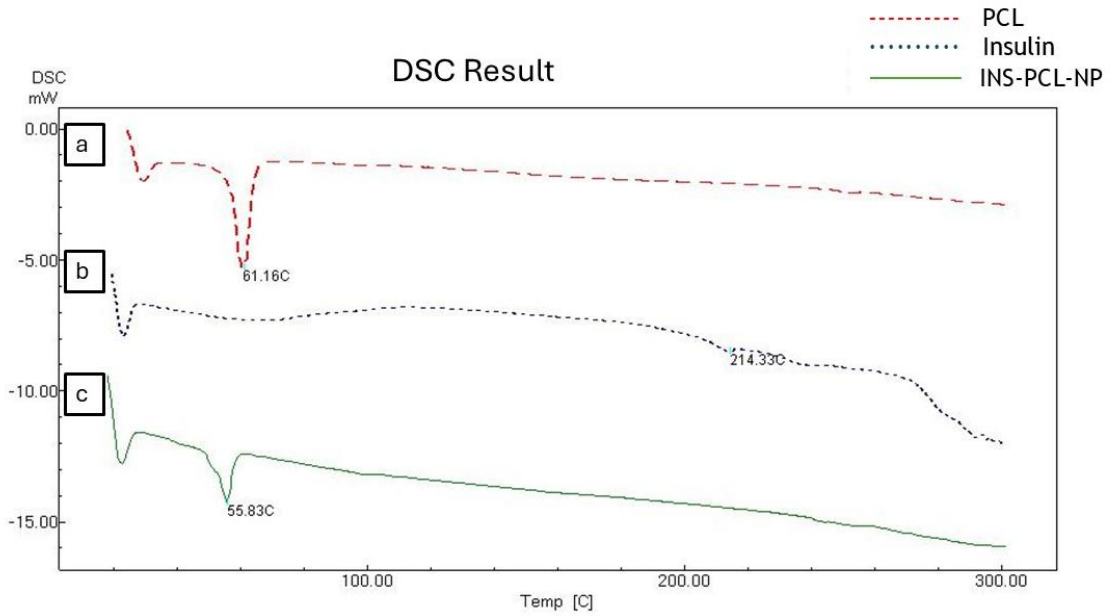


Figure 8. DSC thermograms of a)PCL, b)insulin and c)INS-PCL-NP

In Vitro Drug Release Study

The *in vitro* drug release behavior of INS-PCL-NPs was evaluated. A release of 49.11 ± 2.01% insulin was observed within the first 24 hours from the optimized INS-PCL-NPs. This rapid release is likely attributed to the insulin present on the surface of the nanoparticles. The nanoparticles released a total of 96.09 ± 0.73% insulin (Figure 9) at the end of 168 hour. Insulin release is presumed to occur through a diffusion-controlled mechanism. This controlled release is essential for ensuring the sustained therapeutic effect. In a study, cilnidipine loaded PCL nanoparticles were produced and as a result of in vitro release study, it was observed that approximately 60% cumulative drug release was obtained at the end of 72 hours [30]. In another study hydrophilic hydroxyurea substance was encapsulated in PCL nanoparticles and showed approximately 34% cumulative drug release at the end of 48 hours [31]. In addition, in a study conducted by Kumar and Sawant, exemestane loaded PCL nanoparticles showed approximately 71% cumulative drug release at the end of 240 hours [32]. According to the results of this study, it was seen that PCL nanoparticles were preferred in controlled drug release, and these studies confirmed our results.

In kinetic modeling, an R^2 value close to 1 indicates that the model is suitable for the release behavior. However, since the R^2 value increases with the addition of the variables and causes inaccurate interpretation of the release profiles. In order to prevent this situation, the R^2_{adj} value is used instead of R^2 [33]. In Table 4, it is seen that the Weibull kinetics has the highest R^2_{adj} value with 0.9625. The AIC is the compatibility parameter. A lower value indicates that the formulation release is suitable for use with the kinetic model. The Weibull model was found to have the lowest AIC value with 106.5018. The MSC parameter is known as the model selection criterion and obtained by normalization of the AIC value. A higher MSC value indicates that the model is highly compatible [34]. The highest MSC parameter among the kinetic models was observed in the Weibull model with a value of 2.9444. The findings of the analysis indicate that the kinetic model that most accurately describes the release of insulin from INS-PCL-NPs is the Weibull model. The Weibull model is a kinetic model that is often highly suitable for polymeric matrix-type structures, such as polymeric nanoparticles [35]. The release of insulin from INS-PCL-NPs initiated rapidly and proceeded in a controlled manner, following Weibull kinetics.

Table 4. Assesment of drug release from INS-PCL-NP formulation based on different kinetic models

Mathematical model	Parameters		Mathematical model	Parameters	
Zero order	R^2	0.5088	**Korsmeyer-Peppas**	R^2	0.9346
	R^2_{adj}	0.5389		R^2_{adj}	0.9299
	AIC	144.9291		AIC	115.6884
	MSC	0.5427		MSC	2.3702
First order	R^2	0.8363	**Hixson-Crowell**	R^2	0.7956
	R^2_{adj}	0.8363		R^2_{adj}	0.7956
	AIC	128.3599		AIC	131.9117
	MSC	1.5783		MSC	1.3563
Higuchi	R^2	0.9136	**Weibull**	R^2	0.9675
	R^2_{adj}	0.9136		R^2_{adj}	0.9625
	AIC	118.1306		AIC	106.5018
	MSC	2.2176		MSC	2.9444

R^2: Correlation coefficient, R^2_{adj}: Adjusted correlation coefficient, MSC: Model selection criterion, AIC: Akaike information criterion

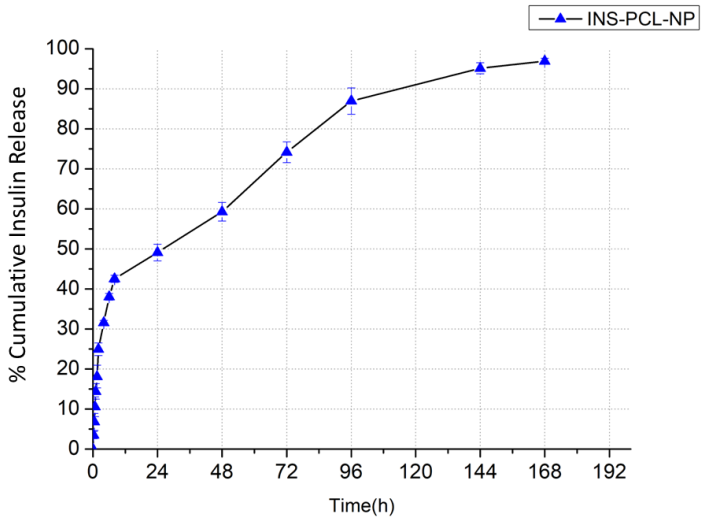


Figure 9. *In vitro* drug release profile of INS-PCL-NPs (n=3)

Conclusion

In this study, insulin-loaded polycaprolactone nanoparticles (INS-PCL-NPs) were successfully developed and optimized for controlled drug delivery in chronic wound healing using the Box-Behnken experimental design. The effects of three independent formulation parameters - PCL concentration, PVA concentration, and sonication time - on key nanoparticle characteristics such as particle size, polydispersity index (PDI), and encapsulation efficiency were systematically investigated. The optimal formulation (X_1 = 2.5%, X_2 = 0.15%, X_3 = 50 sec) demonstrated favorable physicochemical properties, including a desirable particle size, narrow size distribution, and high encapsulation efficiency. In vitro release studies revealed a biphasic insulin release profile characterized by an initial burst release of approximately 49% within the first 24 hours, followed by a sustained release over 168 hours. Drug release kinetics were best described by the Weibull model, indicating a diffusion-controlled mechanism. Overall, the Box-Behnken design proved to be a valuable tool in the rational development of polymeric

nanoparticle formulations. The optimized INS-PCL-NPs exhibit promising potential for enhancing wound healing in chronic diabetic wounds through sustained and localized insulin delivery.

ACKNOWLEDGEMENTS

This study was supported by Gazi University Scientific Research Projects Unit with project number TGA-2023-8155.

AUTHOR CONTRIBUTIONS

Concept: T.T., A.Y., F.T.D., F.A.; Design: T.T., A.Y., F.T.D., F.A.; Control: F.T.D., F.A.; Sources: F.T.D., F.A.; Materials: F.T.D., F.A.; Data Collection and/or Processing: T.T., A.Y.; Analysis and/or Interpretation: T.T., A.Y., F.T.D., F.A.; Literature Review: T.T., A.Y.; Manuscript Writing: T.T., A.Y., F.T.D., F.A.; Critical Review: T.T., A.Y., F.T.D., F.A.; Other: -

CONFLICT OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

The authors declare that the ethics committee approval is not required for this study.

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Ankara Ecz. Fak. Derg. / J. Fac. Pharm. Ankara, 49(3): 915-927, 2025
Doi: 10.33483/jfpau.1601594

DERLEME MAKALE / REVİEW ARTICLE

DUCHENNE KAS DİSTROFİSİNDE YENİ UFUKLAR: NÜKLEER FAKTÖR KAPPA YOLU VE VAMOROLON'UN TEDAVİYE ETKİLERİ

NEW HORIZONS IN DUCHENNE MUSCLE DYSTROPHY: NUCLEAR FACTOR KAPPA PATHWAY AND THERAPEUTIC EFFECTS OF VAMOROLONE

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ÖZ

Amaç: *Duchenne kas distrofisi (DMD), distrofin eksikliği nedeniyle kas dejenerasyonu ve zayıflığına yol açan X'e bağlı bir hastalıktır. Tedavide amaç, hastalığın ilerlemesini yavaşlatmak ve yaşam kalitesini artırmaktır. Nükleer faktör kappa B (NF-κB) yolu, inflamasyonla ilişkili genlerin düzenlenmesinde rol oynar ve bu yolun hedeflenmesi tedavi stratejilerinden biridir. Glukokortikoidler NF-κB yolunu inhibe ederek etki gösterse de, osteoporoz, hipertansiyon ve büyüme geriliği gibi ciddi yan etkilere yol açabilir. 2023'te FDA onayı alan vamorolon sentetik bir kortikosteroiddir. Vamorolon glukokortikoid reseptörlerini (GR) hedefler, ancak glukokortikoid yanıt elemanını (GRE) aktive etmez. Bu sayede, geleneksel kortikosteroidlere göre yan etkileri daha azdır. Bu derleme, NF-κB yolunun DMD tedavisindeki rolünü ve vamorolonun etkililik ve güvenlilik profilini değerlendiren çalışmaları incelemeyi amaçlamaktadır.*
Sonuç ve Tartışma: *Geleneksel glukokortikoidler NF-κB'yi inhibe ederek DMD tedavisinde etkinlik gösterir, ancak ciddi yan etkilere neden olabilirler. Vamorolon, NF-κB yolunu benzer şekilde inhibe ederken GRE aktivasyonunu tetiklemediği için geleneksel glukokortikoidlere kıyasla daha az yan etki oluşturur. Klinik çalışmalar, vamorolon'un etkililiğinin glukokortikoidlere benzer olduğunu, ancak yan etkilerinin daha az olduğunu göstermiştir. Vamorolon, DMD tedavisinde geleneksel glukokortikoidlere alternatif olarak umut vaat eden bir ilaçtır.*
Anahtar Kelimeler: *Duchenne kas distrofisi, glukokortikoidler, nükleer faktör kappa, vamorolon*

ABSTRACT

Objective: *Duchenne muscular dystrophy (DMD) is an X-linked disorder characterized by muscle degeneration and weakness due to dystrophin deficiency. The primary goal of treatment is to slow disease progression and improve quality of life. The nuclear factor kappa B (NF-κB) pathway plays a role in regulating inflammation-related genes and is a target for therapeutic strategies. Although glucocorticoids act by inhibiting the NF-κB pathway, they can cause serious side effects such as osteoporosis, hypertension and growth retardation. Vamorolone is a synthetic corticosteroid that received FDA approval in 2023. Vamorolone targets glucocorticoid receptors (GR) but does not activate the glucocorticoid response element (GRE). This results in fewer side effects than traditional corticosteroids. This review aims to examine studies evaluating the role of the NF-κB pathway in the treatment of DMD and the efficacy and safety profile of vamorolone.*

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Gönderilme / Submitted : 14.12.2024
Kabul / Accepted : 13.04.2025
Yayınlanma / Published : 19.09.2025

Result and Discussion: *Traditional glucocorticoids show efficacy in the treatment of DMD by inhibiting NF-κB, but they can cause serious side effects. Vamorolone similarly inhibits the NF-κB pathway but does not trigger GRE activation, resulting in fewer side effects compared to traditional glucocorticoids. Clinical studies have shown that the efficacy of vamorolone is similar to glucocorticoids, but with fewer side effects. Vamorolone is a promising alternative to traditional glucocorticoids for the treatment of DMD.*
Keywords: *Duchenne muscular dystrophy, glucocorticoids, nuclear factor kappa, vamorolone*

GİRİŞ

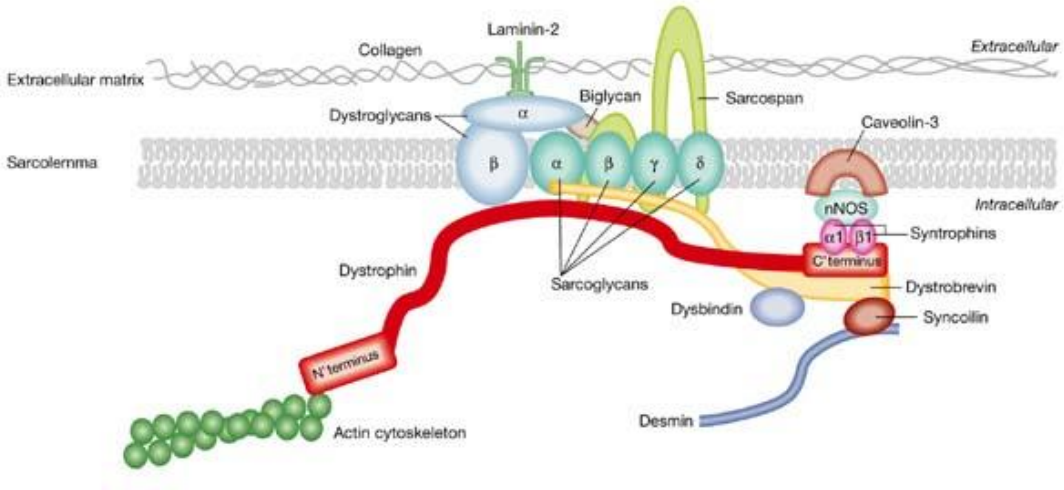
Genetik bir hastalık olan duchenne kas distrofisi (DMD), kaslarda ilerleyici zayıflama ve dejenerasyon ile karakterizedir. DMD'nin tedavisinde kullanılmak için geliştirilmiş çeşitli terapötik yaklaşımlar bulunmaktadır. Hastalığın oluşumunda ve ilerlemesinde önemli bir rol oynayan nükleer faktör kappa B (NF-κB) sinyal yolu, tedavi yaklaşımlarından biri olarak tercih edilmektedir [1]. NF-κB, inflamasyonu ve kas dejenerasyonunu kontrol eden bir transkripsiyon faktörüdür [2]. DMD'de NF-κB yolu, inflamatuar yanıtların artışına ve kas dokusunun dejenerasyonuna neden olarak hastalığın patofizyolojisinde kritik bir rol oynar [3]. Bu nedenle, NF-κB'nin inhibisyonu inflamasyonu azaltarak kas hasarının ilerlemesini yavaşlatır ve bu mekanizma, tedavi yaklaşımlarında önemli bir rol oynar [1].

DMD tedavisinde amaç, hastalığın ilerlemesini yavaşlatmak, kas fonksiyonunu korumak ve yaşam kalitesini artırmaktır. NF-κB yolunun terapötik hedeflenmesi ve vamorolon gibi yeni nesil tedavi seçenekleri bu amaçlara ulaşmada önemli rol oynamaktadır [4]. Vamorolon, glukokortikoid reseptörlerine bağlanarak klasik glukokortikoidlerin antiinflamatuar etkilerini sağlarken, bu ilaçların yol açtığı yan etkileri en aza indirir. Bu özellikleriyle günümüzde vamorolon, DMD yönetiminde yeni bir tedavi yaklaşımı olarak kullanılmaktadır [5]. Ancak, tedavilerin uygulanmasında, hastalığın şiddeti ve bireysel farklılıkların dikkate alınması büyük önem taşımaktadır. Vamorolon gibi ajanların etkililiğini ve güvenliliğini belirlemek için geniş kapsamlı klinik araştırmalar ve uzun dönemli verilerin elde edilmesi gereklidir. Bu derlemede, DMD'nin patofizyolojisi, NF-κB sinyal yolunun hastalık üzerindeki rolü ve tedavideki potansiyel kullanımı ele alınmış; ayrıca DMD için mevcut farmakolojik yöntemler incelenmiş ve özellikle yeni bir ilaç olan vamorolon'un üzerinde durulmuştur.

Duchenne Kas Distrofisi Patofizyolojisi

DMD şiddetli ve ilerleyici kas zayıflığına neden olan bir hastalıktır. Merdiven çıkmada zorluk, paytak bir yürüyüş ve sık düşmeler hastalığın erken dönem semptomlarındandır [3]. DMD'nin literatürdeki ilk tanımı 1851 yılında İngiliz doktor Meryon tarafından yapılmıştır [6]. Meryon, hastalık üzerine yaptığı çalışmalarda birkaç nekropsi gerçekleştirmiş ve hayvanların omuriliklerinin sağlam olduğunu tespit etmiştir. Bu doğrultuda Meryon, hastalığın sinir sisteminden kaynaklanmadığı sonucuna ulaşmıştır. Fakat, yaptığı çalışmalarda vücuttaki kasların körelmiş, yumuşak ve neredeyse kansız olduğunu tespit etmiştir. Kasın mikroskobik incelemesi sonucunda, kas liflerinin parçalandığını ve granüler, yağlı maddeye dönüştüğünü gözlemlemiştir. Bu nedenle, Meryon bu durumu "İstemli kasın granüler dejenerasyonu" olarak adlandırmıştır [7].

DMD, distrofin proteininin yokluğundan veya mutasyonlarından kaynaklanan X'e bağlı bir kas hastalığıdır [3]. DMD hastalarında gözlemlenen genetik mutasyonların yaklaşık %60'ı delesyonlardan, %11'i ise duplikasyonlardan oluşmaktadır. Geriye kalan mutasyonlar, kodlama bölgelerinde veya splice bölgelerinde meydana gelen küçük ölçekli nükleotid değişikliklerini içermektedir. Bu genetik değişiklikler, distrofin proteininin sentezini bozarak hastalığın oluşumunda rol oynamaktadır [8]. Delesyonlar ve duplikasyonlar, ağırlıklı olarak DMD geninin 3-9 ve 45-55 ekzonlarında meydana gelir [9]. Distrofini kodlayan DMD geni, X kromozomunun Xp21.1 pozisyonunda yer almaktadır [1]. Distrofin proteini, aktin hücre iskeletini bazal laminaya bağlayan ve kas hücre zarının stabilitesini sağlayan distrofin-ilişkili protein kompleksi (DAPC) içinde kritik bir rol oynamaktadır [10]. Distrofinin amino ucu F-aktine, karboksil ucu ise sarkolemmada bulunan DAPC'ye bağlanmaktadır (Şekil 1).



Şekil 1. Distrofin proteini [11]

DAPC, distroglikanlar, sarkoglikanlar, integrinler ve kaveolin gibi bileşenlerden oluşur. Bu kompleksin herhangi bir bileşeninde meydana gelen mutasyonlar otozomal kalıtımlı kas distrofilerine neden olmaktadır. Distrofin eksikliğinde, DAPC yapısal bütünlüğünü kaybeder ve bu durum, kompleks bileşenlerinin stabilitesinde ve seviyelerinde belirgin bir azalmaya yol açar. Bunun sonucunda, kas liflerinde ilerleyici bir hasar oluşur ve hücre zarında geçirgenlik artar [11,12]. DMD hastalarının kas biyopsisinde, genellikle nekrotik veya dejeneratif kas liflerinin kümeler halinde bir araya geldiği gözlemlenir. Bu kümelenme, , kas liflerinin hasar gördüğünü ve yıkıldığını gösterirken, kas onarımının yetersiz olduğunu ve kas dejenerasyonunun sürekli devam ettiğini işaret eder [13]. Hastalığın erken evresinde, küçük olgunlaşmamış merkezi çekirdekli lifler de gözlenir. Bu nekrotik lifler makrofajlar ve CD4 lenfositler tarafından çevrelenmiştir. Zamanla, kasların rejeneratif kapasitesi tükenir ve kas lifleri, yavaş yavaş yerini bağ ve yağ dokusuna bırakır [14]. Bu kademeli kas dejenerasyonu, ergenlik döneminde yürüme kaybına ve ilerleyen yaşla birlikte solunum ve kalp yetmezliğine yol açarak DMD hastalarının yaşam beklentisini önemli ölçüde azaltır [12]. DMD'li pediatrik hastalarda kemik kırılganlığı ile birlikte şiddetli osteoporoz bildirilmiştir [15]. Ayrıca pediatrik DMD'de vertebra kırıkları, kronik sırt ağrısına ve omurga deformitesine neden olurken, uzun kemik kırıkları erken ve kalıcı yürüme kaybına zemin hazırlar [16].

Nükleer Faktör Kappa

NF-κB, çoklu bağışıklık ve inflamatuar genlerin ifadesini düzenleyen, yapısal olarak Rel ailesine ait ökaryotik transkripsiyon faktörlerini içeren bir protein ailesidir [17,18]. İlk kez 1986 yılında Sen ve Baltimore tarafından, B hücrelerinde kappa, immünoglobulin arttırıcı bölgelere bağlanabilen bir nükleer faktör olarak tanımlanmıştır [19]. NF-κB ailesi, iki grupta sınıflandırılabilen beş üyeden oluşur [17]. İlk grup, dimerizasyon ve DNA'ya bağlanmak için gerekli olan bir N-terminal Rel homoloji bölgesi (RHD) ve C-terminal transkripsiyonel aktivasyon bölgesine sahip olan RelA (p65), RelB ve c-Rel proteinlerinden oluşur. İkinci grup ise NF-κB1 (p50) ve NF-κB2 (p52) proteinlerini içerir. Bu proteinler, sırasıyla büyük öncül p105 ve p100 olarak sentezlenir ve her biri bir N-terminal RHD ve bir C-terminal ankyrin tekrar bölgesi içerir. Proteolitik işlemle p105 ve p100, sırasıyla p50 ve p52 proteinlerine dönüşür [20].

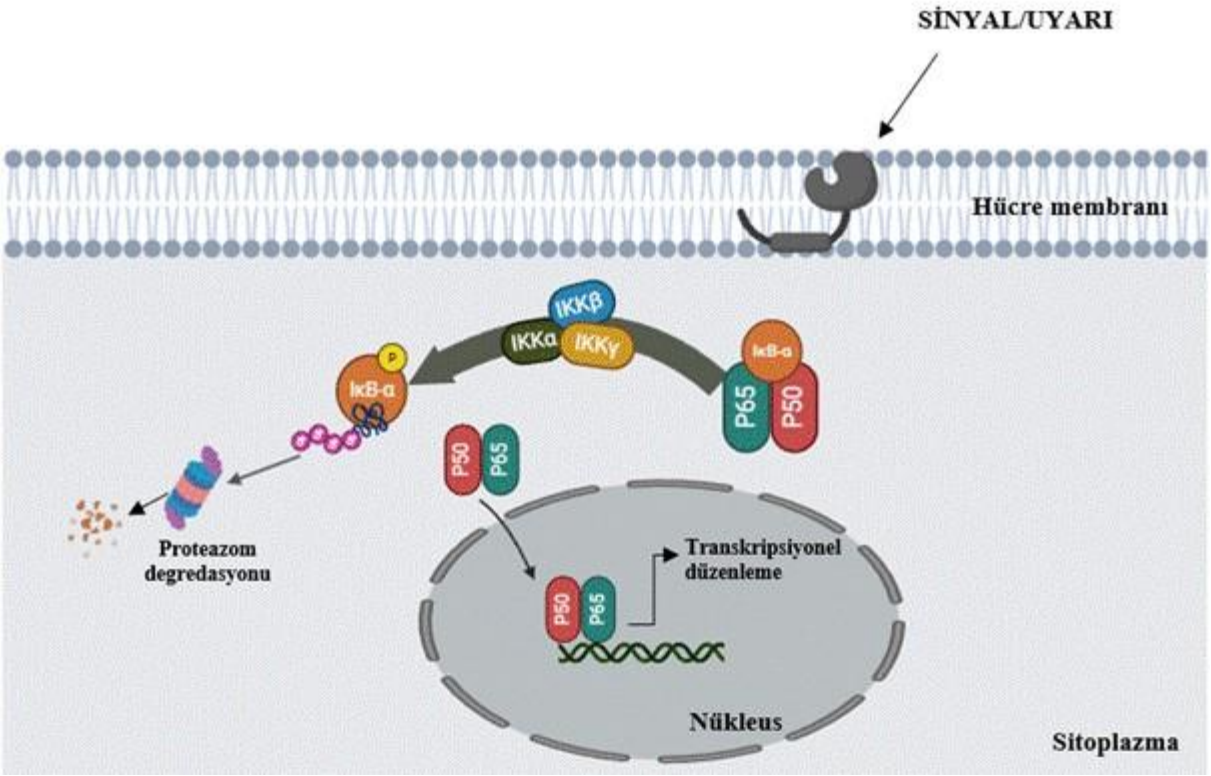
Bu faktörün ilk olarak olgun B hücrelerinde ve plazma hücrelerinde immünoglobulin kappa (IgK) gen transkripsiyonunu aktive ettiği bulunmuştur. Bununla birlikte, NF-κB'nin artık çok çeşitli hücrelerde ifade edilen ve bir dizi çeşitli gen ürününün oluşumunda rol oynayan transkripsiyon faktörü olduğu bilinmektedir [21]. Sitokinler, kemokinler, hücre yapışma molekülleri, büyüme faktörleri, immünoreseptörler, akut faz proteinleri, transkripsiyon faktörleri ve oksidatif stresle ilgili enzimler gibi birçok indüklenebilir gen, NF-κB bağlanma bölgeleri aracılığıyla düzenlenir. Bu bağlanma bölgeleri, genlerin promotörlerinde veya arttırıcı bölgelerinde bulunur ve NF-κB, bu genlerin çalışmasını ve

üretimlerini düzenler. Bu mekanizma, inflamasyon, bağışıklık yanıtları ve hücresel stres tepkilerinin modülasyonunda kritik bir rol oynar [2].

Nükleer Faktör Kappa'nın Sinyalleme Yolları

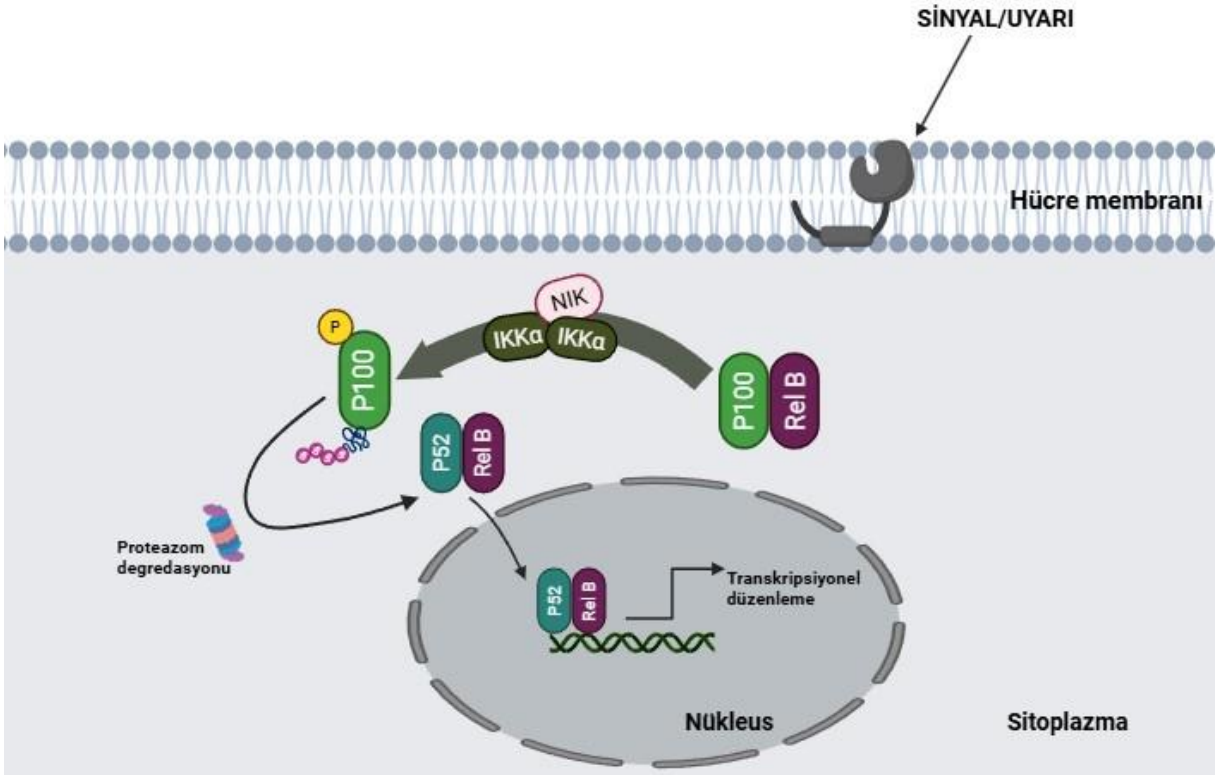
NF-κB çeşitli sinyal yolları aracılığıyla aktive olan bir transkripsiyon faktörüdür. NF-κB'nin aktivitesi, inhibitör IκB proteinleri ile etkileşim yoluyla düzenlenir. NF-κB dimerleri için farklı afinitelere sahip IκB proteini (IκB-α, IκB-β, IκB-γ, IκB-ε) vardır. Bu IκB'ler ayrıca, fosforilasyon ve proteoliz ile farklı şekillerde sinyal verir [22]. NF-κB sinyalleme yolları ikiye ayrılır: Kanonikal (klasik) yol ve kanonik olmayan (alternatif) yol.

Kanonikal (klasik) yol: Kanonikal yolda çeşitli sinyal veya uyarılar membran reseptörü aracılığıyla hücrenin içerisine girer. Bu reseptörler aracılığıyla verilen sinyaller, IκB kinaz (IKK) kompleksinde (IKKα, IKKβ ve IKKγ'dan oluşur) birleşir ve burada fosforilasyon ve aktivasyona neden olur [23]. Aktive olan IKK, NF-κB'ye bağlı bulunan IκB-α'nın fosforilasyonunu ve ardından ubikitin-proteazom yoluyla parçalanmasını tetikleyerek, bağlı halde bulunan NF-κB dimerini serbest bırakır. NF-κB'nin IκB-α'dan ayrılması onu aktif forma çevirir. Aktive olmuş NF-κB, porlar aracılığı ile nükleusa geçer. Burada kendilerine özgü yanıt elemanlarına bağlanarak, transkripsiyonu başlatır (Şekil 2) [23,24].



Şekil 2. Kanonikal (klasik) yol (BioRender uygulaması ile tasarlanmıştır. P, fosfor; IKK, IκB kinaz)

Kanonik olmayan (alternatif) yol: Kanonik olmayan NF-κB sinyal yolağı p100'ün işlenmesine dayanan farklı bir mekanizma yoluyla p52/RelB heterodimer kompleksinin aktivasyonundan sorumlu bir yoldur [25]. NF-κB-indükleyici kinaz (NIK), kanonik olmayan yolun merkezi bir sinyal bileşeni p100 fosforilasyonunu ve işlenmesini tetiklemek için IKKα'yı aktive eder. NIK, IKKα'yı fosforile eder, bunun sonucunda p100 fosforile olur, p100'ün ubikitinasyonunu ve degradasyonunu tetikleyerek p52'yi ve RelB-p52 heterodimerinin nükleer translokasyonunu sağlar. Nükleusta kendilerine özgü yanıt elemanlarına bağlanarak, transkripsiyonu başlatır (Şekil 3) [23,26].



Şekil 3. Non-kanonikal (alternatif) yol **(**BioRender uygulaması ile tasarlanmıştır. P, fosfor; IKK, IκB kinaz; NIK, nükleer faktör kappa indükleyici kinaz)

Duchenne Kas Distrofisi'nde Nükleer Faktör Kappa Yolu

NF-κB, kas kaybına neden olan interlökin-1β (IL-1β), tümör nekroz faktörü-α (TNF-α), metalloproteinazlar gibi çeşitli inflamatuar moleküllere yanıt olarak aktive olur [27]. Son yıllarda yapılan araştırmalar, özellikle DMD'li hastaların iskelet kası kütlesinin düzenlenmesinde NF-κB'nin önemli işlevini açıklığa kavuşturmuş ve NF-κB'nin kas kaybı sürecinde olası bir rolü olduğunu ortaya koymuştur [27]. NF-κB'nin artan aktivitesinin iskelet kası atrofisine neden olduğunu açıklayan birtakım mekanizmalar vardır. Bunlardan ilki, NF-κB'nin, atrofi sırasında spesifik kas proteinlerinin yıkımında rol oynayan ubikitin-proteazom sisteminin çeşitli proteinlerinin ekspresyonunu arttırmasıdır. İkincisi, NF-κB'nin, doğrudan veya dolaylı olarak kas kaybını uyaran, inflamasyonla ilişkili moleküllerin ekspresyonunu arttırmasıdır. Üçüncüsü, NF-κB'nin atrofik iskelet kaslarının rejenerasyonu için gerekli olabilecek miyojenik farklılaşma sürecine müdahale ederek iskelet kası atrofisine neden olmasıdır [28].

Araştırmalar, NF-κB aktivitesinin DMD hastalarının ve mdx farelerinin (distrofin geni eksik fareler) kaslarında arttığını göstermektedir [27,29]. NF-κB'nin aktivasyonunu araştırmak için yapılan bir çalışmada, DMD hastalarının kas örnekleri Western blot ve Elektroforetik Hareketlilik Kayması Testi (EMSA) analizi ile incelenmiş ve hastaların kaslarında NF-κB aktivitesinin arttığı gözlenmiştir [29]. Yapılan in vivo çalışmada, mdx farelerinde NF-κB inhibitörü olan pirolidin ditiyokarbamat (PDTC)'ın DMD'nin neden olduğu kas hasarına karşı koruyucu özellik gösterdiği belirlenmiştir [20]. Başka bir çalışmada mdx farelerinin gastroknemius kasına proteazom inhibitörleri Velvace® (bortezomid) ve MLN273 lokal uygulandığında, a-distroglikan, b-distroglikan, a-sarkoglikan ve distrofin ekspresyonunun arttığı ve NF-κB aktivitesinin azaldığı gözlemlenmiştir [30]. Yapılan bu çalışmalar, NF-κB'nin çeşitli fizyolojik ve patofizyolojik koşullarda iskelet kası kütlesinin kaybıyla bağlantılı en önemli sinyal yollarından biri olduğunu vurgulamaktadır. İskelet kasında NF-κB'nin aktivasyonu, spesifik kas proteinlerinin bozulmasına yol açar, inflamasyonu indükler, fibrozis ve atrofi sonrası miyofiberlerin rejenerasyonunu engeller [28].

Nükleer Faktör Kappa Yolunun Tedavide Kullanımı

1986 yılında NF-κB'nin keşfedilmesinden bu yana, NF-κB sinyal yolunun inflamatuar yanıtın düzenlenmesindeki rolü ve çeşitli hastalıklarla olan ilişkisini ortaya koyan çok sayıda çalışma gerçekleştirilmiştir. Bu araştırmalar, birçok hastalıkta kullanılan ilaçların NF-κB sinyal yolunu modüle ettiğini göstermiştir [31]. Örneğin, bir çalışmada ibuprofenin T hücrelerinde NF-κB aktivasyonunu inhibe ettiği rapor edilmiştir [32]. Benzer şekilde, flurbiprofenin R stereoizomerinin hem in vitro hem de in vivo modellerde NF-κB aktivasyonunu etkili bir şekilde baskılayabildiği gösterilmiştir [33]. Araştırmalarda incelenen diğer non-steroidal antiinflamatuvar ilaçlar (NSAİİ'ler) arasında, asetaminofenin de NF-κB sinyal yolunu etkilediği gösterilmiştir [4]. Özellikle, asetaminofenin, lipopolisakkarit (LPS) ve interferon ile uyarılan makrofajlarda, NF-κB'nin DNA'ya bağlanmasını inhibe etme yeteneğine sahip olduğu saptanmıştır [34]. Başka bir çalışmada, Sülfasalazinin, SW620 kolon hücrelerinde TNF-α, LPS veya forbol esterleri ile indüklenen NF-κB aktivasyonunu inhibe ettiği gösterilmiştir [35]. Sulindak'ın, IKK kinaz aktivitesini azalttığı ve NF-κB aktivasyonunu inhibe ettiği rapor edilmiştir [36].

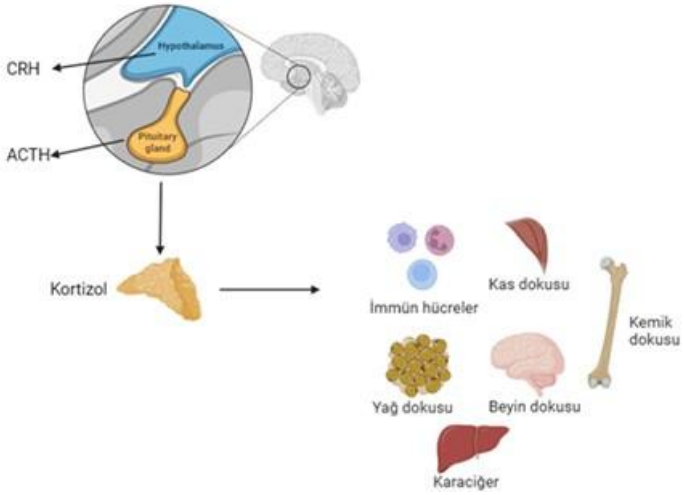
Hem siklooksijenaz (COX) hem de 5-lipoksijenazı inhibe eden tepoksalin'in, HeLa hücrelerinde ve Jurkat T-hücrelerinde NF-κB aktivasyonunu inhibe ettiği belirlenmiştir [37]. Antimalaryal ilaç meflokin'in, IκBα kinaz aktivasyonunu bloke ettiği, IκB-α degradasyonunu azalttığı, p65 fosforilasyonunun azalmasına ve kolorektal kanser hücresinde (CRC) NF-κB hedef genlerinin baskılanmış ekspresyonuna yol açtığı bulunmuştur [38]. 1995 yılında iki ayrı araştırmacı grubu, glukokortikoidlerin IkB-α proteininin ekspresyonunu artırdığını, böylece NF-κB'nin sitoplazmada tutulduğunu ve inaktif hale geldiğini göstermiştir [4]. NF-κB aktivasyonunda IKK kompleksi tarafından Ser32 ve Ser36 serin kalıntılarının fosforilasyonu ile başlatılan IkB-α'nın proteazoma bağlı bozulması aktivasyonda kilit bir adımdır. Laktasistin, bortezomid gibi çeşitli proteazom inhibitörleri güçlü antiinflamatuar ilaçlardır [39]. Hayvan inflamasyon modellerinde, spesifik proteaz inhibitörlerinin, LPS ile indüklenen NF-κB aktivasyonunun yanı sıra TNF-α ve IL-6 üretimini bloke edebildiği gösterilmiştir [4].

Farmakolojik Tedaviler

Glukokortikoidler

Glukokortikoidler, ilk olarak 19. yüzyılda Dr. Thomas Addison tarafından tanımlanmıştır. Addison, kronik yorgunluk, kas dejenerasyonu, kilo kaybı ve ciltte hiperpigmentasyon gibi semptomlarla karakterize bir durumu incelemiş ve bu hastaların adrenal ekstraktlardan terapötik faydalar sağlayabileceğini öne sürmüştür [40]. Bu hastalık günümüzde Addison hastalığı olarak bilinen bir çeşit adrenal yetmezliktir. 1946'da Edward Calvin Kendall adrenal özütlerden dört steroidal bileşik izole etmiş ve bunlara A, B, E ve F bileşikleri adını vermiştir [41]. Bileşik E daha sonra Sarett tarafından sentezlenmiştir. Tedavi edici etki potansiyeli romatolog Philip Hench tarafından romatoid artritli bir hastada denenmiştir [42]. Hench ve Kendall, adrenallerden birkaç steroid hormonu izole etmeyi başaran ve sonunda kortizolün keşfine yol açan Tadeus Reichstein ile birlikte 1950 yılında Nobel Tıp ve Fizyoloji ödülüne layık görülmüştür [43]. Antiinflamatuar etkilerinin keşfedilmesinden bu yana glukokortikoidler çeşitli inflamatuar hastalıkları tedavi etmek için kullanılmıştır [42]. Ayrıca, günümüzde DMD hastalığında en yaygın kullanılan ilaç grubunu oluşturmaktadır.

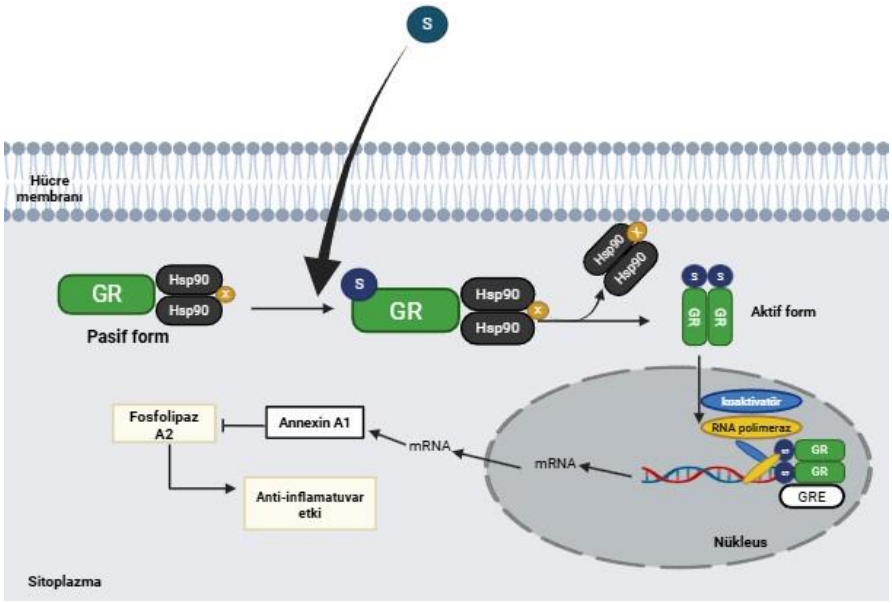
Glukokortikoidler, dokuya özgü transkripsiyonel programları düzenleyerek hem bazal fizyolojik süreçlerin hem de stres yanıtlarının koordinasyonunda rol oynayan endojen hormonlardır. İnsanlarda birincil endojen glukokortikoid kortizoldür, farelerde ise kortikosteron baskın formdur [44]. Glukokortikoidler, 11-hidroksisteroid dehidrogenaz 1 (11-HSD1) tarafından kortizonu kortizole veya deoksikortikosteronu kortikosterona dönüştürerek lokal olarak aktive edilir [45]. Glukokortikoid sentezi hipotalamik-hipofizadrenal (HPA) eksen tarafından düzenlenir. HPA ekseni, otonom sinir sistemi ile birlikte hem fiziksel hem de fizyolojik temel stres yanıt mekanizmasıdır [46]. HPA ekseninin ana aktivatörü kortikotropin salgılatıcı hormondur (CRH). Aktivasyon üzerine CRH, ön hipofize ulaşır ve burada kortikotrof hücrelerden adrenokortikotropik hormon (ACTH) sentezini ve salınımını uyarır. ACTH daha sonra kan dolaşımına salınır [47]. CRH tarafından aktive edilen ACTH adrenal bezin uyarılmasını sağlayarak kortizol salgılatır. Salgılanan kortizol immün hücreler, kas dokusu, beyin dokusu, kemik dokusu, yağ dokusu, karaciğer gibi yapıları etkiler (Şekil 4).



Şekil 4. Kortizol salgılanma döngüsü (BioRender uygulaması ile tasarlanmıştır. CRH, kortikotropin salıverici hormon; ACTH, adrenokortikotropik hormone)

Etki Mekanizması

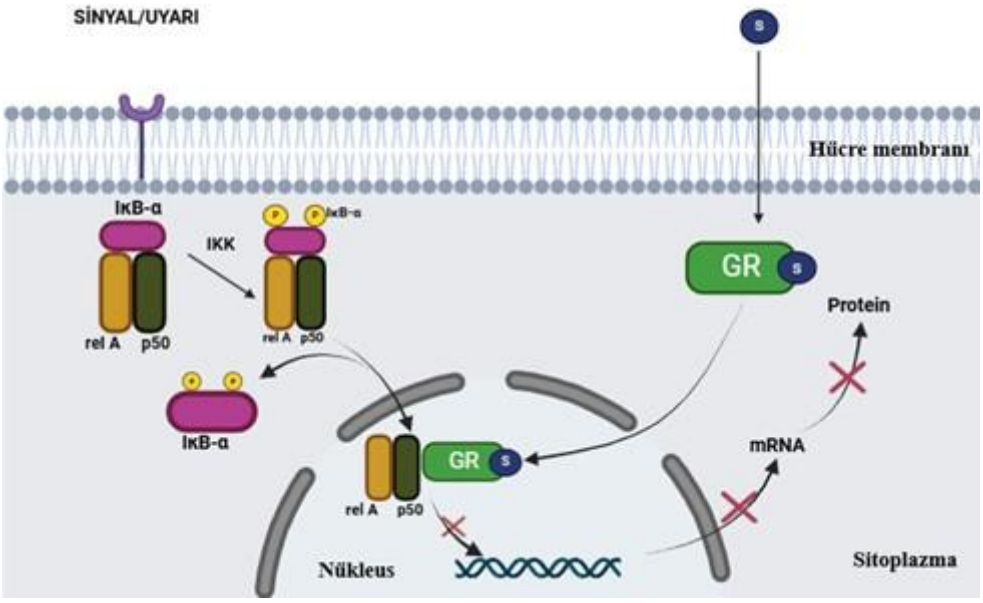
Lipofilik yapılı glukokortikoidler hücre membranından geçerek ısı şok proteinine (hsp90) bağlı olarak inaktif durumda olan glukokortikoid reseptörüne (GR) bağlanır. Ligand reseptöre bağlanınca hsp90 reseptörden ayrılır [48]. Daha sonra reseptör-hormon kompleksi dimerize olup (aktif form) nükleusa geçerek orada kendilerine özgü glukokortikoid yanıt elemanına (GRE) bağlanarak transkripsiyon regülasyonu sağlanır. Glukokortikoidler, Annexin A1 proteinini oluşturarak araşidonik asit kaskadındaki fosfolipaz A2 enzimini inhibe eder ve inflamasyonu baskılar (Şekil 5) [49].



Şekil 5. Glukokortikoidlerin etki mekanizması (BioRender uygulaması ile tasarlanmıştır. S, steroid; GR, glukokortikoid reseptörü; Hsp90, ısı şok protein 90; GRE, glukokortikoid yanıt elemanı)

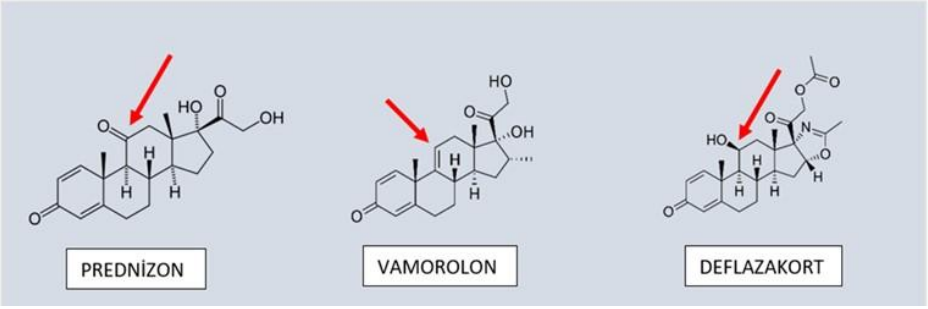
Glukokortikoidler, glukokortikoid reseptörlerini aktive eder ve daha sonra NF-κB'ye bağlanarak inflamatuar süreçlerde rolü olan genler üzerindeki bölgelere bağlanmasını önler. NF-κB'nin aktivasyonu

glukokortikoidler tarafından engellenir [50]. Glukokortikoid-reseptör kompleksleri NF-κB'nin p65 alt birimine bağlanır ve sonuç olarak inflamatuar genlerin NF-κB aktivasyonu önlenir. IkB-α sentezi, glukokortikoid-reseptör komplekslerinin IkB-α geninin promotör bölgesindeki bir glukokortikoid yanıt elementine bağlanmasıyla uyarılır (Şekil 6) [51].



Şekil 6. Glukokortikoidlerin nükleer faktör kappa üzerine etkisi **(**BioRender uygulaması ile tasarlanmıştır. GR, glukokortikoid reseptörü; S, steroid; P, fosfor)

Prednizon/prednizolon ve deflazakort, DMD tedavisinde mevcut standart tedavidir [52]. Kortikosteroid kullanan DMD hastaları sıklıkla fiziksel görünümde değişiklikler, katarakt riskinde artış, duygudurum bozuklukları ve osteoporoz, büyüme yetersizliği, obezite, gecikmiş ergenlik, glukoz intoleransı ve sekonder adrenal yetmezlik gibi endokrin sorunlar yaşamaktadır [53]. Bu yüzden, geleneksel kortikosteroidler kadar etkili olabilen, ancak daha az yan etki profiline sahip ajanlar geliştirilmekte ve araştırılmaktadır. 2023 yılında vamorolon adlı etken madde Gıda ve İlaç İdaresi (FDA) tarafından onay almıştır [54]. Kas distrofisi tedavisi için geliştirilen oral, seçici, dissosiyatif bir kortikosteroid olan vamorolon (AGAMREE®), diğer kortikosteroidler ile aynı reseptöre bağlanır, ancak farklı bir kimyasal yapıya sahiptir. Prednizon/prednizolon ve deflazakortun aksine, vamorolon steroidal C halkası üzerindeki 11β-hidroksil/karbonil parçasına sahip değildir (Şekil 7) [55].

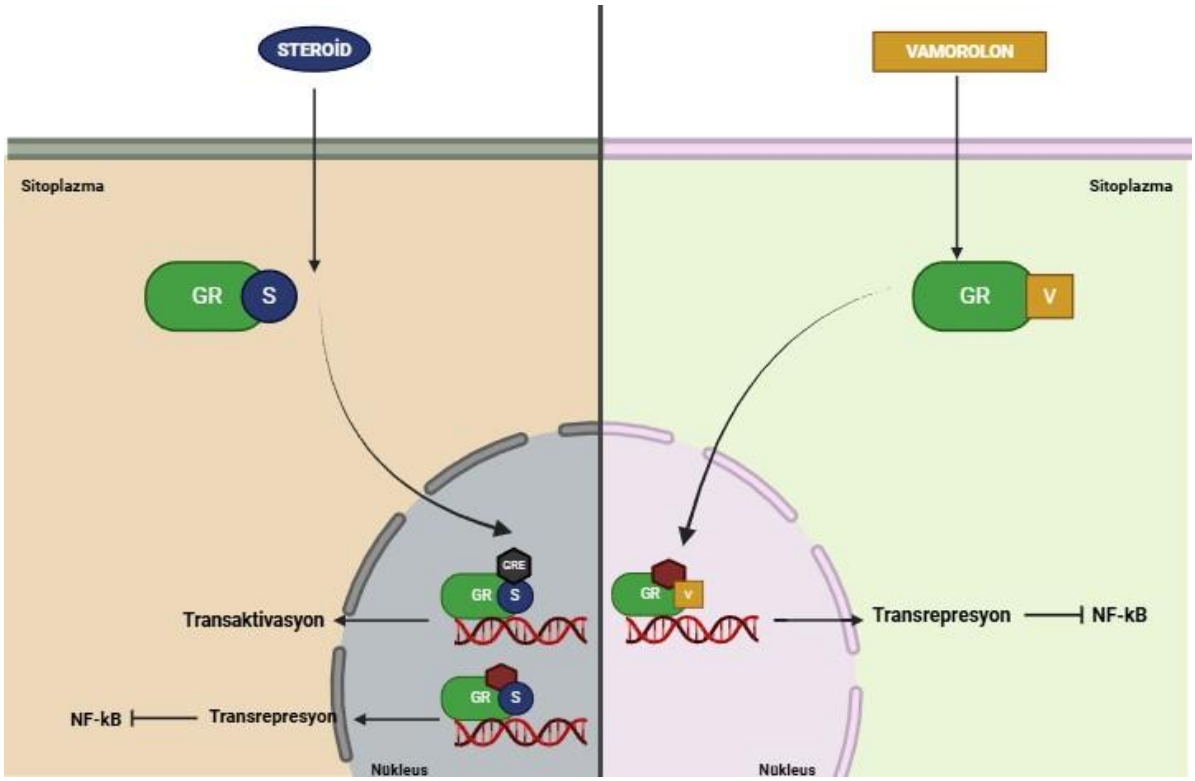


Şekil 7. Prednizon-Vamorolon-Deflazakort Kimyasal Yapısı (BioRender uygulaması ile tasarlanmıştır)

Vamorolon

Vamorolon, ReveraGen BioPharma ve Santhera Pharmaceuticals tarafından DMD tedavisi için geliştirilen bir ilaçtır. 2023 yılında, FDA tarafından Amerika Birleşik Devletleri'nde (ABD) 2 yaş ve üzeri hastalarda DMD tedavisi için onaylanmıştır. Aynı yılın Ekim ayında, Avrupa Birliği'nde (AB) 4 yaş ve üzeri hastalarda DMD tedavisi için olumlu görüş almıştır [54,55]. Vamorolon diğer kortikosteroidlerle aynı reseptöre bağlanır fakat farklı bir kimyasal yapıya sahiptir [56]. Prednizon ve deflazakort gibi kortikosteroidler, mineralokortikoid reseptörünün agonistleridir ve renin-anjiyotensin yolu ile basıncının artmasına neden olur. Fakat vamorolon, eplerenon ve spironolaktona benzer şekilde mineralokortikoid reseptörünün güçlü bir antagonistidir [57]. Bu da distrofik kardiyomiyopati ve kardiyovasküler morbiditeye karşı koruyuculuk sağlar [58].

Vamorolon'un, geleneksel kortikosteroid ilaçlardan farklı etkileri, benzersiz bir etki mekanizmasına dayanmaktadır. Bu mekanizma, GRE aracılığıyla gen transkripsiyonel aktivitelerinin belirli bir kısmının kaybına, mineralokortikoid reseptörü üzerinde güçlü antagonist aktivite göstermesine ve üstün membran stabilizasyon özelliklerine bağlanmaktadır [57]. Vamorolon, kortikosteroidler gibi glukokortikoid reseptörünü hedef alır, ancak geleneksel kortikosteroidlerin aksine GRE transaktivasyonuna yol açmaz [59] (Şekil 8). GRE'ler ciddi yan etkilere yol açan transkripsiyonel yollara aracılık eder. Bu ayırt edici özelliği ile hedef dışı etkilere neden olmadan etkinliğini sürdürmesini sağlayarak, prednizon ve deflazakort gibi günümüzde kullanılan steroidlere göre önemli ölçüde iyileştirilmiş bir güvenlilik profili sağlar [60].



Şekil 8. Vamorolon'un nükleer faktör kappa üzerindeki etkisi (BioRender uygulaması ile tasarlanmıştır. GR, glukokortikoid reseptörü; S, streroid; V, vamorolon; GRE, glukokortikoid yanıt elemanı)

Hayvan çalışmalarında, vamorolon, prednizolon ve deflazakortun kan-beyin bariyerini geçme özellikleri karşılaştırılmıştır. Farelere oral gavaj yoluyla uygulanan bu ilaçların plazma ve beyin konsantrasyonları ölçülmüş ve vamorolon'un beyin-plazma oranının diğer iki ilaçtan daha yüksek olduğu tespit edilmiştir. Bu tespit üzerine vamorolon'un fareler üzerinde depresyon benzeri davranışa

neden olduğu gösterilmiştir. MDR1-MDCK geçirgenlik deneyinde, prednizolon ve deflazakortun P-glikoprotein (P-gp) substratları olduğu ve hücre membranı boyunca geri pompalandığı, vamorolon'un ise daha düşük efflux oranına sahip olduğu, dolayısıyla daha zayıf bir P-gp substratı olduğu gösterilmiştir [52]. Üç kortikosteroidin de farelerde endojen kortikosteron seviyelerini azalttığı, glikoz düzeylerini yükselttiği ve osteokalsin seviyelerini düşürdüğü gözlemlenmiştir. Bu bulgular, vamorolon'un güçlü bir P-gp substratı olmadığını ve merkezi sinir sistemine geçerek potansiyel yan etkileri artırabileceğini ortaya koymaktadır. [52].

Vamorolon'un etkililiği ve güvenliliği, klinik çalışmalarla değerlendirilmiş ve elde edilen sonuçlar umut verici bulunmuştur. Faz I çalışmasında, vamorolon'un güvenliği, tolere edilebilirliği ve farmakokinetiği, 86 sağlıklı yetişkin erkek üzerinde randomize, çift kör, plasebo kontrollü olarak tekli artan doz (SAD) ve çoklu artan doz (MAD) protokolleriyle değerlendirilmiştir. SAD protokolünde, 0.1-20.0 mg/kg doz aralığında yapılan tekli uygulamalarda, plazma konsantrasyonları ve farmakokinetik parametrelerin doz artışıyla birlikte yükseldiği gözlenmiştir. MAD çalışmasında ise 1.0-20.0 mg/kg/gün dozları 14 gün boyunca verilmiş ve benzer şekilde, doz artışıyla birlikte yükselme kaydedilmiştir. Sağlıklı erkeklerde yapılan faz I çalışmasında vamorolon, doz-lineer farmakokinetik ile yaklaşık 2.0 L/saat/kg'lık klerens ve ortalama 2-3 saatlik bir yarılanma ömrü göstermiştir. Aynı zamanda vamorolonun besinle birlikte alınması biyoyararlanımı yaklaşık iki katına çıkarmıştır. Farmakodinamik biyobelirteçlerde ise vamorolon kemik döngüsü biyobelirteçlerinde (osteokalsin, prokollajen tip I, C-terminal telopeptid) glukokortikoidlere kıyasla anlamlı düzelmeler göstermiştir [59].

Faz IIb randomize çalışmada, kortikosteroid tedavisi almamış, genetik olarak doğrulanmış duchenne kas distrofisi (DMD) tanılı 4-7 yaş arası erkek çocukların basamak çıkma süresi (TTCLIMB) ve North Star Ambulatuvar Değerlendirmesi (NSAA) skorları incelenmiştir. Çalışma sonunda, 6 mg/kg/gün vamorolon alan gruptaki çocuklar, 6 dakika yürüme testi ile değerlendirilmiştir. Çalışma, 5 tedavi grubu ile gerçekleştirilmiş ve tedavi süreleri 24-52 hafta arasında değişmiştir. Tedavi grupları, farklı vamorolon dozları (2.0 ve 6.0 mg/kg/gün), prednizon grubu ve prednizon gruplarındaki hastaların vamorolon tedavisi (günde 2 veya 6 mg/kg) almak üzere çapraz geçiş yaptığı hastalardan oluşmuştur. Etkililik değerlendirmeleri, sırtüstü pozisyondan ayağa kalkma süresi (TTSTAND), 6 dakika yürüme testi (6MWT), 10 m koşma/yürüme süresi (TTRW), merdiven çıkma testi ve 10 m koşma/yürüme hızında iyileşme gözlenmiştir. Ancak, bu dozlar daha güçlü etki sağlasa da, belirgin yan etkilerle ilişkilendirilmiştir, özellikle sabah kortizol seviyelerinde daha büyük azalmalar ve cushingoid özellikler, psikiyatrik bozukluklar, kilo artışı, baş ağrısı, ishal ve rinit gibi advers reaksiyonlar daha sık görülmüştür [5,61].

SONUÇ VE TARTIŞMA

DMD, erkek çocuklarda görülen, distrofin proteinin eksikliğinden kaynaklanan bir kas hastalığıdır. Hastalık ilerledikçe kas kütlesi zayıflar ve kalp, solunum sistemi problemleri ortaya çıkar. NF-κB, çoklu bağışıklık ve inflamatuar genlerin ifadesini düzenleyen bir transkripsiyon faktörüdür. NF-κB'yi etkileyen çeşitli ilaçlar bulunmaktadır. Günümüzde DMD için kullanılan glukokortikoidler NF-κB'yi inhibe ederek antiinflamatuar etkiler göstermektedir. Ancak bu ilaçların çeşitli yan etkileri olabilmektedir. Örneğin; hiperglisemi, osteoporoz, katarakt, cushing sendromu. Son yıllarda bu yan etkileri azaltan aynı zamanda DMD hastalığında etkili ilaçlar üzerine araştırmalar yapılmıştır. 2023 yılında FDA tarafından ABD'de 2 yaş ve üzeri hastalarda DMD tedavisi için onay almış olan vamorolon DMD tedavisine yenilikçi bir bakış açısı getirmiştir. Vamorolon, glukokortikoidler gibi aynı mekanizmayla hareket eder fakat farklı olarak GRE'lere bağlanmazlar. Bu özelliği sayesinde glukokortikoidlere kıyasla iyileştirilmiş bir etki profili ortaya koyarlar. Diğer bir faydalı özelliği ise prednizon ve deflazakort gibi kortikosteroidler, mineralokortikoid reseptör agonisti ilaçlardır. Renin-anjiyotensin yolu ile kan basıncının artmasına neden olur. Fakat vamorolon, mineralokortikoid reseptörünün güçlü bir antagonistidir. Bu da kardiyovasküler morbiditeye karşı koruyuculuk sağlayan bir özelliğidir. Vamorolon'un hayvanlarda ve insanlarda yapılmış çalışmaları glukokortikoidlere nazaran daha iyi sonuçlar verdiğini göstermiştir. Her ne kadar glukokortikoidlere nazaran daha iyi sonuçlar göstermiş olsada vamorolon'un yapılmış klinik çalışmalarında cushingoid özellikler, psikiyatrik bozukluklar, kusma, kilo artışı, D vitamini eksikliği, baş ağrısı, ishal, iştah artışı ve rinit gibi

yan etkiler ortaya çıkmıştır. Vamorolon'un NF-κB üzerindeki bu etkileri, gelecek çalışmalarda farklı hastalıklar üzerinde de etkisi olabileceğini düşündürmektedir.

YAZAR KATKILARI

Kavram: G.H., A.B.; Tasarım: G.H., A.B.; Denetim: G.H., A.B.; Kaynaklar: G.H., A.B.; Malzemeler: - ; Veri Toplama ve/veya İşleme: G.H., A.B.; Analiz ve/veya Yorumlama: G.H., A.B.; Literatür Taraması: G.H., A.B.; Makalenin Yazılması: G.H., A.B.; Kritik İnceleme: G.H., A.B.; Diğer: -

ÇIKAR ÇATIŞMASI BEYANI

Yazarlar bu makale için gerçek, potansiyel veya algılanan çıkar çatışması olmadığını beyan ederler.

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Ankara Ecz. Fak. Derg. / J. Fac. Pharm. Ankara, 49(3): 928-939, 2025
Doi: 10.33483/jfpau.1594859

DERLEME MAKALE / REVIEW ARTICLE

HAMİLELİK VE LAKTASYON DÖNEMİNDE RETİNOİK ASİT KULLANIMI

USE OF RETINOIC ACID DURING PREGNANCY AND LACTATION

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ÖZ

Amaç: *Retinoik asit ve türevleri başta akne vulgaris olmak üzere çeşitli cilt hastalıklarında sıklıkla kullanılan etken maddelerdir. Teratojenik etkileri olduğu bilinen bu etken maddelerin gebelik ve laktasyon dönemlerinde kullanımına sıklıkla rastlanmaktadır. Özellikle gebelik öncesi ve gebeliğin organogenez de dahil olmak üzere, bebeğin sağlıklı gelişimi için en riskli olduğu dönemde kullanımıyla karşılaşmak mümkündür. Tüm bu veriler retinoik asitin teratojenik etkileri hakkında yeterince bilgi sahibi olunmadığını göstermektedir. Bu derlemede teratojenite hakkında bilgi verilmiş, retinoik asit türevleri ve bunların teratojenik etkilerinden bahsedilmiştir. Ayrıca gebelik ve laktasyon döneminde topikal ve sistemik retinoik asit kullanımıyla ilgili literatürler incelenmiş ve yapılan araştırmaların sonuçlarına değinilmiştir.*
Sonuç ve Tartışma: *Retinoik asit ve türevlerinin teratojenik etkilerinin bilinmesi oldukça önemlidir. Bu bileşiklerin gebelik ve laktasyon döneminde, sistemik ve topical olarak kullanımına ait veriler yetersizdir. Bu konudaki değerlendirmelerin artması retinoik asit türevlerinin daha güvenli kullanımına olanak sağlayacaktır.*
Anahtar kelimeler: *Hamilelik, laktasyon, retinoik asit, retinoik asit embriyopatisi*

ABSTRACT

Objective: *Retinoic acid and its derivatives are frequently used in various skin diseases, especially acne vulgaris. The use of these active substances, which are known to have teratogenic effects, is frequently encountered during pregnancy and lactation periods. It is especially possible to encounter their use before pregnancy and during the period when pregnancy is the most risky for the healthy development of the baby, including organogenesis. All these data indicate that not enough is known about the teratogenic effects of retinoic acid. In this review, information about teratogenicity, retinoic acid derivatives and their teratogenic effects are discussed. In addition, the literature on the use of topical and systemic retinoic acid during pregnancy and lactation is reviewed and the results of the studies are discussed.*
Result and Discussion: *It is very important to know the teratogenic effects of retinoic acid and its derivatives. There is insufficient data on the systemic and topical use of these compounds during*

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Gönderilme / Submitted : 06.12.2024
Kabul / Accepted : 14.04.2025
Yayınlanma / Published : 19.09.2025

pregnancy and lactation. Increasing evaluations on this subject will allow safer use of retinoic acid derivatives.
Keywords: *Pregnancy, lactation, retinoic acid, retinoic acid embryopathy*

GİRİŞ

Gebelik sırasında ilaç kullanımı sık karşılaşılan bir durumdur [1]. Bu süreçte ilaç kullanımı sadece gebe kadında değil aynı zamanda fetüs üzerinde de endişelere neden olmaktadır [2]. Gebelik döneminde ilaçlara maruziyetin doğum kusurlarına neden olabileceği bilinmektedir. Ancak gebe kadınların bu dönemde hem reçeteli hem de reçetesiz ilaçları, riskleri ve güvenlikleri hakkında fikir sahibi olmadan kullandığı görülmektedir [2]. Mitchel ve arkadaşlarının 30.000 kadın üzerinde yaptıkları çalışmaya göre 1970'lerde doğum öncesi kullanılan farklı türlerdeki ilaçların sayısı ortalama 2.5 iken, 2000'lerde bu oranın 4.2'ye yükseldiği görülmüştür [2]. Ülkemizdeki çalışmalara baktığımızda ilaç kullanımı sebebiyle %9.8 oranında gebelik sonlandırılmıştır [3]. Bu oranlara baktığımızda kullanacağı ilaçlar hakkında gebenin yeteri kadar bilgilendirilmesi önem taşımaktadır [1].

Gebeliklerde yaklaşık %3 oranında konjenital malformasyon riski bulunur ve genellikle genetik kökenli olduğu kabul edilmektedir [3,4]. Teratojenik bir değerlendirme yapılırken bu oran bazal risk olarak kabul edilmektedir [4]. Gebelik, ilaç kullanmadan geçirilmesi gereken bir dönem olarak kabul edilse de özellikle kronik hastalığı olan kadınlar başta olmak üzere, gebelerin bir hastalık teşhisi alması sonucunda ilaç kullanması mecburi hale gelebilmektedir [1]. Bu gibi durumlarda kullanılan ilacın hem anne hem de fetüs açısından yarar/risk değerlendirilmesi yapılarak karara varılmalıdır [5]. İlacın sağlayacağı yarar, zararından fazla ise kullanılması tercih edilebilmektedir [3]. Kaplan ve arkadaşlarının 2015'te yaptığı çalışmada belirttiğine göre gebelerin %90'ından fazlasının en az bir reçeteli veya reçetesiz ilaç kullandığı görülmüştür. Ayrıca bu kullanımların %80'inin organogenez dönemini kapsayacak şekilde ilk trimesterde gerçekleştiği görülmüştür [1]. Bunların yanında tüm gebeliklerin yaklaşık yarısının planlı bir şekilde gerçekleşmediği, bu nedenle de gebeliğin farkedilmediği dönemde ilaçlara maruziyetin de sık olduğu bilinmektedir [1].

1957 yılında sedatif ve antiemetik amaçlarla 50'den fazla ülkede gebeler tarafından kullanılan Talidomid isimli etken madde, bebekler üzerinde meydana getirdiği konjenital malformasyonların farkedilmesiyle beraber 1961 yılından itibaren çeşitli ülkelerde piyasadan çekilmiştir. 10.000'den fazla bebeğin esktremite defektleri başta olmak üzere çeşitli konjenital malformasyonlara sahip olması nedeniyle sağlık alanındaki en büyük facialardan biri olarak kabul edilmektedir. Bu facia ile ilaçların piyasaya sürülmeden önce teratojenik değerlendirmelerinin yapılması konusunda büyük bir farkındalık oluşmuştur ve teratojenite testleri geliştirilmeye başlanmıştır [6]. Ancak ilaçlar onaylanmadan önce yapılan klinik çalışmalarda gebeler, etik nedenlerle yer alamamaktadır. Teratojenite çalışmaları hayvan deneyleri ve klinik gözlemlerle sınırlı kalmaktadır, bu da teratojenik riskler konusunda bilgileri azaltmaktadır [7]. Teratojenite hakkındaki riskin olduğundan fazla gözükmesiyle gebe kadın ilacı kullanmaya çekinebilir ve tedavide başarısızlık oluşabilirken, olduğundan az gözükmesiyle de fetüste malformasyon riski artabilir [8]. Teratojenite, gelişimsel toksisitenin bir belirtisidir ve yavruda yapısal bozukluklara yol açmaktadır veya bu bozuklukların sıklığını arttırmaktadır. Teratojen ise bu kusurlara yol açan etkenin kendisidir. Teratojenler sıklıkla çevresel ajanlardan oluşmaktadır. Tablo 1'de teratojen ajanlar sınıflandırılmıştır [7].

Tablo 1. Teratojen maddelerin sınıflandırılması [7]

Fiziksel ajanlar	X ışınları vb.
Kimyasal ajanlar	İlaçlar, pestisitler, ağır metaller vb.
Enfeksiyöz ajanlar	Kızamıkçık virüsü, zika virüsü vb.
Annenin hastalık ve durumları	Diyabet, preeklampsi (gebelik zehirlenmesi) vb.

Teratojenlerin etkileri çok çeşitlidir. Teratojen ajanların etkilerini aşağıdaki gibi sıralayabiliriz [7].

- Düşük (abortus)

- Büyüme ve gelişme bozuklukları
- Organlarda morfolojik bozukluklar
- İntrauterin gelişimde gecikme
- Prematüre doğum
- Fetal ölüm
- Zihinsel gelişimde gecikme
- Fizyolojik fonksiyonlarda bozukluk (körlük, sağırlık vb)
- Kanser oluşumu
- Erken veya geç olgunlaşma
- Genetik değişim

Teratojenlerin fetüs üzerindeki etkileri; maruz kalınan ilacın türüne, maruziyet miktarına, maruziyet sıklığına, plasentaya ve anne sütüne geçişine, ilacın dağılımına, metabolizmasına, atılımına ve gebeliğin hangi döneminde maruz kalındığına göre değişiklik göstermektedir [7,8]. Maruziyet gebeliğin preimplantasyon döneminde (1-2. haftalar) olduğunda gebelik düşükle sonlanabilir veya bir etki görülmeyebilir. Buna "ya hep ya hiç kuralı" denmektedir. Bundan sonraki dönem ise organogenez dönemi olduğundan en duyarlı dönem olarak kabul edilmektedir. Ardından gelen fetal dönemdeki maruziyetler minör malformasyonlar, büyüme geriliği ve fonksiyon bozukluklarına sebep olabilmektedir [7]. Ayrıca anne sütüne difüzyon aracılığıyla geçebilen ilaçlar da bebek üzerinde teratojen etkiler yaratabilir [8].

Retinoik Asit ve Türevleri

A vitamini (retinol) yağda çözünen vitaminlerden biridir. İzotretinoin (13-cis-retinoik asit) ve tretinoin (all-trans-retinoik asit) A vitamininin doğal türevleridir [9]. Retinoidlerin gelişim, epitel ve iskelet yapılarının normal büyümesi, görme, bağışıklık, üreme ve dış doku sistemleri gibi birçok biyolojik sistemi kontrol ettiği ve bu sistemlerin devamlılığını sürdürdüğü bilinmektedir [10]. Beyin, yüz, kalp, timüs ve omuriliğin gelişimiyle de ilişkili olduğu kanıtlanmıştır [4,9]. Retinolün all-trans formu en bol olanıdır. Vücutta metabolize olmasıyla birlikte 9-cis ve 11-cis dahil aktif retinoid izomerleri üretilmektedir. CYP monooksijenazlar, alkol dehidrogenazlar ve aldehit dehidrogenazlar olmak üzere bir dizi enzim retinolü retinoik asite dönüştürebilir [11].

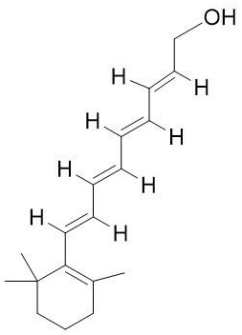
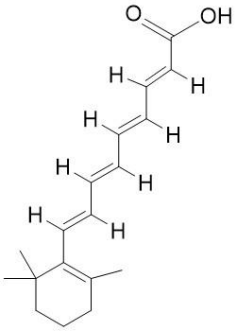
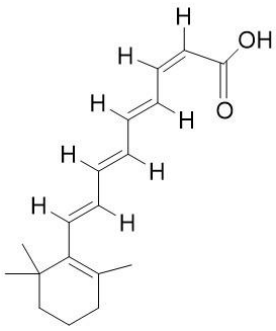
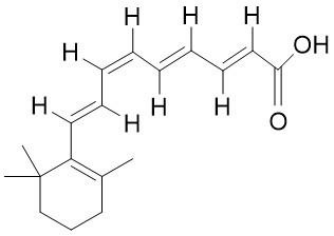
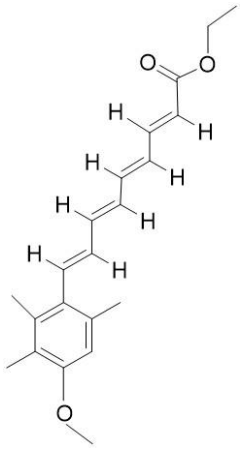
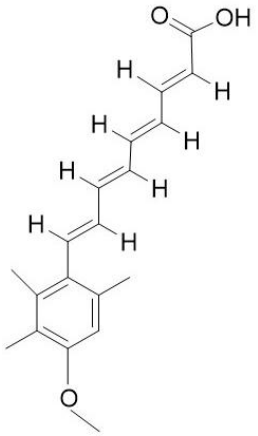
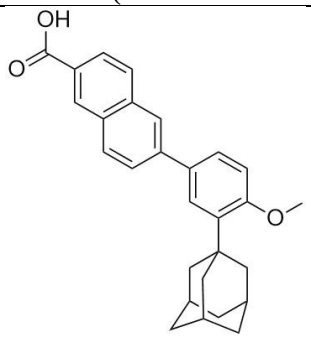
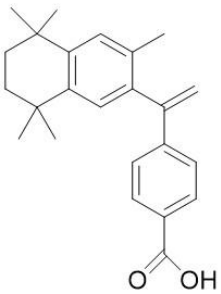
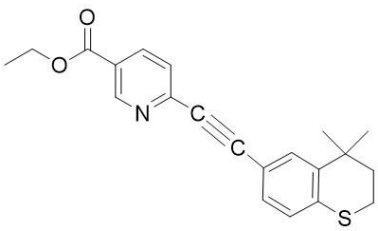
Retinoidler epidermisteki hücrelerin çoğalmasını arttırır, ciltteki keratin tabakasını gevşeterek pullanma sürecini kolaylaştırır. İzotretinoin yağ bezlerinin atrofisine yol açmaktadır. Bu özellikleriyle akne tedavisinde sıklıkla kullanılmaktadır. Retinoik asitin türevlerinden asitretin ve etretinat sedef hastalığının tedavisinde tercih edilen etken maddelerdir. Etretinat yoğun teratojenik etkileri nedeniyle piyasadan çekilmiştir. Asitretinin metabolitlerinden birinin etretinat olduğu bilinmektedir. Sentetik retinoidler arasında yer alan adapalen şiddetli akne vulgaris, çoğunlukla topikal preparatları bulunan tazarotin de sedef hastalığı tedavisinde kullanılmaktadır [9]. İzotretinoin başta olmak üzere retinoidlerin plasentadan kolaylıkla geçebildiği bilinmektedir [4,12]. Topikal preparatlar için düşünüldüğünde bütünlüğü bozuk olan veya ağır inflamasyonlu bir deride sistemik dolaşıma geçen ilaç miktarının da artacağı bilinmektedir. Aynı zamanda dezenfektanlarla beraber kullanılması ve uygulama bölgesinin geniş olması da deriden daha fazla ilaç geçmesine sebep olmaktadır [4]. Tablo 2'de retinoik asit türevlerinin bileşik yapıları verilmiştir.

Retinoik Asitin Kullanıldığı Endikasyonlar

İzotretinoin genç yaş grubunda akne vulgaris başta olmak üzere, güneşe bağlı deri hasarında, sedef hastalığında ve hiperpigmentasyon gibi dermatoljik rahatsızlıklarda sıklıkla tercih edilen sentetik bir retinoid türevidir [13-15]. Aynı zamanda kozmesötiklerde de sıklıkla yer aldığından dolayı, doğurganlık çağındaki kadınlar bu kozmesötiklerin kullanımında birinci adaylar içerisinde yer almaktadır [14]. Kullanıcılara bakıldığında %90'ından fazlasının 13-45 yaş arası ve bunların yarısı da kadınlardan oluşmaktadır [4]. 1980'li yıllardan itibaren kistik ve inatçı akne tedavisinde topikal ve sistemik preparatlarda yer almaktadır [4,9]. İlk topikal retinoid olan tretinoinin ardından yeni ajanlar ve formülasyonların üretilmesiyle lokal yan etkiler azaltılmaya çalışılmıştır. Çeşitli farmakokinetik

çalışmalarla topikal retinoid türevlerinin sistemik emilimlerinin ihmal edilebilir seviyelerde olduğu gösterilmiştir. Ancak bu bilgiler sınırlıdır [15]. Sistemik Alitretinoin kortikosteroid tedavisine yanıt vermeyen kronik şiddetli el egzamasının tedavisinde kullanılmaktadır [9].

Tablo 2. Retinoik asit türevlerinin bileşik yapıları

		
Retinol	Tretinoin (all-trans-retinoic acid)	İzotretinoin (13-cis-retinoic acid)
		
Alitretinoin (9-cis-retinoic acid)	Etretinat	Asitretin
		
Adapalen	Bexaroten	Tazaroten

Etki Mekanizması

Retinoidler hücrelerin üremesini stimüle ederek ve yağ bezlerinde atrofi yaparak etkilerini göstermektedir [4]. Sistemik izotretinoinlerin etkileri aşağıdaki gibi sıralanabilmektedir [13].

- Yağ bezlerinin folikül epitelinde soyulmayı baskılar.
- Hiperkeratinizasyonu azaltır.

- Yağ bezlerinde lipit sentezini ve sebum üretimini inhibe eder.
- Yağ bezlerinin büyüklüğünü azaltır.
- Sebosit adı verilen sebum salgılayan hücrelerde hücre ölümüne yol açar.
- Akne sebebi mikroorganizmaların kolonizasyonunu azaltır.
- Çeşitli inflamatuvar yolakları inhibe ederek antiinflamatuvar etki gösterir.
- Androjen sentezini inhibe eder.
- Skar oluşumunu azaltır.

Retinoik Asit Embriyopatisi

Birçok klinik vaka raporunda retinoidlere prenatal maruziyet sonucu bebeklerde kraniyofasiyal malformasyonlar tespit edilmiş ve düşük riskinin arttığı görülmüştür [10,16]. 1980'li yıllardan beri oral retinoidlerin toksisitesi net bir şekilde bilinmektedir. Gebelik döneminde kullanılmaları sonucunda meydana gelen gelişim defektleri Tablo 3'te özetlenmiştir [4,14]. Bu etkileriyle talidomitten sonra en tehlikeli teratojenlerden biri olduğu kabul edilmektedir [9,14]. FDA gebelik risk kategorilerine göre X grubunda yer almakta ve kesin kontrendikasyon taşımaktadır [4]. Retinoid kullanımı sırasında gebelik önleyici yöntemlerin kullanılması da bu nedenle önem arz etmektedir [14].

Karakteristik retinoik asit embriyopatisinde; kulak anomalileri (işitme kanalı agenezisi veya stenozu), yüz ve palatin defektleri, mikrognati, kardiyovasküler (konotrunkal) defektler ve timusun ve merkezi sinir sisteminin gelişimsel defektleri (göz ve iç kulağın nörolojik hasarından hidrosefaliye kadar) sayılabilir. Ayrıca retinoik asite maruz kalmış ve şuanda 5-10 yaşlarında olan çocuklarda yapılan incelemelerde zihinsel gerilik ve özellikle görsel-mekansal yönelim ve davranışta zayıflıklar gözlenmiştir [16]. Nadir de olsa birkaç çalışmada altıncı sinir felci (göz kasının kontrolünü etkileyen bir sinir sorunu), ateşli olmayan nöbetler, stereotipik (işlevsel olmayan, tekrarlayıcı) hareketler, asimetrik ağlayan yüz ve bilateral inmemiş testisler, doğum sonrası geçici yüz asimetrisi görülen bebekler tespit edilmiştir [17,18].

Retinoidler yağda çözünen vitaminler olduğundan yağ dokuda ve karaciğerde yüksek oranlarda depolanmaktadır. Vücutta kalma süreleri uzamakta ve toksik seviyelere çıkması kolaylaşabilmektedir [4,10]. Dolayısıyla anne retinoid almayı bıraksa bile embriyo depolanmış retinoidden yararlanmaya devam edebilmektedir [10].

Tablo 3. Retinoik asite bağlı doğum kusurları [9,10]

Retinoik asite bağlı doğum kusurları
Spontan düşük
Kulak anomalileri (mikrotia, işitme kanalı stenozu vb.)
Yüz ve damak defektleri
Mikrognati (alt çenenin yetersiz gelişmesi)
Kardiyovasküler defektler
Timüs gelişim eksikliği
Merkezi sinir sistemi defektleri (hidrosefali, mikrosefali, iç kulak ve gözün nörolojik hasarları vb.)
Zihinsel yetersizlikler
Spina bifida
Uzuv defektleri

Retinoidlere maruz kalan gebelerde kaba malformasyonlar için maksimum duyarlılığın 2-5. haftalardaki maruziyet olduğu bildirilmiştir. Ancak gebelik öncesi dönemin, geri kalan organogenez döneminin ve fetal dönemin tamamının risk altında olduğu unutulmamalıdır [10]. Özellikle kulak malformasyonları retinoik asit embriyopatisinde sıklıkla karşımıza çıkmaktadır. Dış kulak kanalı gebeliğin 4. haftasında oluşmaya başlar ve gebeliğin 12. haftasında tamamlanır. 5. haftada ise beynin besleyici arterleri oluşmaya başlar ve gebeliğin 4-5. haftalarındaki maruziyet bu bölgede de malformasyonlara sebep olabilmektedir [12]. Retinoik aside maternal maruziyet fetüs üzerinde bazı nörodavranışsal etkiler de oluşturmaktadır. Davranışsal anomalilerin, ilacın gen ekspresyonunu

değiştirme ve hayvan deneylerinde kanıtlandığı üzere nöral ve glial hücre proliferasyonunu, sinaptogenezi ve apoptozu inhibe etme yeteneğinden kaynaklandığı düşünülmektedir. Ancak insanlar üzerindeki mekanizmalar belirsizliğini korumaktadır [10].

İlk üç aylık dönemde (1. trimester) sistemik olarak kullanılmasıyla doğum kusurları riskinde 10 kat artış görülmektedir, bu nedenle en kritik dönem olduğunu söylemek mümkündür [9,10]. Bu defektler göz önüne alınarak ilaçların kullanımı hakkındaki bilgiler prospektüslerde yer almaktadır. Sistemik izotretinoin kullanımının gebelikten en az 1 ay önce, asitretin ve etretinat gibi sedef hastalığında kullanılan etken maddelerin ise gebelikten en az 2 yıl önce kesilmesi uygun görülmektedir [4]. Bu ilaçları kullanan kadınların, belirtilen süreler içerisinde en az iki farklı kontrasepsiyon yöntemi ile korunmaları önerilmektedir [4,13]. Aynı zamanda ilacı kullanmaya başlayacak hastaya riskler ve bunlardan korunma yöntemleri hakkında bilgi verilerek hasta onam formu imzalatılır, ardından hasta eczaneden bu hasta onam formunu göstererek ilacını alır ve tedavisine başlayabilir [4]. Bu uygulama sistemik kullanımda mevcut olup, topikal kullanım için sadece sözlü uyarı şeklinde bilgilendirme yapılmaktadır [4]. 1980'lerin sonunda birçok ülkedeki multivitamin preparatları üreticileri, 25.000 IU ve üzerinde A vitamini içeren preparatların Teratoloji Derneği tarafından riskli görülmesiyle günlük dozu 6.000 IU'ye düşürmüştür [9].

Geçmiş yıllarda oral retinoik asit kullanımını kısıtlamak ve istenmeyen gebelik oluşmasını önlemek için çeşitli gebelik önleme programları (iPLEDGE gibi) geliştirilmiştir [15]. iPLEDGE, 2006 yılında kurulmuş ve sağlık hizmeti sağlayıcıları, hastalar, eczacılar ve doktorların zorunlu kaydını içeren bir sistemdir. Bu sistem ile doğurgan kadınlarda sistemik izotretinoin tedavisine başlamadan önce, tedavinin her ayı ve tedavi tamamlandıktan 1 ay sonrasına kadar gebelik testleri uygulanmakta ve etkili bir doğum kontrol yöntemi önerisi yapılmaktadır. Böylece tedavi sürecinde istenmeyen bir gebelik oluşumunu önlemek amaçlanmaktadır. Shirazi ve arkadaşları vaka raporunda, tedavi kesildikten 4 hafta sonra meydana gelen gebelikte, embriyopati gözlenmesi nedeniyle sistemin doğum kontrol süresinin revize edilmesi gerektiği yorumunu yapmıştır [19]. Zomerdijik ve arkadaşlarının yaptığı bir çalışmada izotretinoine maruz kalan kadınların çoğunluğunun ilk izotretinoin reçetesi sırasında zaten hamile oldukları görülmüştür. Bu da tedaviye başlamadan önce gebelik testi uygulamalarının eksikliğini göstermektedir. Uygun bir gebelik testi yapılmasıyla bu maruziyet önlenebilir. Ayrıca tedaviye başlayan kadınların yalnızca %59'unun eş zamanlı hormonal yöntemleri kullandığı görülmüştür, bu da kontraseptif yöntemler hakkındaki bilgi eksikliğini göstermektedir. Aynı zamanda bu çalışmada izotretinoin için uygulanan gebelik önleme programlarının (Pregnancy Prevention Program - PPP) başarısı sınırlı görülmüş ve geliştirilmesi gerektiği belirtilmiştir [20]. Kim ve arkadaşlarının yaptığı bir araştırmada 2010-2016 yılları arasında Kore'deki Anne Güvenliği Danışmanlık Merkezi'nde kayıtlı, izotretinoine maruz kalan hamile kadınlar değerlendirilmiştir. Avrupa Birliği ve Amerika tarafından uygulanan gebelik önleme programlarının Kore'deki eksikliği nedeniyle gebelik sırasında izotretinoine maruziyet oranları oldukça yüksek görülmüş ve sonuç olarak Kore'de gebelik önleyici program kurulması gerektiği yorumu yapılmıştır [21].

FDA risk kategorisinde retinoik asitin teratojenitesi hakkında uygulama yolu belirtilmemesi topikal uygulamalarda da risk beklentisi oluşturmaktadır [9]. Bu ayrımın olmamasından dolayı ve sağlık uzmanları teratojenite riskini göz ardı etmek istemediğinden, ilaçların prospektüsünde topikal izotretinoin kullanımının kontrendike olduğu belirtilmektedir [13,9]. Retinoid embriyotoksisitesinin zaman içerisinde maruz kalınan toplam miktarına bağlı olduğu görülmüştür. Sıçanlar üzerinde yapılan bir denemede tretinoinin teratojenitesi maruz kalma süresiyle ilişkilendirilmiştir [11].

Gebelikte randomize kontrollü klinik çalışmalar etik nedenlerden dolayı yapılamamaktadır. Bu nedenle yaptığımız araştırmalarda epidemiyolojik çalışmalardan yararlanmış bulunmaktayız.

Gebelikte Sistemik Retinoik Asit Kullanımı

Çeşitli ülkelerde doğum öncesi izotretinoin kullanımıyla bağlantılı doğum kusuru bulunan çocuklar rapor edilmiştir. Raporlar sonucunda ABD'ndeki Teratoloji Derneği gibi bilimsel kuruluşlar bu risklere dikkat çekmiş ve doğum kontrol programlarının oluşturulmasına yardımcı olmuştur. İzotretinoin kullanımı sırasında oluşan gebeliklerin birçoğu gelişimsel kusur endişesiyle sonlandırılmaktadır [9]. Kanada'da 1984-2002 yılları arasında 13-45 yaşlarında izotretinoin kullanan kadınlar üzerinde yapılan bir araştırmada 8609 kadından 90'ı hamile kalmıştır. Bunlardan 76'sı

hamileliğini sonlandırmış, 3'ü spontan düşük yapmış, 2'si doğum sırasında neonatal ölümle karşılaşmıştır. Kalan dokuz canlı doğan bebeğin ise yalnızca birinde (%11) yüz ve boyun bölgesinde konjenital anomali görülmüştür [22]. Başka bir çalışmada fetüs ve yenidoğanlara ilişkin veriler incelendiğinde 44 gebelikten beşinde malformasyon tespit edilmiştir. İki fetüste sırasıyla hamileliğin beşinci ve yedinci haftalarına kadar izotretinoin kullanılmış ve meydana gelen vermis agenezisi gebelikleri sonlandırma nedeni olarak gösterilmiştir. Diğer 3 yenidoğanda ise yaşamın ilk günlerinde minör malformasyon tespit edilmiştir [23]. Bir başka çalışmada asitretin kullanan kadınlarda 8 gebelik tespit edilmiş, 2'sinin gebeliği yarıda kesilmiştir. Dördü spontan düşükle sonuçlanmış, ikisi de canlı doğmuştur. Düşük yapılan fetüslerden birinde retinoide bağlı tipik malformasyonlar tespit edilmiştir. Canlı doğan iki bebeğin sağlıklı olduğu ancak birinde yüksek frekanslarda işitme bozukluğu olduğu görülmüştür. Aynı çalışmadaki verilere göre, hamilelik sırasında etretinat tedavisi gören 75 kadından 29'unda canlı doğum, bunların 6'sında tipik retinoid ve 3'ünde spesifik olmayan doğum kusurları gösterilmiştir [24]. Tablo 4'te izotretinoine bağlı teratojenik etkilere yer verilmiştir.

Tablo 4. İzotretinoin teratojenitesinin sınıflandırılması [25]

İzotretinoin teratojenitesi
Dış anormallikler
Kulakta anotia (kulak kepçesinin olmaması), mikropina, dış işitsel kanalın küçük olması veya hiç olmaması
Gözlerde mikroftalmi
Yüz dismografisi
Yarık dudak
İç anormallikler
Merkezi sinir sisteminde serebral malformasyonlar, hidrosefali, mikrosefali, kranyal sinir eksikliği
Kardiyovasküler sistem
Timüs bezi anormalliği
Paratiroid hormonu eksikliği
Yetersiz IQ
Kendiliğinden kürtaj
Erken doğum

Hollanda'da, uygulanan gebelik önleme programına rağmen gebe kalan kadınların izotretinoine maruziyetini tahmin etmek ve izotretinoine maruziyet sonucunda meydana gelen olumsuz fetal veya neonatal sonuçları analiz etmek amaçlı bir çalışma gerçekleştirilmiştir. Gerçekleştirilen bu çalışma, Avrupa Birliği'nde yapılan 200.000'den fazla gebeliğin bulunduğu ilk nüfus tabanlı araştırma olarak kabul edilmiştir. Araştırma kapsamındaki 51 gebelik (53 fetüs) gebelik önleme programına rağmen izotretinoine maruz kalmıştır. Bu 51 kadının 45'i gebelik süresinde izotretinoine maruz kalmıştır, kalan 6'sı ise izotretinoin kesilmesinden sonraki 30 gün içerisinde hamile kalmıştır. Gebelikte 16. haftaya kadar olan kendiliğinden ve isteğe bağlı düşükler çalışmaya dahil edilmemiştir. Bu nedenle çalışmanın mevcut izotretinoin embriyopatisini olduğundan daha az tahmin ettiği düşünülmektedir. Çalışmanın sonucunda, sistemik izotretinoinin tartışmasız olarak embriyotoksisiteye neden olduğu ve istenmeyen fetal ve neonatal olayların riskini arttırdığı düşünülmektedir [20].

Schaefer ve arkadaşları Berlin Klinik Teratoloji Enstitüsü'ne 1993-2008 yılları arasında kaydedilen izotretinoine maruz kalan gebelikleri değerlendirmiştir. Kontrendike dönemde sistemik retinoine maruz kalan 108 gebelik tespit edilmiştir. Sonucu bilinen gebeliklerin %76'sı ilaç riskinden korktukları için elektif olarak sonlandırılmıştır. 5 gebelikte kendiliğinden düşükler meydana gelmiştir. 18 canlı doğumdan 1'inde majör (ventriküler septal defekt), 2'sinde minör doğum defekti (prematüre bir bebekte kalıcı foramen ovale, hafif kalça displazisi ve doğum sonrası geçici yüz asimetrisi) görülmüştür. Doğum kontrolü oranlarında ise hastaların %70'inin hiçbir yöntem kullanmadığı, kalan %30'unda ise doğum kontrol yöntemlerinin başarısız olduğu tespit edilmiştir. Aynı zamanda yapılan incelemede, gebelik sonlandırma eğiliminin, en son izotretinoin alımındaki gebelik haftasına göre doğrusal bir artış gösterdiği görülmüştür [16].

Alay ve arkadaşları 2009-2020 yılları arasında teratolojik danışma merkezine başvuran gebe kadınları iki gruba ayırmıştır; gebelik öncesi ve sırasında sistemik izotretinoine maruz kalanlar, gebelik öncesi izotretinoine maruz kalanlar. Kontrol grubu ise herhangi bir teratojene maruz kalmayan gebe kadınlardan seçilmiştir. Doğum defektleri karşılaştırıldığında ise gebelik öncesi ve sırasında izotretinoin maruziyeti ile kontrol grubu arasında istatistiksel farklar gözlenmiştir. Gebelik öncesi maruziyet ve kontrol grupları arasında anlamlı bir fark bulunamamıştır. Gruplar canlı doğum açısından değerlendirildiğinde, canlı doğum ile izotretinoin maruziyeti arasında ilişki bulunmuştur. Gebelik öncesi ve sırasında izotretinoin maruziyeti olan gruptaki 42 canlı doğumun 12'sinde, gebelik öncesi izotretinoin maruziyeti olan gruptaki 66 canlı doğumun 4'ünde ve kontrol grubundaki 70 canlı doğumun 1'inde konjenital anomali tespit edilmiştir. Bu aradaki fark karşılaştırıldığında konjenital anomali sıklığı ile izotretinoine maruz kalma süresi arasında doğrusal bir ilişki olduğu gözlenmiştir. Konjenital anomalilerin dağılımlarına bakıldığında ise kontrol grubunda kulak, merkezi sinir sistemi ve genitoüriner sistem anormallikleri görülmemiştir. Bu nedenle bu etkilerin gebelik öncesinde ve sırasındaki maruziyetten kaynaklandığı düşünülmektedir. Ayrıca izotretinoine maruz kalmış bebeklerde daha önce literatürde belirtilmeyen fenotipik özellikler değerlendirilmiştir. Bunlar içerisinde; altıncı sinir felci, ateşli olmayan nöbetler, stereotipik hareketler, asimetrik ağlayan yüz ve bilateral inmemiş testisler listelenmiştir. Ancak bunların genetik varyasyondan veya tesadüfi olarak ortaya çıkabileceği de vurgulanmıştır [18].

Choi ve arkadaşlarının oral izotretinoin kullanımının etkilerini tartıştığı meta-analiz çalışmasında rahim içi izotretinoin maruziyetinden sonra fetüsteki majör malformasyon riskinin belirtilenlerden daha düşük olabileceğini ileri sürmüştür. Majör malformasyon oranlarının yüksek belirtilmesiyle izotretinoine maruz kalmış hamile kadınların yanlış algı ve aşırı korku ile gebeliği sonlandırmak isteyebilecekleri belirtilmiş, bu nedenle konu hakkında daha uzun vadeli ve kapsamlı çalışmalar yapılması gerektiğini vurgulamıştır. Düşük sayısının fazla olması, canlı doğum sayısını azaltmakta, bu da canlı doğumlarda rastlanılan konjenital anomalilerin sıklığını düşürmektedir. Bu nedenle çalışmalardaki verilerin tam olarak doğru sonuçları yansıtmayacağı düşünülmektedir [26].

Sarıcı ve arkadaşlarının 2012 yılında yazdığı raporda kulak ve asimetrik ağlayan yüz anomalileri bulunan bir bebek incelenmiştir. Anne bir ayı gebelik sırasında olmak üzere 3 ay boyunca izotretinoin kullanmış, gebelik testi sonucuna göre ilacı kesmiştir. Bebeğin fetal ultrasonografide kardiyak anomalileri saptanmıştır. Doğumdan sonra bebeğin sağ kulağında kulak anormalliği ve ağlayan yüzde asimetri farkedilmiştir [17].

Shirazi ve arkadaşlarının yazdığı bir vaka raporunda 4 hafta boyunca sistemik izotretinoin almış ve bıraktıktan sonra da 4 hafta boyunca kontrasepsiyon uygulamış, ardından hamile kalmış bir annenin bebeği incelenmiştir. Yenidoğanda, şüpheli izotretinoin maruziyeti nedeniyle izole bilateral mikrotia görülmüştür. Bu vaka incelemesinin sonucunda da izotretinoinin vücutta birikim yapabileceği düşünülerek kontrasepsiyonun ilaç bırakıldıktan sonra 1 aydan daha uzun süre devam etmesi gerektiği yorumu yapılmıştır. Ayrıca bazı yazarların ise izotretinoinin vücudun yağ dokusunda birikme eğilimi olması nedeniyle tedavinin tamamlanmasından sonra en az 3 ay gebelikten kaçınılması gerektiği yorumu yaptığı görülmüştür [19]. Literatürdeki başka bir çalışmada annelerin izotretinoin tedavisinin kesilmesinden sonraki 30 gün içerisinde gebe kaldığı canlı doğumları incelediğinde, bebeklerin %10'unda malformasyonlar olduğu görülmüştür. Bir diğer çalışmada ise izotretinoin tedavisinin kesilmesinden 3 ay sonrasında meydana gelen gebelikte bile embriyopati tespit edildiğini bildirmiştir [19].

Gebelikte Topikal Retinoik Asit Kullanımı

Hamilelik döneminde androjen seviyelerinde artışlar yaşanması, akne gelişmesine veya kötüleşmesine sebep olabilir. Bu nedenle tedavi için topikal retinoik asit preparatları tercih edilebilmektedir [27,28]. Genel olarak deriden emilen tretionin miktarının çok düşük olduğu kabul edilmektedir. Ancak literatürde topikal tretinoin kullanımıyla ilişkili 4 vaka raporu bulunmaktadır. Bu raporlarda retinoid embriyopatisi ile uyumlu doğum kusurları yer almaktadır [27].

Literatürdeki bir olguda gebeliğin 41. haftasında 4090 gr ağırlığında doğan, sağ kulağı ve dış kulak yolu olmayan bir bebek incelenmiştir. Aileye sorgulama yapıldığında annenin gebelikten önce ve gebeliğin 2-3 ayı boyunca yüzü ve sırtının geniş bir yüzeyini kaplayacak şekilde topikal tretinoin

preparatı (%0.025) kullandığı öğrenilmiştir. Retinoik asit embriyopatisinde görülen kulak kepçesinin malformasyonları bu yapının embriyogenez döneminde erken oluşmasından kaynaklanmaktadır. Bu nedenle olgudaki kulak malformasyonu retinoik asit embriyopatisinin çok tipik bir özelliğidir ve tesadüfen meydana gelmiş olma olasılığını ortadan kaldırmaktadır [12]. Literatürdeki başka bir olguda ise dış kulak yolu kapalı ve olması gerekenden küçük kulağı olan bir bebek incelenmiştir. Akraba ilişkisi olmayan sağlıklı ebeveynlerin öyküsü sorgulandığında annenin son menstrual kanamasından önceki ay ve gebeliğinin ilk bir haftasında tretinoin krem (%0.05) kullandığı görülmüştür [28]. Başka bir olguda gebe kalmadan önce ve gebeliğin ilk 5 haftasında %0.05 tretinoin içeren alkol bazlı bir krem kullanan annenin bebeği değerlendirilmiştir. Bebekte kulak malformasyonu, hipoplastik skapula, eksik klavikula, hipoplastik humerus, kısa radius ve ulna, kısa sternum, el redüksiyon defekti görülmüştür. Yapılan bazı hayvansal modellemelerde tretinoinin uzuv küçülmesi kusurlarının oluşmasında izotretinoinden 10 kat daha teratojenik olduğu görülmüştür [29]. Bir başka olguda aort koarktasyonu, hipoplastik el, hipertelorizm ve küçük kulak kanalları ile doğan bebeğin aile öyküsü sorgulandığında, annenin gebe kalmadan önce ve gebeliğin ilk 2 ayı boyunca alkol bazlı topikal tretinoin (%0.05) ve topikal benzoil peroksit kullandığı görülmüştür [30].

Loureiro ve arkadaşlarının 1983-2003 yılları arasında yaptığı çalışmada, gebeliğin ilk trimesterinde topikal tretinoine maruz kalan 106 kadın ileriye yönelik takibe alınmıştır. Gebelik kaybı, majör yapısal kusurlar, doğum öncesi ve sonrası büyüme dahil olmak üzere doğum sonuçları, hamilelik sırasında topikal tretinoin maruziyeti olmayan 389 kadın ile karşılaştırılmış ve anlamlı bir fark bulunamamıştır. Çalışma değerlendirmesi, ilk trimesterde topikal tretinoin maruziyetinin herhangi bir olumsuz gebelik sonucu riski oluşturmadığı yönünde olmuştur [31]. Topikal tretinoin üzerine yapılan başka bir çalışmada Jick ve arkadaşları topikal tretinoine maruz kalan 215 kadın ile benzer yaşlarda, ilaca maruz kalmamış 430 kadını karşılaştırmıştır. Topikal tretinoinin majör malformasyonları arttırdığına dair bir risk olmadığı sonucuna varılmıştır [32]. Shapiro ve arkadaşlarının yaptığı bir çalışmada gebelik yaşı, sigara ve alkol bakımından benzer olan, topikal tretinoine maruz kalmış 94, kalmamış 133 kadın karşılaştırılmıştır. Canlı doğum, düşük veya isteğe bağlı sonlandırma oranlarında anlamlı bir fark bulunamamıştır [33]. Hoogdalem yaptığı bir çalışmada topikal tretinoin preparatlarının klinik farmakokinetik verilerini gözden geçirmiştir. Bileşiğin topikal uygulama sonrası sistemik yararlanımı düşük bulunmuştur [34]. Ciddi şekilde iltihaplı derilerde ve dezenfektan ile birlikte kullanımı sonucunda emilim oranının artacağı bildirilmiştir [29,34]. Bunlarla birlikte gebelikte güvenli kullanımına ilişkin fikir eksikliği olduğunu özellikle belirtmiştir [34]. Gebeliğin 13. haftasına kadar adapalen etken maddesi kullanan bir vaka raporunda ultrasonografik olarak anoftalmi ve göz sinirlerinde agenezi tespit edilmiştir [35]. Fransa'da yapılan bir çalışmada topikal retinoid (tretinoin, izotretinoin, adapalen) tedavisi uygulanan 94 gebelik değerlendirilmiştir. Spontan düşük bilgisi görülmemiş ve teratojenite ile ilgili herhangi bir veri kanıtlanamamıştır. Ancak tedavi süresi ve maddelerin ayrımı gibi detaylar çalışmada yer almamaktadır [9].

Tazarotenin farmakokinetik özelliklerinin araştırıldığı bir çalışmada çeşitli veriler elde edilmiştir. Perkütanöz emiliminin sınırlı olduğu ve uygulanan ilacın %6'sından azının kan dolaşımına ulaştığı bildirilmiştir. Tazaroten hızla tazarotenik asit ve lipofilik olmayan diğer metabolitlerini oluşturmaktadır. Tazaroten ve metabolitleri idrar ve dışkı yoluyla kandan hızla elimine edilmektedir. Bu özellikleri sayesinde topikal tazaroten uygulanmasının minimum seviyede risk gösterdiği sonucuna varılmıştır. Hamilelik sırasında topikal tazaroten ile tedaviye maruz kalan bebekler arasında sağlıklı çocuklar rapor edilmiştir [36]. Topikal retinoidlerin sistemik emilimi üzerine yapılan çalışmalarda plazma seviyelerinin çok düşük olduğu gösterilmiştir [14]. Sağlıklı gönüllülerde tretinoinin perkütan emiliminin uygulanan dozun %2'si kadar olduğu ve endojen tretinoin kan konsantrasyonu ile karşılaştırıldığında çok düşük seyrettiği tahmin edilmektedir [30]. Terapötik kullanım koşullarında topikal retinoid uygulamasının yol açtığı plazma konsantrasyonlarındaki artışın, endojen (doğal oluşan) retinoid plazma seviyelerinden çok daha az olması beklenmektedir. 235 topikal retinoid maruziyetinin incelendiği bir çalışmada ise yenidoğanlar kontrol grubuyla karşılaştırıldığında %1-%3 'ten daha yüksek bir risk görülmemiştir. Ayrıca görülen malformasyonların retinoik asit embriyopatisiyle ilişkili olduğuna dair bir kanıt bulunamamıştır [14].

Topikal retinoidlere maruz kalan toplam 654 hamile kadını ve 1375 kontrol grubunu içeren bir meta-analiz çalışmasında, spontan düşük, düşük doğum ağırlığı, prematüre doğum gibi majör konjenital

malformasyon verileri değerlendirildiğinde ilaç kullanımına bağlı anlamlı bir artış gözlenmemiştir. İnsan derisi üzerinde yapılan çalışmalarda topikal retinoidlerin cilt tarafından emiliminin ve vücutta birikiminin; retinoid tipine, taşıyıcısına ve formülasyondaki retinoid dozuna bağlı olduğu düşünülmektedir [15].

Genel olarak tretinoin, adapalen, tazaroten gibi topikal retinoidlerin gebelikteki güvenliğine ilişkin farklı raporlar mevcuttur. Bu preparatların kullanımından kaynaklanan malformasyon vakaları literatürde yer almaktadır ancak tesadüfi meydana gelmiş olabileceği düşünülmelidir [25]. Bu veriler topikal retinoid kullanımını güvenli kılmamakla birlikte gebeliğin ilk üç ayında yanlışlıkla retinoide maruz kalan gebeler için güvence sağlamaktadır. Ancak topikal retinoidlerin yarar/risk oranı belirsizliğini koruduğundan dolayı gebelik sırasında kullanımı kesinlikle tavsiye edilmemektedir [14,15].

Laktasyonda Sistemik ve Topikal Retinoik Asit Kullanımı

Toksik potansiyelleri ve uzun yarı ömürleri nedeniyle laktasyon döneminde retinoik asitlerle sistemik tedavi önerilmemektedir. İzotretinoin üreticisi de emzirilen bebek üzerinde herhangi bir olumsuz etkisi görülmemesine rağmen laktasyon döneminde kullanılmamasını tavsiye etmiştir [25]. Tretinoin, izotretinoin gibi retinoidlerin topikal uygulamayla birlikte emzirilen bebeğe kayda değer miktarda ulaşmadığı gözlenmiştir [9]. Bir başka kaynakta topikal tretinoin ve adapalenin düşük sistemik emilimi nedeniyle laktasyon döneminde uyumlu olduğu düşünülmektedir. Ancak tazarotenin laktasyon dönemindeki güvenliği belirsizliğini korumaktadır [25]. Çeşitli kaynaklarda yer alan bilgilere göre etretinat annenin ağırlığına bağlı olarak %1 oranında anne sütüne geçmekte ve bu nedenle emziren kadınlarda da kullanımı risk oluşturmaktadır [9].

SONUÇ VE TARTIŞMA

Retinoik asit ve türevleri akne vulgaris ve sedef başta olmak üzere deri hastalıklarında kullanılan etken maddelerdir. Etkilerini; yağ bezlerinde sebum üretimini azaltarak, hiperkeratinizasyonu azaltarak, akne sebebi mikroorganizmaların kolonizasyonunu ve inflamatuvar yolakları inhibe ederek gösterirler. Doğurganlık çağındaki kadınlar tarafından sıklıkla kullanılması, ilacın kullanım sürecinin gebeliğe denk gelmesi riskini taşımaktadır. Retinoik asit türevlerinin plasenta ve anne sütüne geçme riskleri vardır. Bu da anne karnındaki veya emzirme dönemindeki bebeğin ilaca maruzuyeti anlamına gelmektedir. Teratojenik etkileri olduğu bilinen bu etken maddelerin belirtilen dönemlerde kullanımı risk taşımaktadır. Literatürdeki birçok incelemede sistemik retinoidlerin gebelik döneminde kullanımının teratojenik etkileri kanıtlanmıştır. Topikal retinoidlerin gebelik döneminde kullanılması ile ilgili veri sayısı daha az olmakla birlikte teratojenik etki açısından şüpheli yaklaşılması gerekmektedir. Literatürdeki laktasyon döneminde ait veriler oldukça azdır, bu nedenle yine risk teşkil etmektedir. Laktasyon dönemine ait yapılacak incelemelerin artması gerekmektedir. Mevcut verilere göre retinoik asit türevlerinin gebelik ve laktasyon döneminde kullanılması; düşük, kulak, yüz, damak anomalileri, kardiyovasküler ve uzuv defektleri, zihinsel yetersizlikler, merkezi sinir sistemi defektleri, timüs gelişim eksikliği gibi birçok teratojenik etkiye sebep olabilmektedir. Bu riskler göz önüne alınarak ilaç kullanımının gerekliliği tartışılmalıdır. Aynı zamanda gebe olmayan ancak doğurganlık çağındaki kadın hastalara ilaç tedavisi uygularken de riskler ve kontrasepsiyon yöntemleri hakkında bilgilendirme yapılmalıdır. Hastalara ilaç kullanım süresince gebe kalmaması konusunda uyarı yapılmasında fayda vardır.

YAZAR KATKILARI

Kavram: G.D., S.T.; Tasarım: G.D., S.T.; Denetim: G.D., S.T.; Kaynaklar: G.D., S.T.; Malzemeler: G.D., S.T.; Veri Toplama ve/veya İşleme: G.D., S.T.; Analiz ve/veya Yorumlama: G.D., S.T.; Literatür Taraması: G.D., S.T.; Makalenin Yazılması: G.D., S.T.; Kritik İnceleme: G.D., S.T.; Diğer: -

ÇIKAR ÇATIŞMASI BEYANI

Yazarlar bu makale için gerçek, potansiyel veya algılanan çıkar çatışması olmadığını beyan ederler.

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Ankara Ecz. Fak. Derg. / J. Fac. Pharm. Ankara, 49(3): 940-962, 2025
Doi: 10.33483/jfpau.1537612

DERLEME MAKALE / REVIEW ARTICLE

ALOPESİ AREATA TEDAVİSİNDE POTANSİYEL BAZI TIBBİ BİTKİ TÜRLERİNİN İNCELENMESİ

EXAMINATION OF SOME POTENTIAL MEDICINAL PLANT SPECIES IN THE TREATMENT OF ALOPECIA AREATA

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ÖZ

Amaç: *Alopesi areata (AA), vücut bağışıklık sisteminin saç foliküllerine saldırması sonucu saç kaybına neden olan otoimmün bir hastalıktır. Günümüzde AA tedavisinde çeşitli yöntemler kullanılmakta, ancak hastaların doğal ürünlere olan ilgisinin artması nedeniyle bitkisel tedavi yöntemleri ön plana çıkmaktadır. Bu çalışmada, AA tedavisinde terapötik potansiyele sahip bitki türlerinin etkinliğinin ve uygulanabilirliğinin incelenmesi amaçlanmıştır.*

Sonuç ve Tartışma: *AA tedavisinde doğal bitki ekstrelerinin kullanımı, son yıllarda önemli bir ilgi odağı haline gelmiştir. Çalışmada ele alınan bitkilerin birçoğu, anti-enflamatuvar ve immünomodülatör özellikleri ile AA tedavisinde potansiyel faydalar sağlayabilecek niteliktedir. Çok sayıda bitki türü saç foliküllerindeki hücrelerin çoğalmasını teşvik etmek, saç derisindeki kan dolaşımını artırmak, 5α-redüktaz enzimini inhibe etmek, büyüme faktörlerinin salınımını tetiklemek veya enflamatuvar sitokinlerin seviyesini düşürmek gibi çeşitli mekanizmalarla saç büyümesinin teşvik edilmesine katkıda bulunmaktadır. Bununla birlikte bahsi geçen bitkilerin etki mekanizmalarının aydınlatılması için daha fazla çalışma gereklidir.*

Anahtar Kelimeler: *Alopesi areata, bitki ekstresi, saç uzaması*

ABSTRACT

Objective: *Alopecia areata (AA) is an autoimmune disease that causes hair loss due to the body's immune system attacking hair follicles. Although various treatments are currently used for AA due to the side effects caused by conventional treatments, patients' interest in natural products has increased, bringing herbal therapeutic approaches to the forefront. This study aims to examine the efficacy and applicability of plants with therapeutic potential in the treatment of AA.*

Result and Discussion: *In recent years, the use of natural plant extracts in the treatment of AA has gained significant attention. Many of the plants discussed in this study have anti-inflammatory and immunomodulatory properties, making them potentially beneficial in the treatment of AA. A large number of plant species contribute to the promotion of hair growth by stimulating the proliferation of cells in hair follicles, increasing blood circulation in the scalp, inhibiting the 5α-reductase enzyme, triggering the release of growth factors, and reducing the levels of inflammatory cytokines. However, further studies are needed to clarify the mechanisms of action of the aforementioned species.*

Keywords: *Alopecia areata, herbal extract, hair growth*

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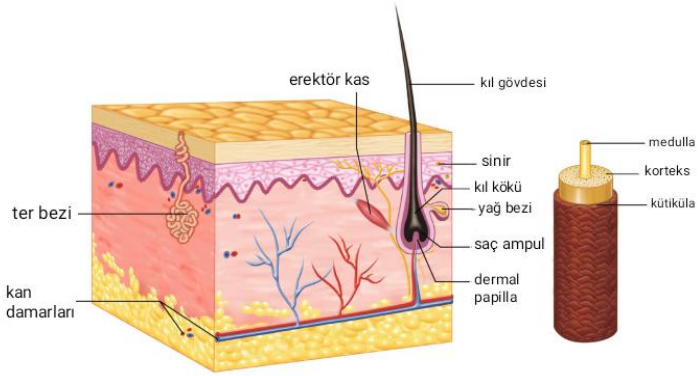
Gönderilme / Submitted : 28.08.2024
Kabul / Accepted : 17.04.2025
Yayınlanma / Published : 19.09.2025

GİRİŞ

Alopesi areata, kafa derisinde ve vücudun başka yerlerinde saç dökülmesine neden olan yaygın bir otoimmün cilt hastalığıdır [1]. AA, genellikle kafa derisinde bir veya daha fazla küçük, yuvarlak, belirgin sınırlara sahip ve pürüzsüz leke oluşturan ve akut başlayan saç kaybı ile karakterize edilen yaygın bir saç dökülme durumudur [1,2]. Bu durum, kafa derisinin tamamen saç dökülmesine (*alopecia totalis*) veya vücudun tamamen saç dökülmesine (*alopecia universalis*) kadar ilerleyebilir. Kafa derisi en sık etkilenen bölge olmasına rağmen, sakal veya saç taşıyan herhangi bir bölge tek başına veya kafa derisi ile birlikte etkilenebilir [1]. AA, etnik kökenlere bakılmaksızın tüm yaş gruplarındaki erkek ve kadınlarda görülse de, genellikle çocukluk çağında ortaya çıkar [1,3]. Dünya çapında 147 milyonun üzerinde insan, hayatlarının bir döneminde AA geçirmiş, geçiriyor veya geçireceği tahmin edilmektedir [1]. AA hastaları sıklıkla belirgin psikolojik sağlık sorunları, özsaygı eksikliği yaşar ve eşlik eden psikiyatrik hastalıkların ortaya çıkma riski daha fazladır [3]. AA'nın tanısı genellikle klinik olarak konulur, ancak bu durum için biyopsi tanısal niteliktedir [4,5]. Tedavi zordur ve etkilenen bireylerde saçın yeniden çıkmasını hedefler. Hastalığa yönelik çeşitli tedavilerin etkinliği hastalar arasında değişebilir, bu da evrensel bir tedavi algoritmasının uygulanmasını zorlaştırır. Hastalar bireysel olarak ele alınmalı ve nihai sonuç saçın kozmetik olarak yeniden çıkmasına dayanmalıdır. İdame tedavisi, kabul edilebilir saç çıkması elde eden hastalarda önemlidir. Hastanın yüksek motivasyona ve tedaviyi yapan doktorla iyi bir uyuma ihtiyacı vardır [6]. Doğal veya sentetik bazlı ürünler de dahil olmak üzere birçok tedavi seçeneği üzerinde araştırma yapılmaktadır. Sentetik ürünler, insan sağlığına zarar verebilecek birçok yan etkiye neden olabilir. Bu nedenle doğal ürünler giderek popülerlik kazanmakta ve formülasyonda bitki ekstrelerinin kullanımı artmaktadır [1].

Saç Yapısı ve Fizyolojisi

Saç, örtü sisteminin bir bileşeni olup saç folikülünün içinde bulunduğu dermal tabakaya kadar uzanır. İnsanlarda yaklaşık beş milyon saç folikülü bulunur; bu foliküller soğuktan ve UV radyasyonundan koruma sağlar, sebum üretir ve büyümesi veya yapısı dengesiz olduğunda önemli bir psikolojik etkiye sahip olabilir. Mikroskobik düzeyde, her saçın uzunluk, renk, çap ve kesit şeklindeki çeşitliliği, etnik gruplar ve bireyler arasında görülen karakteristik profilleri oluşturur [7]. Saç, cildin ektoderminden türetilmiştir. Vücut üzerindeki koruyucu uzantılardır ve yağ bezleri, ter bezleri ve tırnaklar ile birlikte deri yapısının yan ürünleri olarak kabul edilirler. Saç, embriyolojik gelişim sırasında epidermisten türetildiği için epidermal türev olarak da bilinir [1]. Her saç teli iki ayrı yapıdan oluşur. Birincisi derinin dışında görünen kısmı içeren kıl gövdesi (cansız kısm); medulla, korteks ve kütikül olmak üzere üç katmandan oluşmaktadır, ikincisi ise deri yüzeyinin altında yer alan kıl köküdür (canlı kısm). Ayrıca saç folikülünde bir iç ve dış kök kılıfı bulunmaktadır [7,8]. Saç kökünde bulunan dermal papilla ise, saçın döngüsel bölümünü yenileyen ve saç gövdesini oluşturan epitelyal kök hücreleridir [9]. Saç büyümesi, yaş ve beslenme alışkanlıklarının yanı sıra vasküler, endokrin ve sinirsel uyaranlarla da düzenlenir. Saçlı derideki saçlar yaklaşık olarak 1 cm/ay veya 15 cm/yıl oranında uzar [10].



Şekil 1. Saç anatomisi [11]

Saç Büyüme Döngüsü

Deri gibi saç da kök hücrelerin hızla bölünmesi ve farklılaşmasıyla oluşur; kök hücrelerinden oluşan keratinositler göç eder, düzleşir, ölür ve bu şekilde keratinize hücreler oluşturur. Cilt yüzeyinde açığa çıkan son saç ürünü tamamen keratinden oluşacaktır. Saç folikülünün büyümesi döngüseldir [7]. Saç büyüme döngüsü anajen (büyüme), katajen (gerileme), telojen (dinlenme) ve eksojen fazı (dökülme) olmak üzere dört aşamadan oluşmaktadır [12].

1. Anajen fazı: Büyüme aşaması

Saç büyüme aşamaları anajen fazı ile başlar. Bu aşama, kafa derisindeki saçlar için yaklaşık 3 ila 5 yıl süren en uzun aşamadır. Anajen fazında, saç kökleri, kesilene veya ömrünün sonuna ulaşıp dökülene kadar büyümeye devam edecek olan saçları dışarı iter. Sağlıklı bireyde herhangi bir zamanda, kafa derisindeki saçların yaklaşık yüzde 90'ı anajen aşamasındadır [8]. Yaşla birlikte anajen fazın uzunluğu azalır, bu da zamanla saçların daha zayıf olmasına ve incelmesine neden olur [12].

2. Katajen fazı: Geçiş aşaması

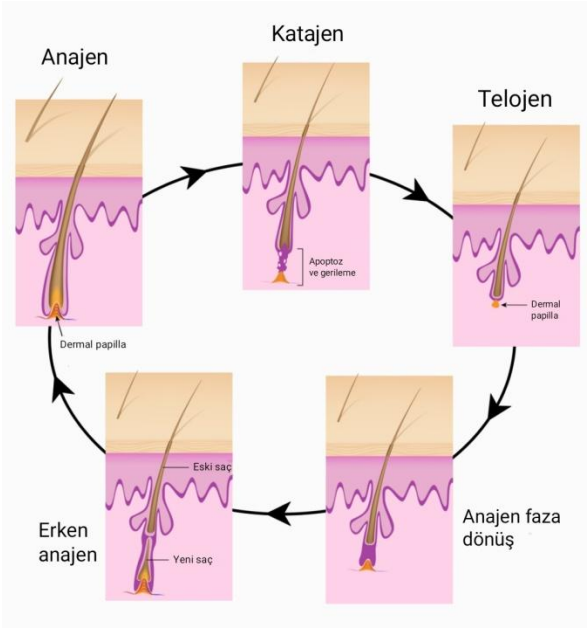
Katajen fazı, yaklaşık iki hafta süren, anajenden telojene geçişi temsil eder. Katajen faz boyunca, saç folikülleri geriler ve saç foliküllerindeki dermal papilladan ayrılır, bu da folikülün ampulünde epitelyal hücre apoptozuyla sonuçlanır. Katajeni takiben dermal papilla, saç folikülünün çıkıntısına doğru yukarı doğru hareket eder. Katajen sırasında dermal papilla çıkıntıya ulaşamaz ise folikülün telojen fazına girmesine ve sonuç olarak saç dökülmesine neden olur [12].

3. Telojen fazı: Dinlenme aşaması

Telojen fazı genellikle yaklaşık 3 ay sürer. Saçlı derideki saçların tahmini olarak yüzde 9'u bu aşamadadır. Telojen fazındaki saçlar uzamaz ancak genellikle dökülmezler. Eski saçlar dinlenirken, saç folikülünün tabanında yeni saçlar gelişmeye başlar ve eski saçları dışarı iter. Ancak anajenik foliküller dinlenme fazına erken girerse telojen effluvium (TE) olarak bilinen aşırı dökülme ve incelme meydana gelebilir [12].

4. Ekzojen fazı: Dökülme aşaması

Eksojen, telojenin sonlanmasını ve anajenin başlatılmasını anlatır. Bu dönemde yeni çıkan saçlar yukarıya doğru büyümeye devam ederek eski saçları dışarı doğru iter ve sonuçta dökülme gerçekleşir [12]. Sağlıklı bireylerde her gün saçlı deriden 50-60 kadar saç dökülür ve bu foliküllerden yeni saçlar çıkmaya başlar [8].



Şekil 2. Saç büyüme döngüsü [13]

Alopesi Nedir?

Alopesi, saç dökülmesi veya kellik durumunu ifade eden tıbbi terimdir. Vücudun bazı veya tüm bölgelerinde, çoğunlukla da kafa deriside saç kaybına neden olan tıbbi bir durumdur. Saç dökülmesi; genetik yatkınlık, çevresel tetikleyiciler, kimyasal maddelere maruz kalma, ilaçlar, beslenme eksikliği, aşırı stres veya uzun süren bir hastalık gibi çeşitli faktörlerden kaynaklanabilir [14]. Alopesi, saç dökülmesinin şekline ve nedenlerine bağlı olarak çeşitli kategorilere ayrılmaktadır. Bunlardan en yaygın görülenler; androgenetik alopesi, telogen effluvium, erkek tipi ve kadın tipi saç dökülmesi ve alopesi areata'dır [14,8].

Alopesi Areata Patogenezi

Alopesi areata (AA) patogenezinin tam resmi henüz belirlenmemiş olmasına rağmen, son araştırmalar hastalık mekanizmasının aydınlatılmasında büyük ilerleme kaydetmiştir. Birçok dolaylı kanıt, AA'nın temelde bağışıklık hücreleri aracılığıyla olduğu ve aslında otoimmün bir hastalık olabildiği fikrini desteklemektedir [15]. Anajen kıl folikülü genellikle bağışıklık açısından ayrıcalıklı bir bölgedir, ancak bu durum AA'da bozulur. Enflamatuvar yanıta aracılık eden bağışıklık hücreleri, telojen fazına erken girişle distrofik kıl folikül döngüsüne yol açar. Otoreaktif T hücreleri, saç folikülüne sızarak kök hücre kısmını koruyarak kalıcı organ tahribatını önler ve yeniden saç büyümesine izin verir. CD8+ T hücre yoğunluğu ve hastalık şiddeti arasında bir ilişki bulunmaktadır. Hayvan modellerinde yapılan çalışmalar da AA'da T hücrelerinin rolünü desteklemektedir [3]. Son araştırmalar, saç kaybı fenotipinin başlıca CD8+ lenfositler tarafından tetiklendiğini, ancak hastalık mekanizmasının CD4+ lenfositler tarafından yönlendirildiğini göstermektedir [15]. Saç folikülünden türeyen otoantijenler bu süreçte de rol oynayabilir. Aktif faz alopesi areata'da, CD8+ ve CD4+ T hücrelerini içeren peribulbar lenfositik enflamasyon, katajen ve telojen foliküllere kayma ve küçülme bulunmaktadır. Kronik fazda, foliküllerin çoğunluğu küçülmüş durumda ve katajen veya telojen fazındadır ve peribulbar enflamasyon minimal ya da bazen hiç yoktur. Son zamanlarda, AA'dan etkilenen farelerde ve insanlarda AA lezyonlarında mast hücrelerinin varlığı ve CTL'lerle yakın ilişkide olduğu tespit edilmiştir. Bu durum, AA patogenezinde mast hücreleri ile CTL'ler arasında potansiyel bir çapraz iletişim olasılığını göstermektedir. İnsan lökosit antijeni I ve II lokusları, doğal ve edinilmiş bağışıklıkta yer alan genler ve oksidatif stres, AA'da rol oynayan faktörler arasında bulunmaktadır[3]. Genetik yatkınlık hastalık gelişiminde önemli bir katkı sağlasa da, hastalık başlangıcı ve fenotipik sunum muhtemelen karmaşık çevresel etkileşimler tarafından modifiye edilir [15]. Ayrıca, çeşitli epidemiyolojik çalışmalara göre, egzama, astım ve alerjik rinit dahil atopi hastalığı olan bireylerde AA gelişme riskinde artış bildirilmiştir [3].

Klinik Değerlendirme

AA nüfusun yaklaşık %2'sini etkiler ve klinik görünümleri çok çeşitlidir, saç kaybının iyi sınırlı yamalardan yayılmış alopesiye kadar uzanır. Hastalık başlangıç yaşı ve hastalığın yayılma derecesi, tedaviye yanıtı etkileyen başlıca faktörlerdir. AA'nın birkaç alt tipinin farklı belirtileri vardır; bunlar arasında ophiasis, sisaipho, ani grileşme tipi ve yayılmış formlar yer alır. AA genellikle ani başlangıçlı, belirgin sınırlı saç dökülme lekeleri olarak ortaya çıkar, özellikle kafa derisinde belirgin iltihap veya yara izi belirtileri olmaksızın. Aktif hastalığı olan hastalarda özellikle lezyonun çevresinde pull testi pozitif olabilir. Genellikle hastalar asemptomatiktir, ancak bazen saç dökülmeden önce karıncalanma, kaşıntı ve disestezi bildirilir. Ciddi etkilenen bireylerde, AA tüm saçları içerecek şekilde ilerleyebilir (*alopecia totalis* [AT]), veya tüm saçları ve vücut saçlarını içerebilir (*alopecia universalis* [AU]). Erkekler, kafa derisi alopesisine kıyasla başlangıçta sakal etkilenmesi ile daha sık karşılaşabilir (39.3% karşısında 50.5%). Hasta değerlendirmesi, folikülü, saç telini ve çevre deriyi daha yakından incelemek ve biyopsi örneğini almak için en uygun alanı belirlemeye yardımcı olmak amacıyla trikoskopi içermelidir. Klinisyenler, AA'nın yaygın ve karakteristik bir göstergesi olarak kabul edilen ünlem işareti şeklindeki saçlara bakmalıdır; ünlem işareti saçları, ucu tabana göre daha kalın olan kırık saçlardır. Distrofik ve kırık saçlar da sıkça bulunur ve AA'ya özgü olmasa da, anajen foliküllerde mitotik aktivite durduğunda ortaya çıkar. Hastaların %10 ila %20'sinde düzenli çukurluklar, uzunlamasına çıkıntılar, kırılganlık veya çizgilenme gibi tırnak anormallikleri görülür [3].

Alopesi Areata Tedavisi

AA'nın tedavisi hastanın yaşı, hastalığın şiddeti ve süresine bağlıdır. Tedavi seçenekleri arasında intralezyonel ve topikal kortikosteroidler, topikal immünoterapi, topikal minoksidil, antralin ve fototerapi (oral veya topikal psoralen ile ultraviyole A ışığı) yer almaktadır. Ancak bunların hiçbiri saç yeniden büyümesini garanti eden bir tedavi değil ve hiçbiri AA tedavisi için FDA onaylı değildir [16]. Baricitinib 2022 yılında ve ritlecitinib 2023 yılında AA indikasyonu için FDA onayını alan, oral janus kinaz (JAK) inhibitörleri olan, ciddi AA vakalarının tedavisi için kullanıma sunulan iki sentetik ilaçtır [17].

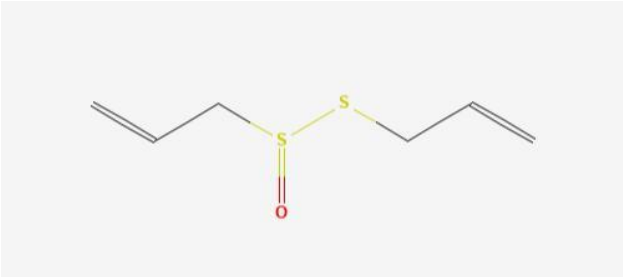
Alopesi Areata Tedavisinde Potansiyel Tıbbi Bitkiler

Allium Sativum L.

Kullanılan kısım: Allii sativi bulbus

Allium sativum L. veya sarımsak Liliaceae familyasına ait bir türdür ve birçok farmakolojik etkilere sahiptir [18]. *A. sativum*'dan elde edilen ekstrelerin ve izole edilmiş bileşiklerin, antikarsinojenik, antioksidan, antidiyabetik, renoprotektif, anti-aterosklerotik, antibakteriyel, antifungal, antiprotozoal ve antihipertansif aktiviteler dahil olmak üzere çeşitli biyolojik özelliklere sahip olduğu rapor edilmiştir. Sarımsağın ayrıca immünomodülatör ve anti-enflamatuvar aktivitelere sahip olduğu da iyi bilinmektedir [19]. Geleneksel tıpta gıda zehirlenmesi, kolera, ateş, baş ağrısı, dizanteri, kan basıncını düşürme gibi çeşitli hastalıkların tedavisinde ve uyarıcı, idrar söktürücü, balgam söktürücü ve terletici bir madde olarak kullanılmaktadır [20]. *A. sativum* alliin, allisin, ajoenler, vinildithiinler gibi kükürt içeren fito-bileşenler ve kersetin gibi flavonoitler açısından zengindir [19].

Sarımsağın biyolojik olarak en aktif kükürt içeren bileşiği olan allisin [S-(2-propenil)-2-propen-1-sülfinotiyoat], sarımsağın kokusundan ve tadından sorumludur [21,22]. Alliin (S-alil-L-sistein sülfoksit), ezilmiş sarımsak dişlerinde bulunan toplam tiyosülfinatların yaklaşık %70'ini temsil eden allisin'in ana prekürsörüdür [23].



Şekil 3. Allisin bileşiğinin kimyasal yapısı [24]

Topikal sarımsak ekstresinin AA tedavisindeki etkinliğini belirlemek amacıyla yapılan bir çalışmaya, kafa derisinde tek veya çoklu AA yamaları şikayeti olan 10 hasta (toplam 18 yama) dahil edildiği bildirilmiştir. Tüm yamaların iki ay boyunca günde iki kez ezilerek hazırlanmış ham sarımsak ekstresi ile topikal tedavi edildiği rapor edilmiş, saç büyümesi iki haftada bir değerlendirilmiştir. Çalışma sonuçlarına göre, tüm hastaların tedaviye yanıt verdiği ve AA yamalarının tamamının tedavi edildiği bildirilmiştir. Bununla birlikte tüm hastalarda eritem, sekiz yamada kaşıntı ve beş yamada yanma hissi gözlemlendiği tespit edilmiş ancak bu yan etkilerin ihmal edilebilir düzeyde oldukları açıklanmıştır. Böylece *A. sativum* ham ekstresinin AA için etkili ve hızlı bir topikal tedavi olduğu, bu türün ucuz ve ulaşılabilir olmasının AA tedavisinde avantajlı bir rol oynamasına neden olduğu kayıt edilmiştir [25].

Başka bir çalışma, AA tedavisinde topikal %5 sarımsak jeli ve betametazon krem kombinasyonunun etkinliğini değerlendirmek amacıyla randomize ve plasebo kontrollü bir klinik deney olarak tasarlanmıştır. Çalışma materyali olarak, sarımsak ezilmiş ve filtrasyon yoluyla sarımsak ekstresi elde edilerek ardından jel formülasyonu haline getirilmiştir. Çalışma, 40 hasta üzerinde yürütülmüştür;

bu hastalar rastgele olarak ya sarımsak jel grubuna ya da kontrol grubuna ayrılmıştır. Katılımcılar 5 – 56 yaş aralığında belirlenmiş ve tümü çalışma prosedürünü tamamlamışlardır. Üç aylık tedavi süresinin sonunda, sarımsak jeli uygulanan grupta, saç sayısında ve terminal saç yoğunluğunda kontrol grubuna göre istatistiksel olarak anlamlı bir artış gözlenmiş ve saçsız yamaların büyüklüğünde anlamlı bir azalma tespit edildiği bildirilmiştir. Tedaviye iyi ve orta derecede yanıt veren hasta sayısı sırasıyla 19 ve 1 olarak raporlanmıştır ve bu sonuçlar kontrol grubundaki sonuçlara kıyasla önemli ölçüde daha yüksektir. Çalışma süresince herhangi bir komplikasyon rapor edilmemiştir. Sonuç olarak, topikal sarımsak jeli uygulamasının AA hastalarında etkili ve iyi tolere edilen bir tedavi yöntemi olabileceği ve uzun vadeli terapötik faydalar sunabileceği gösterilmiştir [26].

Sarımsak ayrıca A, C ve B-kompleks vitaminleri; histidin, arginin, sistein, metiyonin ve fenilalanin gibi amino asitler; flavonoitler; ve yüksek miktarda potasyum, çinko, kükürt ve selenyum içerir [27-29]. İnsan dermal papilladan türetilen eksozomların saç döngüsünde anagen fazı indükleyip uzattığı gösterilmiştir. Sarımsak ekstrelerinin AA'daki olası terapötik etkisinin anti-enflamatuvar, immünomodülatör ve antioksidan özelliklerle ilişkili olduğu düşünülmektedir. Bitki kaynaklı eksozomların saç büyümesindeki rolü henüz araştırılmamış olsa da, Hep G2 hücrelerinde yapılan bir çalışmada sarımsak eksozomlarının IFN-γ ve IL-6 seviyelerini düşürerek belirgin anti-enflamatuvar etki gösterdiği bildirilmiştir [29]. Sarımsağın 5α-redüktaz enzimini de güçlü bir şekilde inhibe ederek gösterdiği antiandrojenik aktivitenin, androjenik alopesi üzerindeki etkisini açıklayabileceği kaydedilmiştir [28,29].

A. sativum bileşiminde yer alan diallil disülfidin immün sistemi modüle edici özelliğinden ötürü AA tedavisinde terapötik etkiye katkıda bulunabileceği ayrıca *A. cepa* gibi farklı *Allium* türlerinin de antimikrobiyal, immünomodülatör ve anti-enflamatuvar etkilere sahip çok sayıda farklı organosülfür bileşik taşıdığı bildirilmektedir [30].

***Allium Cepa* L.**

Kullanılan kısım: Allii cepae bulbus

Allium cepa L. türü Liliaceae familyasına ait gıda olarak tüketilen ve aynı zamanda diüretik, stimülan ve ekspektoran özellikleriyle tanınan bir bitkidir [31,32]. Yüzyıllardır çeşitli yerli kültürlerde mikrobiyal enfeksiyonlar, solunum, mide-bağırsak, cilt ve kalp-damar bozuklukları, diyabet, renal kolik, romatizma, cinsel iktidarsızlık, adet ağrısı ve baş ağrısı gibi çeşitli rahatsızlıkların tedavisi için tüketilmektedir [33]. *A. cepa* türünün su, karbonhidratlar, proteinler, lektin, yağlar, vitaminler, mineraller, flavonoidler, organosülfür ve fenolik bileşikler içerdiği bildirilmiştir [34].

AA tedavisinde topikal ham soğan suyunun etkinliğini, musluk suyu ile karşılaştırmak amacıyla tasarlanan bir çalışmada, hastalar soğan suyu (n=23) ve musluk suyu (n=15) grupları olarak ikiye ayrılmıştır. İki grup da tedaviyi günde iki kez iki ay boyunca uygulamış, ham soğan suyuyla tedaviye başladıktan iki hafta sonra saçın yeniden büyümesinin başladığı belirtilmiştir. Dört haftada, saç yeniden büyümesinin 17 hastada görüldüğü ve altı haftada, saç yeniden büyümesinin 20 hastada (%86.9) gözlendiği ve bu oranın erkeklerde kadınlara göre anlamlı şekilde daha yüksek olduğu bildirilmiştir. Musluk suyu ile tedavi edilen kontrol grubunda, tedavinin 8. haftasında, cinsiyet farkı olmaksızın, saç yeniden büyümesinin sadece 2 hastada (%13) görüldüğü, ham soğan suyunun kullanımının, musluk suyuna kıyasla saç yeniden büyüme konusunda anlamlı şekilde daha iyi sonuçlar verdiği ve AA için etkili bir topikal tedavi olabileceği gösterilmiştir. *A. sativum* ve *A. cepa*'nın kimyasal bileşenlerinde başta allisin (diallil disülfit) olmak üzere birçok benzerlik bulunduğu da rapor edilmiştir [35].

Başka bir çalışmada, *A. cepa* suyunun ve minoksidil formülasyonunun saç büyümesini teşvik edici aktiviteleri karşılaştırılmıştır. Saç büyüme düzeni; grup I (kontrol grubu), grup II (*A. cepa* test grubu) ve grup III (minoksidil standart grup) için gözlemlenmiştir. Grup I'de saç büyümesinin 10. günde başladığı, grup II'de 6. günde başladığı ve grup III'te 5. günde başladığı bildirilmiştir. Saçın tamamının büyümesi sırasıyla grup I için 24. gün, grup II için 20. gün, grup III için 18. günde gözlemlendiği açıklanmıştır. *A. cepa*'nın saç büyümesini tetikleyici aktivitesinin, minoksidile benzer düzeyde etkili olduğu gösterilmiştir [36].

***Hibiscus Rosa-Sinensis* L.**

Kullanılan kısım: Hibiscus rosa-sinensis folium

Hibiscus rosa-sinensis L. Malvaceae familyasının dünyada yaygın olarak yayılış gösteren bir türüdür. Yaprakları, kabukları, kökleri ve çiçekleri Hint geleneksel tıp sisteminde çeşitli hastalıkların tedavisinde ilaç olarak kullanılmaktadır. *H. rosa-sinensis* bitkisinin farklı kısımlarının antioksidan, antimikrobiyal, antidiyabetik, antiülser, hepatoprotektif, antigenotoksik ve anti-enflamatuvar özelliklere sahip olduğu ve birçok hastalığın tedavisine yardımcı olduğu çeşitli araştırmalarla kanıtlanmıştır [37]. Fitokimyasal analizler, bu bitkinin tanenler, antrakinonlar, fenoller, flavonoidler, alkaloidler, terpenoidler, saponinler, kardiyak glikozitler, protein, serbest amino asitler, karbonhidratlar, indirgeyici şekerler, müsilaj, uçucu yağlar ve steroidler içerdiğini göstermiştir [38]. Hindistan'da özellikle diüretik çay olarak tüketilen *H. rosa-sinensis* türünün çiçeklerinden Soxhlet yöntemi ile elde edilen sulu ekstrelerin albino farelerde humoral immün yanıtı hücresel immün yanıta göre daha fazla aktive ettiği ve bu immünmodülatör etkinin türün bileşiminde yer alan flavonoit veya alkaloitlerden kaynaklı olabileceği belirtilmiştir [39]. Hipoglisemik etkileri bilinen H. rosa-sinensis türünün sulu yaprak ve etanolik çiçek ekstrelerinin diyabetik sıçanlarda trgliserid seviyelerini düşürüp, HDL kolesterol seviyelerini arttırdığı kayıt edilmiştir [40].

H. rosa-sinensis'in yaprak ve çiçek ekstrelerinin saç büyümesini teşvik edici aktivitelerinin *in vivo* olarak değerlendirilmesine yönelik yapılan bir çalışmada, yaprak ve çiçeklerin ayrı olarak petrol eteri ile ekstraksiyonunun yapıldığı, çözücü buharlaştırıldıktan sonra geriye kalan ham ekstrenin %1 oranında sıvı parafin içinde çözündürüldüğü ve fareler üzerine topikal olarak uygulandığı belirtilmiştir. Farelerin; çiçek ekstresi ile tedavi edilen, yaprak ekstresi ile tedavi edilen ve tedavi almayan grup (kontrol grubu) olmak üzere üçe ayrıldığı, tedavinin 42 gün devam ettiği ve tedavi süresi boyunca morfolojik ve histolojik değerlendirmeler yapıldığı açıklanmıştır. Bu çalışmanın sonuçlarına göre, yaprak ekstresinin saç folikülü üzerinde olumlu etkisinin olduğu ve çiçek ekstresine kıyasla daha fazla saç büyüme arttırıcı etki gösterdiği bildirilmiştir. Dolayısıyla *H. rosa-sinensis,* AA tedavisinde potansiyel bir alternatif olarak görülmektedir [41].

Başka bir çalışma, *Aloe vera* jeli içindeki *Maharanga bicolor* ve *H. rosa-sinensis* ekstrelerinin saç büyümesini teşvik etme aktivitesini belirlemek amacıyla yapılmıştır. Minoksidil sülfat ve *M. bicolor* ve *H. rosa-sinensis*'in petrol eteri ekstresinin farklı konsantrasyonlarının 30 gün boyunca farelerin tıraşlı bölgelerine uygulandığı belirtilmiştir. %2 Minoxidil pozitif kontrol olarak kullanılırken, boş formülasyon negatif kontrol olarak kabul edilmiştir. Saç büyüme düzeni, saç büyüme başlangıcı, saç büyüme tamamlanması ve saç uzunluğu değerlendirilmiş, *M. bicolor* (2%) formülasyonunun 6. günde, *H. rosa-sinensis* (2%) formülasyonunun 8. günde ve boş örneğin 11. günde saç büyüme başlangıcı gösterdiği bildirilmiştir. Her bir ekstrenin %2'sini içeren formülasyon ile tedavi edilen grubun, 19. günde en kısa saç büyüme tamamlanma süresine sahip olduğu ve güçlü saç büyüme düzeni sergilediği, *M. bicolor* ve *H. rosa-sinensis* türleri ile hazırlanan petrol eteri ekstrelerinin saç büyümesini uyarıcı aktivitelerinin minoksidile kıyasla daha yüksek olduğu gösterilmiştir [42].



Şekil 4. *Hibiscus rosa-sinensis* L. [30]

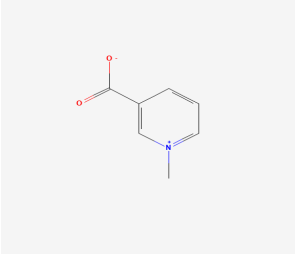
***Trigonella Foenum-Graecum* L.**

Kullanılan kısım: Trigonellae foenugraeci folium ve Trigonellae foenugraeci semen

Trigonella foenum-graecum L. veya çemen otu, Fabaceae familyasına ait tek yıllık otsu aromatik bir bitkidir. Hipoglisemik ve hipokolesterolemik etkilerinin yanı sıra immünomodülatör ve antikanser aktiviteye sahiptir [43]. Bu bitki, doğumu kolaylaştırmak, sindirime yardımcı olmak ve metabolizmayı iyileştirmek için genel bir tonik olarak kullanılmıştır [44]. Yaprak ve gövdenin sulu, petrol eteri ve diklorometan ekstrelerinin ön analizi, alkaloitler, flavonoidler, tanen, saponin, reçine ve steroidlerin varlığını göstermiştir [45].

T. foenum-graecum yaprak ekstrelerinin saç büyümesini uyarıcı etkilerini araştırmak amacıyla yapılan bir çalışmada, toz halindeki yaprakların ekstreleri, çeşitli çözücülerde (petrol eteri, kloroform, metanol, etanol ve damıtılmış su) sıcak ve soğuk ekstraksiyon yöntemleri kullanılarak elde edilmiş ve tüm ekstreler arasında minoksidil ile karşılaştırıldığında sadece etanol ve petrol eteri ekstrelerinin *in vivo* deneylerde saç büyümesi ve uzunluğu üzerinde önemli etkiler gösterdiği bildirilmiştir. Bu etkilerin, antioksidan aktivite ve serbest radikal temizleme özelliklerine sahip olan zengin fenolik içeriğine bağlı olabildiği belirtilmiştir. Bu bitkinin yaprak ekstresinde, sağlıklı saç ve tırnak büyümesini desteklediği bilinen yüksek miktarda çinko ile belirli protein ve aminoasitlerin bulunmasının saç büyümesini teşvik edici etkilerine önemli ölçüde katkıda bulunabileceği ayrıca, *T. foenum-graecum* yapraklarında bulunan nikotinik asit benzeri bir alkaloid olan trigonellinin, kafa derisinde kan dolaşımını arttırarak saç büyümesini uyardığı düşünülmektedir [46].

Başka bir çalışma, *T. foenum-graecum*'un tohumları ve *Butea monosperma*'nın çiçeklerinin saç büyümesini artırıcı etkisini değerlendirmek amacıyla yapılmıştır. Albino sıçanlarda kemoterapiyle uyarılan alopesi modeli kullanılmıştır. Bu iki bitkinin saç büyümesi üzerindeki etkileri; saç boyu, saç sayısı, total serum protein seviyeleri ve testosterone seviyeleri değerlendirilerek araştırılmıştır. Her iki türün boş örnekle tedavi edilen kontrol grubuna kıyasla olumlu sonuçlar verdiği ve saç büyümesini teşvik edici etkiye sahip olduğu belirtilmiştir. *T. foenum-graecum*'un saç büyümesini destekleyebilecek önemli miktarda protein ve çeşitli amino asitler içerdiği ve *Butea monosperma*'nın saç dökülmesini önleyen ve serbest radikal temizleyici aktivitesi ile antioksidan etkinliği olan yüksek miktarda flavonoit içerdiği bildirilmiştir [47].



Şekil 5. Trigonellin bileşiğinin kimyasal yapısı [48]

Asarum Sieboldii Miq. (Syn. *Asiasarum Sieboldi* Miq.)

Kullanılan kısım: Asarum sieboldii radix

Asarum sieboldii Miq., Aristolochiaceae familyasına ait tıbbi bir bitkidir [49]. *A. sieboldii* türü Kore ve Çin tıbbında yaygın olarak tedavi amacı ile kullanılmaktadır. Türün aftöz stomatit, lokal ağrı, diş ağrısı ve diş eti iltihabı gibi hastalıklara karşı analjezik ve anti-enflamatuvar etkileri ile beyin hücre hasarına karşı koruyucu etkiler gibi bir dizi farmakolojik aktivitesi olduğu bildirilmektedir [50,51]. Bu bitkinin köklerinin etil asetatta çözünebilen fraksiyonundan tetrahidrofurofurano lignanları, fenilpropanoitler ve alkamitler izole edildiği ve safrol, metil öjenol, metoksitoluen ve 3-benzodioksol türevlerini içerdiği bildirilmiştir [52,53].

Saç dökülmesi tedavisinde geleneksel doğu tıbbında kullanılan bitki ekstrelerini inceleyen bir çalışmada, 45 farklı bitki ekstresi *in vivo* olarak incelenmiş ve *A. sieboldii* kök ekstresinin telojenden anajene geçişi uyarmada hem hücresel çoğalmayı hem de protein sentezini artırmada en etkili tür olduğu tespit edilmiştir. Bu çalışmada kök ekstresinin insan dermal papilla hücrelerinde saç büyümesini uyarıcı etkiye sahip olan VEGF geninin ekspresyonunu arttırdığı gösterilmiştir. Aynı çalışmada, C57BL/6

farelerinin sırtlarına 30 gün boyunca topikal olarak uygulanan, *A. sieboldii* ekstresinin, kontrol tedavisine göre daha erken telogenden anajene geçişe neden olduğu açıklanmıştır. Bu sonucun doğrulanması için C3H farelerine aynı rejimin 45 gün boyunca uygulandığı ve yine telogenden anajene geçişin indüklendiği bildirilmiştir. Histolojik çalışmalara göre, *A. sieboldii* kök ekstresinin, kontrol tedavisi ile karşılaştırıldığında saç foliküllerinin derinliğini ve boyutunu belirgin bir şekilde artırdığı gösterilmiştir. Bu sonuç, *A. sieboldii* ekstresinin erken anajen başlangıcını teşvik ettiği ve saç büyümesini uyardığı düşüncesini desteklemektedir. Sonuç olarak yapılan çalışmalarda, *A. sieboldii* kök ekstresinin *in vitro* kültürlenmiş dermal papilla hücrelerinde büyüme faktörlerinin ekspresyonunu düzenlediği ve hayvan modellerinde önemli saç büyüme tetikleyici etkilere sahip olduğu kaydedilmiştir [53].

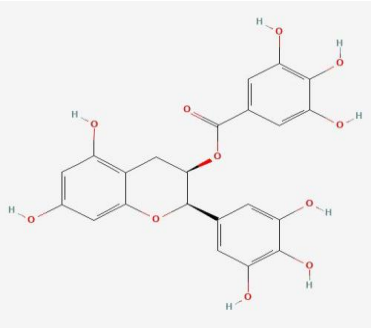
***Vitis Vinifera* L.**

Kullanılan kısım: Vitis viniferae semen

Vitis vinifera L. veya üzüm, Vitaceae familyasının *Vitis* cinsine ait bir türüdür. Bazı ülkelerde üzüm, anemi, alerji, yara bakımı, soğuk algınlığı, grip, gaz giderici olarak ve bronşit tedavisi gibi geleneksel tıpta kullanıldığı rapor edilmiştir [54]. Flavonoitler, üzüm çekirdeğinde en bol bulunan bileşenlerdir ve bunu fenolik asitler, resveratrol, tirozol ve siringaldehit takip etmektedir. Ayrıca, steroidler, terpenoidler, antosiyaninler, emodinler ve glikozitler içermektedir [55,56].

V. vinifera meyve kabuğu ve çekirdek ekstrelerinin antifungal ve 5α-redüktaz inhibitör aktivitelerini değerlendirip saç büyümesi üzerinde etkilerini araştırmak amacıyla yapılan bir çalışmada, bu ekstrelerin çeşitli mantarlara karşı güçlü antifungal aktivite sergilediği gösterilmiştir. Ayrıca, etanolik ekstrelerin, insan saç folikülü dermal papilla hücrelerinin (HFDPC) çoğalmasında bir artışa neden olduğu ve HFDPC üzerinde *in vitro* olarak analiz edilen 5α-redüktaz inhibisyonu yoluyla saç dökülmesini azaltıcı aktivite gösterdiği belirtilmiştir. Bu aktivitelerin *V. vinifera*'nın çekirdeğinde yaygın olarak bulunan epigallokateşin-gallat (EGCG) ve kateşol varlığına bağlı olduğu açıklanmıştır [57].

V. vinifera meyve çekirdek ekstrelerinde bulunan prosiyanidin oligomerlerinden özellikle (-)-epikateşin, prosiyanidin B-2 ve prosiyanidin C-1'in hem saç kültürlerinde hem de C3H farelerinde topikal kullanım için test edildiği ve hem epitelyal hem mezenkimal hücrelerde keratinosit ve fibroblast artışını %250'lere varan oranlarda arttırma sureti ile topikal uygulama sonrasında artan bir saç çoğalmasının kayıtlı olduğu bir araştırmada bu etkinin saç hücresi döngüsündeki telogen-anagen faz dönüşümüne bağlı olabileceği rapor edilmiştir. Çalışmada ayrıca incelenen diğer flavonoit bileşiklerden hiçbirinin prosiyanidinlerden daha yüksek proliferatif aktivite göstermediği cilt hücreleri arasında sadece saç keratinositleri veya epidermal keratinositler gibi epitel hücrelerinin prosiyanidin oligomerlerine yanıt verdiği bildirilmektedir [58].



Şekil 6. Epigallokateşin-gallat (EGCG) bileşiğinin kimyasal yapısı [59]

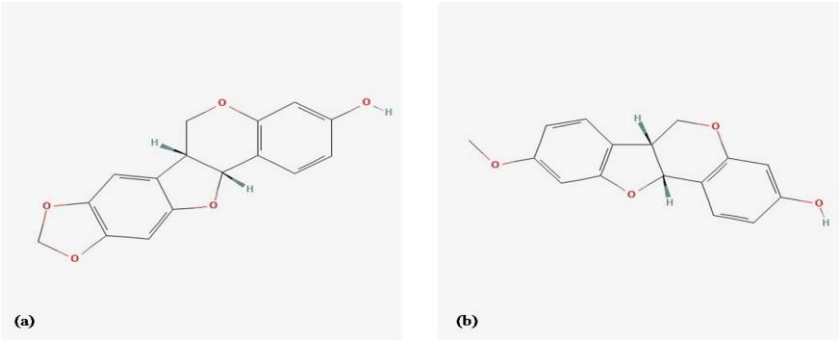
***Sophora Flavescens* Ait.**

Kullanılan kısım: Sophorae flavescentis radix

Sophora flavescens Ait., Fabaceae familyasına ait tıbbi bir bitkidir. Geleneksel Çin tıbbında, *S. flavescens* ateş, dizanteri, sarılık, astım, egzama, enflamatuvar bozukluklar, ülserler ve cilt yanıkları ile ilişkili hastalıkların tedavisinde yaygın olarak kullanıldığı bildirilmiştir [60]. Son zamanlarda yapılan *in vitro* ve *in vivo* çalışmalar, *S. flavescens*'ten elde edilen en az 50 saf bileşiğin ve ham ekstrenin, geniş kapsamlı antitümör, antimikrobiyal, antipiretik, antinosiseptif ve anti-enflamatuvar farmakolojik aktiviteler sağladığını göstermiştir [61]. Bu bitkinin alkaloitleri olarak oksimatrin, matrin, losmatrin, soforanol ve sofokarpin sayılabilir. Bunun yanında bioflavon olan norkurarinon, kuraridinol, soforaflavanon, formonetin ve yağ asitleri içerdiği bilinmektedir [62].

Saç büyümesi üzerinde *S. flavescens*'in *in vivo* etkisini araştıran bir çalışmada kurutulmuş metanolik kök ekstresinin topikal uygulanmasından sonra telojenden anajene daha erken dönüşümü indüklediği belirtilmiştir. Bu çalışmanın kapsamında, kök ekstresinin *in vitro* dermal papilla hücrelerinin büyümesi üzerinde belirgin etkisinin olmadığı ancak IGF-1 ve KGF gibi büyüme faktörlerinin mRNA seviyelerini indüklediği açıklanmıştır. Bu nedenle *S. flavescens* ekstresinin büyüme faktörlerinin düzenlenmesi yoluyla etki ettiği düşünülmektedir. Ayrıca bu türün tip II 5α-redüktaz aktivitesi üzerinde güçlü inhibitör etkisinin olduğu ortaya konulmuştur [62].

Aynı amaçla yapılan başka bir çalışmada *S. flavescens*'in kök ekstresinin düşük konsantrasyonda saç keratinositlerinin çoğalmasını uyardığı, daha yüksek konsantrasyonda insan saç folikülünün organ kültüründe saçın uzaması üzerinde belirgin bir etkiye sahip olduğu bildirilmiştir. Bu etkilerden sorumlu, iki pterokarpan türevi olan L-maackiain ve medikarpinin, aktif bileşikler olarak tanımlandığı belirtilmiştir [63].



Şekil 7. (a) L-maackiain ve (b) medikarpin'in kimyasal yapıları

Citrullus Colocynthis (L.) Schrad.

Kullanılan kısım: Colocynthidis fructus

Citrullus colocynthis (L.) Schrad. Cucurbitaceae familyasına ait bir türdür. Bu bitkinin meyveleri, nutrasötik potansiyellerinin yanı sıra astım, bronşit, kanser, kolik, soğuk algınlığı, öksürük, diyabet, dizanteri ve sarılık gibi çeşitli kullanım alanları olduğu rapor edilmiştir [64]. Esas olarak, kukurbitasin E, I ve L glikozitleri içerir [65]. Ayrıca, fitosterol glikozit, sitrullol, pektin ve albüminoitler içermektedir [66]. Bu türün antioksidan, hipoglisemik, antibakteriyel, anti-enflamatuvar, analjezik, hepatoprotektif, antidiyabetik, hipolipidemik, antineoplastik, profibrinolitik, anti-alerjik ve bağışıklık uyarıcı gibi birçok biyolojik özelliği vardır [67].

C. colocynthis Schrad geleneksel olarak kullanılan bir saç toniğidir. Bu bitkinin petrol eteri ve etanol ekstrelerinin *in vivo* saç büyümesi üzerindeki etkilerini değerlendirmek için yapılan bir çalışmada, ekstrelerin yağlı merhem bazına dahil edildiği ve topikal olarak uygulandığı bildirilmiştir. Minoxidil %2 solüsyonunun standart olarak kullanıldığı belirtilmiştir. Saç büyümesinin başlaması ve saç büyüme döngüsünün tamamlanması için gereken süre kaydedilmiş, tedavi edilmeyen kontrol grubu ile karşılaştırıldığında, petrol eteri ekstreleri ile tedavide saç büyümesinin başlama süresinin yarı yarıya azaldığı ve saçın tamamen büyümesi için gereken sürenin de önemli ölçüde azaldığı gösterilmiştir. Meyvenin petrol eteri ekstresinin standart minoksidile kıyasla daha fazla sayıda saç folikülünü anajen faza getirmede başarılı olduğu ayrıca ekstrenin anajenik saç foliküllerinin korunmasına yardımcı olduğu

ve dolayısıyla, anajenik fazdaki saç folikülü popülasyonunun yüzdesinin kontrol grubuna göre belirgin bir iyileşme gösterdiği rapor edilmiştir [68].

***Phyllanthus Emblica* L. (Synonym *Emblica Officinalis* Gaertn.)**

Kullanılan kısım: Phyllanthi fructus

Phyllanthus emblica L., Euphorbiaceae familyasına ait bir bitkidir [69]. Bu tür, son derece besleyicidir ve C vitamini, mineraller ve amino asitler açısından önemli bir besin kaynağı olarak rapor edilmiştir. *P. emblica*'nın antimikrobiyal, anti-enflamatuvar, analjezik, antipiretik, hepatoprotektif ve antiülserojenik aktivitelere sahip olduğu bildirilmiştir [70]. Geleneksel tıpta diyare, sarılık ve enflamasyon tedavisinde kullanılmıştır [71]. Ayrıca, alkaloidler (phyllantidine, phyllantine), gallotanenler, karbonhidratlar, pektin, fenolik asit, gallik asit, ellajik asit, emblikol ve çeşitli amino asitler içermektedir [72].

P. emblica, Hint geleneksel tıp sistemlerindeki en önemli tıbbi bitkilerden biridir. Bu bitki, güçlü bir 5α-redüktaz inhibitörü ve saç büyüme destekleyicisidir. *P. emblica* yağının saç büyümesini desteklemedeki etkinliğini belirlemek amacıyla yapılan bir çalışmada, insan denekler üzerinde gerçek bir deneysel araştırma tasarımı kullanılmıştır. Çalışmaya alınan alopesi hastalarının rastgele 2 gruba ayrıldığı, A grubuna *P. emblica* yağı uygulandığı ve B grubuna hint yağı uygulandığı belirtilmiştir. Her iki gruptaki saç uzunluğundaki ortalama değişim ve saç büyüme hızı, haftalık olarak bir şerit metre kullanılarak saç uzunluğunun ölçülmesiyle izlenmiştir. A Grubunda (*P. emblica*) saç büyüme hızı 1,58 cm/ay, B grubunda (Hint Yağı) ise 1,1 cm/ay olduğu bildirilmiştir. Bu çalışmayla *P. emblica* yağının alopesi hastalarında saç büyümesini desteklemede etkili olduğu gösterilmiştir. Ayrıca Hint yağının da saç büyümesini destekleyici etki gösterdiği, ancak *P. emblica* yağının deney boyunca daha önemli bir saç büyüme hızına sahip olduğu görülmüştür [73].

***Eclipta Alba* (L.) Hassk (Synonym: *Eclipta prostrata* (L.) L.)**

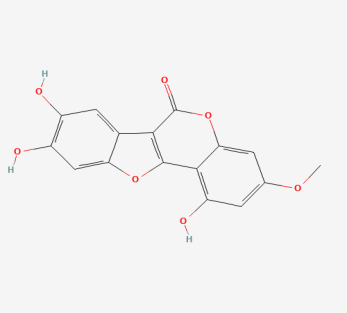
Kullanılan kısım: Ecliptae herba

Eclipta alba (L.) Hassk, Asteraceae familyasına ait beyaz çiçekli bir bitkidir [74]. *E. alba,* saç büyümesini teşvik ettiği iyi bilinen bir Ayurveda bitkisidir [75]. Ayrıca, antihepatotoksik, analjezik, antioksidan, antihiperglisemik, antiagresif ve yara iyileştirici özelliklere sahip olduğu bilinmektedir [76]. Geleneksel kullanımları arasında, uykusuzluk için yatmadan önce yaprakların yağlı ekstresinin saç derisine uygulanması ve akrep sokması tedavisinde bitkinin dekoksiyonunun kullanılması yer almaktadır. Bununla birlikte astım, bronşit, ateş, mide ve karaciğer rahatsızlıkları, sarılık, ülser, yara ve lökodermada kullanımı kayıtlıdır [77].

E. alba petrol eteri ve etanol ekstrelerinin saç büyümesi üzerindeki *in vivo* etkisini değerlendirilmek amacıyla yapılan bir çalışmada, ekstrelerin yağ içinde su (S/Y) kremine dahil edildiği ve topikal olarak uygulandığı bildirilmiştir. Saç büyümesinin başlaması ve saçın tamamen büyümesi için gereken sürenin (gün olarak) kaydedildiği belirtilmiştir. Minoksidil %2 solüsyonunun topikal olarak uygulandığı ve karşılaştırma için pozitif kontrol olarak kullanıldığı açıklanmıştır. *E. alba* ekstresiyle tedavinin saç büyümesinin başlama süresini, kontrol grubuna kıyasla yarıya azalttığı gösterilmiştir. Saçın tamamen büyümesi için gereken sürenin de önemli ölçüde azaldığı açıklanmıştır. Saç büyümesinin kantitatif analizi ile, petrol eteri (%5) ekstresiyle tedaviden sonra anajen fazdaki saç folikülü sayısının (69±4) kontrole (47±13) kıyasla daha yüksek olduğu gösterilmiştir. %2 ve %5 petrol eteri ekstreleri ile yapılan tedavinin, pozitif kontrol minoksidil %2 tedavisinden daha iyi sonuçlar verdiği açıklanmıştır [75].

Başka bir çalışmada *E. alba, A. sieboldii* ve *Panax ginseng* ekstrelerinin etkisi, saç büyüme potansiyeli açısından *in vivo* ortamda araştırılmıştır. Bu araştırmada çıplak fareler adı verilen, anormal keratinizasyon nedeniyle genetik olarak saçtan yoksun olan mutant farelerin kullanıldığı belirtilmiştir. Araştırılan türlerin metanol ekstrelerinin farelerin sırtına iki saç büyüme neslinin tamamlanmasına kadar her gün topikal olarak uygulandığı bildirilmiştir. Tüm ekstreler arasında, *E. alba* ile tedavi edilen farelerin saç büyüme alanının belirgin bir şekilde görünür olduğu ve aynı zamanda sırtın maksimum kısmını kapladığı, türün güçlü bir şekilde saç büyümesini teşvik edici potansiyele sahip olduğu bildirilmiştir [78]. Bu gözlemlere göre, *E. alba* ile tedavinin geçici olarak saç foliküllerinin keratinositini normalleştirmeye yardımcı olduğu ve artan saç kapsama alanıyla tamamen oluşmuş görünür saç

folikülleri oluşturduğu öne sürülmektedir. Saç foliküllerinin sayısının ve büyüklüğünün artması, saç büyümesinin telojenden anajene geçişinin bir göstergesi olarak kabul edilmektedir [79,80]. HPLC analizi, *E. alba*'nın metanol ekstresinin başlıca wedelolakton içerdiği göstermiştir. Bunun yanında bulunan demetilwedelolakton ve saponinler gibi çeşitli biyoaktif fitokimyasalların, türün ana tıbbi etkilerinden sorumlu olduğu bildirilmiştir [78].



Şekil 8. Wedelolakton bileşiğinin kimyasal yapısı [81]

Argania Spinosa (L.) Skeels

Kullanılan kısım: Argania spinosae semen

Argan veya *Argania spinosa* (L.) Skeels Sapotaceae familyasına ait bir ağaçtır [82]. Türün tohumlarından elde edilen argan yağı, cilt enfeksiyonlarında, cilt nemlendirmesinde ve saç dökülmesine karşı kullanılan birçok kozmetik ürünün bileşimine giren doğal bir hammaddedir [83]. Argan pres küspesi (APC), argan yağı ekstraksiyonunun bir yan ürünüdür ve argan çekirdeklerinin %50-70'ini temsil eder [84,85]. APC, fenolik bileşikler ve saponinler açısından zengindir ve önemli miktarda fitosterol içermektedir [86]. APC ekstrelerinin, saç dökülmesine karşı aktivite sergilediği ve cilt beyazlatıcı etki gösterdiği bildirilmiştir [87].

APC ekstrelerinin insan saç folikülü dermal papilla hücreleri (HFDPC) üzerindeki etkilerini inceleyerek saç büyümesi üzerine potansiyel etkisini değerlendirmek ve moleküler mekanizmayı belirlemek amacıyla yapılan bir çalışmada, %50 veya %80 sulu etanol kullanılarak hazırlanan ekstrelerin HFDPC'lere uygulandığı, ardından hücre çoğalma testleri, mitokondriyal biyogenez testi ve oksidatif stres testi ile değerlendirildiği bildirilmiştir. Bu çalışmanın sonuçlarına göre, APC'nin HFDPC'lerin çoğalmasını ve ATP içeriğini arttırdığı, böylece anajen fazına hazırlık olarak büyümelerine yol açtığı, oksidatif stresi azalttığı ve mitokondriyal biyogenezi iyileştirdiği açıklanmıştır. Ayrıca, enflamasyon, kolajen parçalanması ve oksidatif stres ile ilişkili genlerin ekspresyonunun azaldığı gösterilmiştir. Bu mekanizma ile APC ekstrelerinin saç büyümesini uyarma etkisine sahip olduğu belirtilmiştir. Bu çalışmanın sırasında GC/MS analizi kullanılarak APC'nin %50 ve %80 etanol ekstresinde saf yağ asitleri, türetilmiş şeker zincirleri ve tokoferoller, skualen ve spinasterolün varlığı tespit edilmiştir [88].

Ginkgo Biloba L.

Kullanılan kısım: Ginkgo biloba folium

Ginkgo biloba L. Ağacı Ginkgoaceae familyasına ait bir türdür. Modern farmakolojik çalışmalar, *G. biloba* yaprak, tohum ve ekzokarpının ham ekstrelerinin, kardiyovasküler ve serebrovasküler hastalıklar, ateroskleroz, kanser, astım, non-alkolik yağlı karaciğer ve diyabet komplikasyonları tedavisinde yardımcı olabileceklerini göstermiştir [89]. Ayrıca, klinik olarak Alzheimer hastalığı ve bilişsel eksiklikler gibi santral sinir sistemi bozukluklarını tedavi etmek için reçete edildiği bilinmektedir [90]. *G. biloba*'nın ana kimyasal bileşenleri; diterpenoidler, ginkgolidler A, B, C, bilobalid-A, kemferol, kersetin, isorhamnetin, fenolik asitler ve polisakkaritlerdir [89,91].

Suda çözünen *G. biloba* yaprak polisakkaritlerinin (WGBP) ayırma ve arıtma teknolojisini optimize etmek, bileşim özelliklerini analiz etmek, *in vivo* saç büyümesini teşvik edici etkisini

gözlemlemek, biyolojik aktivitelere sahip polisakkarit fraksiyonunu belirlemek ve anti-enflamasyon mekanizmasını araştırmak amacıyla yapılan bir çalışmada, asidik polisakkaritlerin (WGBP-A2) izole edildiği ve AA'lı farelerin sırtına uygulandığı bildirilmiştir. Bu tedavinin sonucu olarak farelerinin cilt dokusundaki VEGF ve HGF içeriğinin önemli ölçüde arttığı, serumdaki enflamatuvar faktörlerin azaldığı gösterilmiştir. WGBP-A2 den dört fraksiyonun elde edildiği, rhamnogalakturonan I tipi polisakkarit fraksiyonunun (WGBP-A2b) *in vitro* etkilerinin araştırıldığı belirtilmiştir. Hücresel düzeyde, WGBP-A2b ile tedavi edilen insan göbek damarı endotel hücrelerinde (HUVEC'lerde) enflamatuvar yolak ile ilişkili faktör proteinler olan p-p65 ve p-IκBα, TNF-α ve IL-1β ekspresyonlarının azaldığı açıklanmıştır. Sonuç olarak, *G. biloba*'nın asidik polisakkaritlerinin saç büyümesini teşvik edici bir etkiye sahip olduğu, bu etkinin saç foliküllerinin etrafındaki enflamasyonu hafifletmesine ve VEGF ve HGF'nin ekspresyonunu düzenlemesine bağlı olabileceği ortaya konulmuştur. Dolayısıyla AA tedavisinde ve önlenmesinde etkili bir tür olabileceği öne sürülmektedir [92].

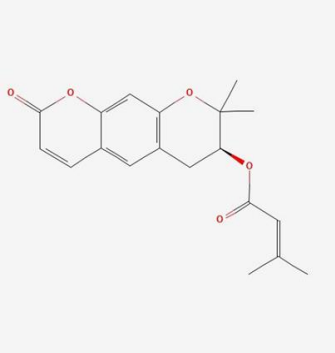
***Angelica Gigas* Nakai**

Kullanılan kısım: Angelicae gigantis radix

Angelica gigas Nakai, Asteraceae familyasına ait tıbbi bir bitkidir. Hematojenik potansiyele sahip olması nedeniyle geleneksel olarak dolaşım bozukluklarını, anemiyi ve kadın rahatsızlıklarını tedavi etmek için kullanılan önemli bir türdür. Bu bitki kumarinler, uçucu yağlar ve poliasetilenler gibi çeşitli bileşikler içerir [93].

Kumarinler arasında, *A. gigas* kök ekstresinin ana bileşeni olan, piranokumarin türevi dekursin; anti-enflamatuvar, antikanser ve antioksidan gibi çeşitli biyolojik aktivitelere sahip olduğu için kapsamlı bir şekilde araştırılmıştır [94,95].

A. gigas etanolik kök ekstresinin ve dekursinin *in vivo* saç büyümesi üzerindeki destekleyici etkilerini ve mekanizmalarını araştırmak amacıyla yapılan bir çalışmada, yüksek performanslı sıvı kromatografisi uygulanarak bu bitki ekstresinin %7.3 dekursin içerdiği gösterilmiştir. Fare sırt derisindeki kılların traş edildiği ve deneklerin üç gruba ayrıldığı, ardından distile su, %0.15 dekursin ve %2 kök ekstresinin topikal olarak uygulandığı belirtilmiştir. %2 kök ekstresinin %0.15 dekursin içerdiği için ticari dekursin %0.15 konsantrasyonunda kullanılmıştır. 17 gün boyunca günde iki kez topikal olarak uygulandıktan sonra saç köklerindeki morfolojik değişiklikler incelendiği ve cilt dokusundaki enflamasyonda rol oynayan sitokin düzeylerinin karşılaştırıldığı bildirilmiştir. Sonuçlara göre, dekursin ve *A. gigas* ekstresinin saç büyümesi üzerinde destekleyici etki sağladığı gösterilmiştir. Saç büyümesi, kontrol grubuna kıyasla tedavilerden sonraki yedinci günden itibaren önemli ölçüde artmış tedavilerden 17 gün sonra saç büyümesinin tamamlandığı gözlemlenmiştir. Ayrıca, proinflamatuvar sitokinler olan tümör nekroz faktörü TNF-α ve interlökin IL-1β'nın protein seviyeleri ve immünoreaktivitelerinin önemli ölçüde azalırken, anti-enflamatuvar olan IL-4 ve IL-13 seviyeleri ve immünoreaktivitesi kontrol grubundakilerle karşılaştırıldığında önemli ölçüde arttığı açıklanmıştır. Sonuç olarak, dekursin içeren *A. gigas* kök ekstresi ile tedavinin, pro- ve anti-enflamatuvar sitokinleri düzenleyerek saç büyümesini desteklediği bildirilmiştir [96].



Şekil 9. Dekursin bileşiğinin kimyasal yapısı [97]

Rosmarinus Officinalis L.

Kullanılan kısım: *Rosmarini folium, Rosmarini aetheroleum*

Lamiaceae familyasına ait bir tür olan *R. officinalis* (biberiye) türünün vatanı Akdeniz bölgesidir ancak adaptif yapısı nedeniyle dünyanın her yerinde kolaylıkla yetiştirilmektedir. Halk hekimliğinde biberiye, böbrek sancısı, dismenore, solunum bozuklukları, depresyon ve saç dökülmesinin hafifletilmesi de dahil olmak üzere çeşitli terapötik etkilerinden ötürü kullanılmaktadır. Bunun yanı sıra diyabet ve hipertansiyon tedavisi için de geleneksel kullanımı kayıtlı olan bir türdür. Alman Komisyon E, biberiye yaprağını dispepsi tedavisi için biberiye yağını ise haricen eklem ağrısı ve minor peripheral dolaşım bozukluklukları için onaylamıştır. Bileşiminde rosmarinik asit, ursolik asit, kafeik asit gibi fenolik asitler, luteolin, apigenin ve diosmin gibi flavonoitler ve değişen oranlarda başta karnozik asit olmak üzere diterpen ve oleanolik asit ve ursolik asit gibi triterpenik bileşikler, özelikle uçucu yağında α-pinene, 1,8-sineol, borneol, kafur, kampfenve limonen gibi birçok biyoaktif fitokimyasal bileşen içerir. Bu çeşitli bileşenler sayesinde anti-enflamatuvar, antioksidan, anti-mikrobiyal, antiproliferatif, antitümör, zayıflamaya yardımcı biyolojik aktiviteler gösterdiği kayıtlıdır [98].

R. officinalis yaprak özütünün (RO-ext, 2 mg/gün/fare) topikal uygulaması, testosteron tedavisiyle indüklenen saç yeniden uzaması kesintisi yaşayan farelerde saç yeniden uzamasını iyileştirdiği, sırt bölgeleri traş edilen farelerdesaç büyümesini desteklediği bildirilmiştir. *R. officinalis* yaprak ekstresinin etki mekanizması araştırılmış ve sırasıyla 200 ve 500 µg/ml'lik konsantrasyonlarda 5α-redüktaz enzimini %82.4 ve %94.6 oranında inhibe ettikleri gösterilmiştir. Biyoaktivite rehberli fraksiyonlama yardımı ile 5α-redüktaz inhibisyonundan sorumlu bileşiğin 12-metoksikarnosik asit olduğu tanımlanmıştır. Ek olarak, *R. officinalis* ekstresi ve 12-metoksikarnosik asit, LNCaP hücrelerinin androjene bağlı proliferasyonunu sırasıyla 5 µg/ml ve 5 µM'de %64.5 ve %66.7 oranlarda inhibe ettiği ve *R. officinalis* yaprak ekstresinin saç büyümesi için ümit verici bir doğal hammadde olduğu bildirilmiştir [99].

Bir diğer çalışmada, UVB'ye maruz kalan farelere 21 gün boyunca topikal olarak parafin yağı, %2 minoksidil veya çeşitli *R. officinalis* uçucu yağının çeşitli konsantrasyonları uygunlanmış, saç uzunluğu ve folikül çapı ile deri vasküler endotelyal büyüme faktörü (VEGF) seviyesi üzerindeki etkilerini değerlendirilmiştir. 21. Gün sonunda yapılan ölçümler ile %10'luk uçucu yağın saç uzunluğunu, folikül çapını ve VEGF seviyesini minoksidil ve parafin gruplarına oranla önemli ölçüde artırabildiği ve bu etkinin uçucu yağ bileşiminde yer alan antioksidan bileşiklerden kaynaklanabileceği kayıt edilmiştir [100].

Biberiyenin saç derisindeki dolaşımı artırdığı ve muhtemelen saç büyümesini desteklediği, tarihsel kullanıma göre bildirilmiştir. Sınırlı sayıda araştırma, AAtedavisinde biberiye uçucu yağının farklı bitki tür uçucu yağlar ile kombine formlarının yararlı olabileceğini göstermekle birlikte veriler yetersiz kalmakta ve kesin bir değerlendirme yapılabilmesi için ek çalışmalara ihtiyaç bulunduğu bildirilmektedir [101].

Lavandula Angustifolia L.

Kullanılan kısım: *Lavandulae flos, Lavandulae aetheroleum*

Çeşitli tıbbi, kozmetik ve aromaterapi uygulamalarında yaygın olarak kullanılan *Lavandula angustifolia* L., Akdeniz bölgesine özgü Lamiaceae familyasında yer alan çok yıllık aromatik bir bitki türüdür [102]. Lavanta uçucu yağı, stres azaltıcı, zihinsel rahatlamayı artırıcı, uyku düzenleyici, iltihap giderici ve antiseptik özelliklerinin yanı sıra, mide bulantısı, sindirim bozuklukları ve şişkinlik gibi rahatsızlıkların tedavisinde yaygın olarak kullanılır [103]. Lavanta yağının ana bileşenlerinin linalool, linalil asetat, lavandulol, 1,8-sineol, osimen ve terpinen-4-ol olduğu kayıtlıdır. Lavanta uçucu yağının güçlü antioksidan, antimikrobiyal, antifungal, antispazmodik, antikanser, sedatif ve analjezik aktivitelere sahip olduğu bildirilmiştir [104].

Lavanta uçucu yağının (LO) farelerde saç büyümesine olan etkilerini belirleme amacı ile yürütülen bir *in vivo* çalışmada deney hayvanları normal grup (N: tuzlu su), taşıyıcı kontrol grubu (VC: jojoba yağı), pozitif kontrol grubu (PC: %3 minoksidil), deney grubu 1 (E1: %3 LO) ve deney grubu 2 (E2: %5 LO) olmak üzere ayrılmıştır. Test bileşiği solüsyonları, günde bir kez, haftada 5 kez, 4 hafta boyunca farelerin sırtlarına uygulama başına 100 µl olacak şekilde topikal olarak uygulanmıştır.

Hematoksilen ve eozin ile boyanan deri dokularında saç folikülü sayısı, dermal kalınlık ve saç folikülü derinliğindeki değişiklikler ve mast hücresi sayıları ölçülmüştür. PC, E1 ve E2 gruplarında, N grubuna kıyasla önemli ölçüde artmış saç folikülü sayısı, derinleşmiş saç folikülü derinliği ve kalınlaşmış dermal katman ile birlikte mast hücresi sayısında önemli ölçüde azalma görüldüğü ve LO'nun morfolojik ve histolojik olarak saç büyümesini belirgin şekilde teşvik edici etkiye sahip olduğu gösterilmiştir [105].

AA'lı yetişkinlerde aromaterapötik uygulamaların etkinliğinin değerlendirildiği randomize, çift kör, kontrollü bir klinik çalışmada denekler (n=84) 2 gruba ayrılarak ilk gruba *Thymus vulgaris* (2 damla, 88 mg), *Lavandula angustifolia* (3 damla, 108 mg), *Rosmarinus officinalis* (3 damla, 114 mg) ve *Cedrus atlantica* (3 damla, 94 mg) yağlarının 3 ml jojoba ve 20 ml üzüm çekirdeği yağı kombinasyonundan oluşan taşıyıcı yağ içerisindeki karışım, kontrol grubuna ise sadece taşıyıcı yağ kafa derisine masaj yaptırılarak kullandırılmıştır. Kombine tedaviyi 7 aylık günlük kullanan deneklerin %44'ünün kontrol grubundaki hastalara (%15) kıyasla anlamlı iyileşme gösterdiği kayıt edilmiştir. Bununla birlikte çalışmanın örneklem büyüklüğünün anlamlı bir farkı tespit etmek için yetersiz olduğu, randomizasyon sürecinin tanımlanmadığı, bir kombinasyon ürününün kullanımı nedeni ile herhangi bir spesifik yağa dair sonuçlara varılmasının mümkün olmadığı, çalışma çift-kör olarak tasarlanmış olsa da, aromatik yağ karışımlarının kokusunun tanımlanabilir oluşu sebebi ile metodolojik yönden uygun olmayan yönleri bulunduğu değerlendirilmiştir [106]. İkinci bir benzer çalışmada, aynı kombinasyon 40 hastada formülasyona çuha çiçeği yağı eklenerek değerlendirilmiş ve müdahale grubu 12. haftada hastaların %60'ı orta düzeyde yeniden büyüme elde ederken, taşıyıcı yağ grubundaki iyileşmenin %30 olduğu, lokalize AA tedavisinde aromaterapinin plaseboya göre anlamlı düzeyde etkin ve güvenli bulunduğu saptanmış, her iki çalışmada da herhangi bir yan etki bildirilmemiştir [107].

SONUÇ VE TARTIŞMA

Saç fiziksel koruma, yalıtım, sebum dağılımı, duyusal algı vb. gibi çeşitli cilt işlevlerine katkıda bulunmanın yanı sıra insan toplumunda öz saygıyı, yaşam kalitesini, çekiciliği ve sosyal etkileşimleri büyük ölçüde etkilemektedir [108]. Saç derisini doğrudan etkileyen ve belirgin sınırlara sahip lekeler oluşturan saç dökülmesinin bir türü olan AA bağışıklık sisteminin saç köklerine saldırdığı bir otoimmün hastalık olarak kabul edilmekte, anksiyete ve depresyon gibi psikolojik hastalıklar ile de ilişkilendirilmektedir [109]. AA cinsiyet fark etmeksizin tüm yaş gruplarındaki bireylerde görülmekte, genetik, bağışıklık tepkileri, hormonal dengesizlikler, iltihaplanma, artan stres, yetersiz beslenme ve ilaçlar gibi çeşitli faktörlerin AA ile ilişkili saç dökülmesinde etkin rol oynadığı düşünülmektedir [110]. AA'nın farmakolojik tedavisinde potasyum kanallarını açarak saç gövdesinde epitelyal hücrelerin proliferasyon ve VEGF ekspresyonunu arttırma yolu ile etki eden topikal minoksidil, immünmodülatör, antralin [111], saç foliküllerinde T hücresi aracılı bağışıklık yanıtı baskılayarak etki gösteren intralezyonal ve oral kortikosteroidler, pulse steroid tedavileri ve triamsinolon [112], difensipron (DPCP), dinitroklorobenzen (DNCB), skuarik asit dibütil ester (SADBE) gibi topikal duyarlandırıcı immünoterapi ajanları, 5α-redüktaz tip 2 enzim inhibitörü olan non steroidal antiandrojenler olan flutamid ve finasterid [113], T-lenfosit ve monositlerden sitokin salınımını özellikle IL-2'yi inhibe ederek etki eden oral siklosporin ve sülfasalazin [114] sayılabilir. Bunun yanı sıra fotokemoterapi ve biyolojik tedaviler gibi farklı tedavi seçenekleri bulunmaktadır. AA tedavisinde tüm bu tedaviler sınırlı etkililiğe sahiptir ve sıklıkla yüksek yan etki riski ile ilişkilidir [115]. Örneğin dirençli AA tedavisinde yaygın kullanılan topikal immünoterapi ajanlarından skuarik asit dibutilesterin yan etkileri arasında uygulama bölgesinde kızarıklık, şişme ve kaşıntı, kabarcıklanma, ciltte yanma, döküntü ve bazı vakalarda kalıcı kontakt dermatit ve şiddetli anjiyoödem kayıtlıdır [116]. Otuz yılı aşkın zamandır androgenetik alopeside etkinliği gösterilmiş minoksidilin AA tipi saç dökülmesinde etkinliğinin araştırıldığı toplam 892 klinik araştırma sonucu değerlendiren bir meta analiz raporunda, %5 minoksidil ile AA tedavisinin klinik ve istatistiksel olarak güvenli olduğu kanıtlanmış olmakla birlikte [117,118] hirsutizm, lokal iritasyon, kaşıntı, cilt kuruluğu ve eritemlere neden olabildiği bildirilmiştir [119]. Oral kortikosteroidlerin akne, obezite, hafif hipertansiyon ve katarkta yatkınlık oluşturma gibi yan etkileri bulunmaktadır [8]. Flutamidin saç döngülerini olumsuz etkilediği, hepatik disfonksiyon ve meme hassasiyetine yol açtığı [120] difensipiron tedavisinde hafif veya şiddetli alerjik kontakt dermatit,

otoekzematizasyon, hipopigmentasyon, hiperpigmentasyon, vitiligo, grip benzeri bulgular ve lenfadenopati gelişebildiği, antralinin ise irritant kontakt dermatite neden olduğu bildirilmektedir [121].

AA tedavisinde doğal ürünler farmakolojik ajanların neden olduğu yan etkiler sebebi ile giderek daha fazla popülerlik kazanmakta ve formülasyonlarda bitki türlerinin kullanımı artmaktadır. Aralarında epigallokateşingallat (EGCG), kafein, kapsaisin, kurkumin, prosiyanidinler, ginsenozitler gibi saflaştırılmış birçok fitokimyasal ile birlikte melatonin, vitaminler, demir, çinko, bakır ve çok sayıda bitki ekstresi, uçucu ve sabit yağların saç uzamasını teşvik edici etkileri bildirilmiştir [122]. Bunlar arasında özellikle kapsaisinin AA tedavisinde klobetazolden daha etkili olduğu kayıtlıdır [123]. Ancak AA tedavisinde bitki türlerinin kullanıldığı klinik çalışmalarda kanıt kalitesi düşüktür ve literatürde sınırlı sayıda çalışma bulunmaktadır. Bununla birlikte çeşitli aktif fitokimyasalların ve bitki ekstrelerinin hücresel düzeyde büyüme faktörlerinin ifadesini, bazı sinyal yollarını veya hücre döngüsü ilerlemesini indükleyerek saç döngüsünün anagen fazını başlatabileceği veya uzatabileceği çok sayıda mekanizma önerilmiştir [124]. Bu kapsamda alopesi tedavisinde saç büyüme döngüsü üzerine olumlu etkileri araştırılmış bitki türleri üzerinde araştırma sonuçları literatürde yer almaktadır. *Rumex japonicas, Carthamus tinctorius, Plumbago zeylanica* türlerinden hazırlanan etanolik ektsrelerin dermal papilla hücrelerinin canlılığını arttırdıkları, insan veya hayvan donörlerinden elde edilen saç kökleri kullanılarak yapılan *ex vivo* deneylerde *Cucumis melo, Orthosiphon stamineus* ve *Panax ginseng* gibi türlerden elde edilen ekstrelerin, saç kökü ve matristeki keratinositlerin çoğalmasını destekleyerek saç foliküllerinin büyümesini desteklediği kayıt edilmiştir [124]. Bunun yanı sıra alopesi etyolojisi ile ilişkilendirilen enflamatuvar yanıt nedeni ile anti-enflamatuvar ve immünomodülatör etkili bitkilerin alopesinin tedavisinde etkili olabileceği bu nedenle içeriğinde terpenoidler, flavonoitler, amino asitler ve polifenolik bileşikler açısından zengin *Arctium lappa* kökleri, *Sophora japonica* meyve, *Acorus calamus* kök, *Urtica dioica* yaprak, *Humulus lupulus* meyve ekstreleri bulunan ve orta düzeyde anti-enflamatuvar ve immünomodülatör aktivite gösterdikleri *in vivo* çalışmalarla gösterilmiş bitki türlerininde alopesi tedavisinde etkili olabilecekleri düşünülmektedir [125]. Sabit yağların kullanıldığı araştırma sonuçları %35 hint yağı (*Ricinus communis* L.) içeren losyonun 1 ay boyunca topikal olarak uygulandığı sağlıklı tavşanlarda saç büyümesi ve saç yenilenmesi üzerinde olumlu etki gösterdiğini, shea yağı (*Butyrospermum parkii*) ve hint yağı içeren bir merhemin ise sağlıklı tavşanlara topikal olarak uygulanmasının saç büyümesini teşvik ettiği ve saç kütlesinde artışa neden olduğunu bildirmiştir [126,127]. *Citrus sinensis, Linum usitatissimum, Nigella sativa, Trigonella foenum-graecum* ve *Zingiber officinale* ile hazırlanan infüzyona hint yağı ve E vitamini eklenerek elde edilen saç serumunun topikal uygulamasının tavşanlarda saç büyümesini ve saç ağırlığında artışa yol açtığı bir başka araştırma sonucu mevcuttur [128]. Bununla birlikte bu ve benzeri kombine ürünlerin kullanıldığı çalışmalarda aktiviteden sorumlu fitobileşenin ve/veya ekstrelerin hangileri olduğunu tespit etmek oldukça güçtür.

Bu çalışma kapsamında değerlendirilen bitki türlerinin AA ve saç büyümesini teşvik edici etkileri aşağıdaki şekilde özetlenebilir. *Allium sativum* türünün AA üzerine etkilerinin araştırıldığı klinik çalışmalar %5'lik sarımsak jelinin %0.1'lik betametazondan anlamlı derecede daha yüksek olduğunu gösterdiği ve hastalarda herhangi bir komplikasyon gözlemlenmediği kaydedilmiştir [26]. *A. cepa* ham suyunun AA'da %5'lik minoksidil ile eş düzey etkili olduğunu, hastaların %86.9'unun iyileşme sağladığı ve hafif eritem dışında herhangi bir yan etkiye rastlanmadığı kaydedilmiştir [35]. *Hibiscus rosa sinensis* yapraklarının Aloe vera jeli içerisindeki %2'lik petrol eterli ekstresi ile yapılan *in vivo* çalışmada yaprak ekstresinin %2 minoksidil ile karşılaştırılabilir düzeyde saç büyümesini uyarıcı aktivite gösterdiği bildirilmiştir [42]. *Trigonella foenum graecum* %10'luk etanol ekstresi ile tedavi edilen saçsız alanın saçla kaplanma oranı %100 olduğu belirtilmiş oysaki bu oran %5 minoksidil grubunda %75 olarak kaydedilmiştir [46]. *Asarum sieboldii* ile ilgili yapılan *in vivo* çalışmada kök ekstresi %40 etanol içinde %1'lik bir çözelti hazırlamak üzere çözündürülmüştür. Otuz gün süren topikal tedaviden sonra, *Asarum sieboldii radix* ekstresi, %40 etanol kullandırılan kontrol grubuna kıyasla daha erken bir şekilde telojenden anajene geçişi uyarmıştır [53]. *Vitis vinifera* çekirdek ekstresinin saç büyümesini destekleyici aktivitesini araştıran *in vitro* bir çalışmada, insan saç folikülü dermal papilla hücreleri üzerinde minoksidil ile üzüm çekirdek ekstresinin 5α-redüktaz inhibitör etkinlikleri karşılaştırılmıştır. Sonuç olarak, 0.06 mg/ml ve 0.3 mg/ml konsantrasyonlarındaki üzüm çekirdeği ekstresi, 5α-redüktazın inhibisyonunda 0.2 mg/ml minoksidile kıyasla önemli ölçüde daha yüksek bir

etkinlik gösterdiği bildirilmiştir [57]. *Sophora flavescens* ile ilgili yapılan *in vivo* çalışmada, kök metanol ekstresinin %1'lik çözeltisinin otuz gün süren tedavinin sonunda anagen fazının erken başlamasını indüklediği bildirilmiştir [62]. *Citrullus colocynthis* ile ilgili yapılan *in vivo* çalışmada, merhem bazına ilave edilen %2 ve %5 meyvenin petrol eteri ekstreleri, %2 ve %5 etanol ekstreleri ile %2 minoksidilin alkol çözeltisi etkinlikleri karşılaştırılmıştır. Sonuçlar, *Citrullus colocynthis* meyvesinin petrol eteri ekstresi ile tedavinin, saç büyümesinin başlama ve tamamlanması açısından minoksidil ile karşılaştırıldığında daha iyi bir etkinliğe sahip olduğunu ileri sürmektedir [68]. *Phyllanthus emblica* ile ilgili yapılan klinik bir çalışmada, meyve etanolik kuru ekstresinin hindistan cevizi yağı ile kaynatılarak hazırlanan karışım (10 mg/ml) ile hint yağının saç folikülleri üzerindeki etkisi çalışmaya dahil edilen 20 alopesi hastası iki gruba ayrılarak incelenmiş, *Phyllanthus emblica* yağının alopesi hastalarında saç büyümesini desteklemede hint yağından daha etkili olduğu gösterilmiştir [73]. *Argania spinosa* ile ilgili yapılan *in-vitro* çalışmada, argan pres küspesi (APC) %50'lik ve %80'lik etanolik ekstreleri 5 µg/ml konsantrasyonunda HFDPC hücrelerine eklenmiş ve pozitif kontrol olarak 0.1 µM minoksidil kullanılmıştır. Sonuçlar, APC ekstrelerinin saç büyümesinde rol oynayan genlerin ekspresyonunu arttırmada ve enflamasyonda rol oynayan genlerin ekspresyonunu baskılamada minoksidilden daha iyi sonuçlar verdiği kaydedilmiştir [88]. *Ginkgo biloba* yaprak sulu ekstrelerinin farelere 600 mg/kg/gün dozunda oral yoldan verildiği *in vivo* çalışmada, kontrol grubuna göre, *Ginkgo biloba* ekstresi ile tedavi edilen farelerinin cilt dokusundaki büyüme faktörlerinin önemli ölçüde arttığı, serumdaki enflamatuvar faktörlerinin azaldığı gösterilmiştir [92].

Ele alınan çalışmalara göre saç büyümesini destekleyici aktivite açısından minoksidilden daha üstün tıbbi bitkiler; *Allium cepa, Trigonella foenum-graecum, Vitis vinifera, Citrullus colocynthis, Eclipta alba* ve *Argania spinosa* olarak tespit edilmiştir.

AA tedavisinde bitki ekstrelerinin, aromatik ve sabit yağların, fitobileşenlerin, çeşitli vitamin ve minerallerin, mikrobesin kombinasyonlarının, dahili destek ürünlerinin ve bunların yanı sıra psikolojik yükün yüksek olduğu dermatolojik problemlerde önemli olan başa çıkma stratejileri olarak hipnoz ve psikoterapi gibi farkındalık temelli müdahalelerin kullanıldığı görülmektedir.

Bitkisel kaynaklı tedaviler, kimyasal ilaçların oluşturduğu yan etkilere neden olmadığı ve iyi bir etkinliğe sahip olduğu için AA tedavisinde tercih edilebilecek güvenli bir tedavi seçeneği oluşturmaktadır. Bitkisel ürünler; saç folikülündeki hücresel çoğalmayı uyarmak, saç derisindeki kan dolaşımını iyileştirmek, 5α-redüktaz enzimini inhibe etmek, büyüme faktörlerini indüklemek veya enflamatuvar sitokinlerin seviyesini azaltmak suretiyle veya bilinmeyen bir mekanizma ile etkilerini göstermektedir. Bu derlemede AA tedavisinde potansiyel terapötik etkiye sahip olan tıbbi bitkilere değinilmiştir.

YAZAR KATKILARI

Kavram: T.Ş., H.B.İ.; Tasarım: T.Ş., H.B.İ.; Denetim: T.Ş., H.B.İ.; Kaynaklar: - ; Malzemeler: - ; Veri Toplama ve/veya İşleme: - ; Analiz ve/veya Yorumlama: T.Ş., H.B.İ.; Literatür Taraması: T.Ş., H.B.İ.; Makalenin Yazılması: T.Ş., H.B.İ.; Kritik İnceleme: T.Ş., H.B.İ.; Diğer: -

ÇIKAR ÇATIŞMASI BEYANI

Yazarlar bu makale için gerçek, potansiyel veya algılanan çıkar çatışması olmadığını beyan ederler.

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Ankara Ecz. Fak. Derg. / J. Fac. Pharm. Ankara, 49(3): 963-980, 2025
Doi: 10.33483/jfpau.1487011

DERLEME MAKALE / REVIEW ARTICLE

İNSÜLİN DİRENCİ VE ALZHEİMER HASTALIĞI ARASINDAKİ PATOJENİK BAĞLANTI: FARMAKOLOJİK MÜDAHALELER

THE PATHOGENIC LINK BETWEEN INSULIN RESISTANCE AND ALZHEIMER'S DISEASE: THE PHARMACOLOGICAL INTERVENTIONS

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ÖZ

Amaç: *Alzheimer hastalığı, dünya çapında milyonlarca insanı etkileyen, en zorlu ve yıkıcı nörodejeneratif hastalıklardan biridir. Bu hastalık, hastaları ve ailelerini derinden etkileyen önemli bir halk sağlığı yükü oluşturmaktadır. Alzheimer hastalığının patofizyolojisini çeşitli genetik ve çevresel faktörler etkilemektedir. Ayrıca, Alzheimer hastalığı, metabolik sendrom, obezite ve tip 2 diyabet gibi insülin direnci bozukluklarıyla yüksek oranda ilişkilidir. Bu durum, Alzheimer hastalığı ile insülin direnci bozuklukları arasında, hem genetik hem de genetik olmayan faktörlerden kaynaklanabilecek potansiyel bir patofizyolojik bağlantının varlığını işaret etmektedir.*
Sonuç ve Tartışma: *Bugüne kadar, Alzheimer hastalığının farmakolojik tedavileri, hastalığın patofizyolojik faktörlerini durdurmaktan ziyade semptomları yönetmeye odaklanmıştır. Bununla birlikte, Alzheimer hastalığının tedavisinde umut verici bir yaklaşım olarak, hastalık ile insülin direnci bozuklukları arasındaki ortak patojenik yolakların farmakolojik müdahalelerle hedeflenmesine yönelik ilgi giderek artmaktadır. Bu derleme, Alzheimer hastalığı ile insülin direnci arasındaki patofizyolojik bağlantı ve bu bağlantıyı hedef alan farmakolojik tedaviler hakkındaki literatürü incelemektedir.*
Anahtar Kelimeler: *Alzheimer hastalığı, diyabet, insülin direnci, nörodejenerasyon, tedavi*

ABSTRACT

Objective: *Alzheimer's disease is one of the most challenging and devastating neurodegenerative diseases affecting millions worldwide. Alzheimer's disease enforces a significant public health burden, profoundly impacting patients and their families. Several genetic and environmental factors influence Alzheimer's disease pathophysiology. Alzheimer's disease is highly associated with insulin resistance disorders like metabolic syndrome, obesity, and type 2 diabetes mellitus. This frequently reveals a common underlying pathophysiological link between insulin resistance disorders and Alzheimer's disease, potentially stemming from both genetic and non-genetic factors.*
Result and Discussion: *To date, Alzheimer's disease pharmacological treatments focus on managing symptoms rather than halting pathophysiological factors. However, there is growing interest in targeting the shared pathogenic pathways between Alzheimer's disease and insulin resistance disorders with pharmacological interventions as a promising approach to treating Alzheimer's disease. This review examines the literature on the pathophysiological link between Alzheimer's disease and insulin resistance and the pharmacological treatments aimed at targeting this link.*
Keywords: *Alzheimer's disease, diabetes, insulin resistance, neurodegenerative, therapeutics*

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Gönderilme / Submitted : 20.05.2024
Kabul / Accepted : 18.04.2025
Yayınlanma / Published : 19.09.2025

GİRİŞ

Alzheimer hastalığı, günümüz sağlık hizmetlerinde acil zorluklar yaratan, dünya çapında milyonlarca insanı etkileyen ve halk sağlığı sistemleri, bakıcılar ve aileler üzerinde derin etkiler yaratan ilerleyici bir nörodejeneratif hastalıktır. Alzheimer hastalığı, bilişsel işlevleri, hafızayı ve günlük yaşam aktivitelerini bozarak, sonunda ciddi bir engelliliğe ve günlük aktivitelerin gerçekleştirilebilmesi için başkalarının yardımına ihtiyaç duyulmasına yol açar. ABD Hastalık Kontrol ve Önleme Merkezleri'nin (CDC) altta yatan ölüm nedenlerine ilişkin 2018-2021 raporuna göre, Alzheimer hastalığı yedinci en yaygın ölüm nedeni olarak yer almaktadır [1]. Küresel nüfus yaşlanmaya devam ettikçe, Alzheimer hastalığının prevalansının dramatik bir şekilde artması beklenmekte, bu da daha derin bir anlayışa, etkili tedavilere ve önleyici stratejilere olan acil ihtiyacı vurgulamaktadır [2]. Alzheimer hastalığının ilerlemesini durdurmak veya tersine çevirmek amacıyla on yıllardır süren yoğun araştırmalara ve çeşitli tedavi denemelerine rağmen, hastalık halen tedavi edilememektedir [2,3]. Bu mevcut durum, hastalığın etiyolojisi, patofizyolojisi ve potansiyel terapötik müdahaleler konusunda daha fazla araştırmaya acil ihtiyaç duyulduğunu göstermektedir. Alzheimer hastalığında rol oynayan patofizyolojik faktörlerin hedeflenmesi, hastalığın ilerleyici seyrinin önlenmesine ve bu seyrin tersine çevrilmesine yönelik önemli bir katkı sağlayabilir.

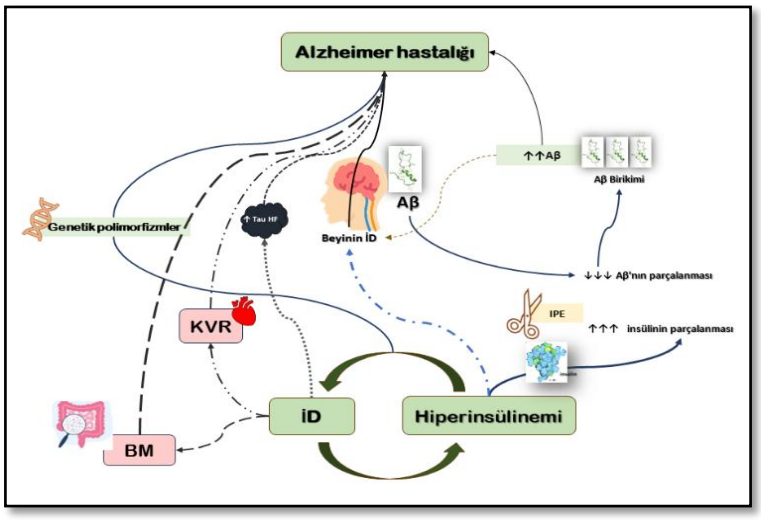
Beta amiloidlerin (Aβ) nöronların dışında birikmesi ve tau proteinlerinin nöronların içinde hiperfosforilasyonu ve birikmesi, Alzheimer hastalığının iki ana patojenik özelliğidir [4-7]. Bununla birlikte, Alzheimer hastalığının diğer dikkat çeken özellikleri arasında beyin atrofisi, özellikle hipokampal ve neokortikal atrofi, beyaz madde hiperintensiteleri ve asetilkolin ile katekolaminler gibi nörotransmiterlerin seviyelerinin azalması sayılabilir. Alzheimer hastalığı, hem genetik hem de genetik olmayan faktörlerin etkili olduğu bir hastalıktır [8-19]. Genetik olmayan risk faktörleri arasında yaşlanma, iskemik inme ve koroner arter hastalığı gibi kardiyovasküler hastalıklar, tip 2 diyabet, metabolik sendrom, obezite, depresyon, özellikle menopoz dönemindeki kadınlar, bilişsel hareketsizlik, dislipidemi, sigara kullanımı, madde bağımlılığı ve sağlıksız beslenme alışkanlıkları yer almaktadır [3,11,13,14,20-24]. Bu faktörler, Alzheimer hastalığının gelişiminde oksidatif stres, mitokondriyal fonksiyon bozukluğu, kronik inflamasyon, azalmış glukoz metabolizması ve enerji kullanımı ile beyin insülin direnci dahil olmak üzere çeşitli patolojik yolları aktive edebilir [7,25-28].

Metabolik sendrom, obezite ve tip 2 diyabet gibi insülin direnci bozuklukları, Alzheimer hastalığı ile güçlü ilişkiler göstermektedir [25,29-33]. Ancak, bu ilişkinin altında yatan kesin mekanizmaların açıklanması gerekmektedir. Paylaşılan genetik faktörlerin veya diğer patojenik unsurların Alzheimer hastalığı ile insülin direnci bozuklukları arasındaki bağlantıya katkıda bulunması olasıdır [16,25,34-37]. Ayrıca, genetik ve genetik olmayan faktörlerin etkileşimi bu ilişkiyi etkileyebilir. Genetik ve çevresel faktörler arasındaki etkileşimler, Alzheimer hastalığının etiyolojisinin karmaşıklığını ve genetik duyarlılığın çevresel faktörlerle birlikte anlaşılmasının önemini vurgulamaktadır. Alzheimer hastalığı ile insülin direnci arasındaki ortak patolojik yollar, terapötik müdahaleler için potansiyel bir hedef sunmaktadır. Bu nedenle, bu derlemede Alzheimer hastalığı ile insülin direnci arasındaki ortak patojenik yolaklar ve bu bağlantıyı hedef alan farmakolojik tedaviler araştırılmıştır. Bu amaca ulaşmak için, Alzheimer hastalığı ile insülin direnci bozuklukları arasındaki patojenik bağlantılar ve bu bağlantılara müdahale edebilecek farmakolojik yaklaşımlar hakkında mevcut literatür gözden geçirilmiştir. Literatür taraması, PubMed aracılığıyla MEDLINE veritabanı kullanılarak gerçekleştirilmiştir.

Alzheimer Hastalığı ile İnsülin Direnci Arasındaki Patojenik Bağlantı

Bozulmuş insülin sinyali Alzheimer hastalığının gelişiminde kritik bir faktördür [28,38,39]. Alzheimer hastalığı, insülin direnci ve insülin eksikliğinin hastalık patogenezine önemli katkıda bulunması nedeniyle tip 3 diyabet olarak değerlendirilmektedir [40]. İnsülin direnci, insüline verilen yanıtın azalmasıdır. Bu durum, genetik ve çevresel faktörlerden kaynaklanabilmektedirt. İnsülin direnci, normal kan şekeri seviyelerini korumak için artan insülin sekresyonu (hiperinsülinemi) ile telafi edilir, bu da bir insülin direnci ↔ hiperinsülinemi döngüsü yol açar [41-43]. Moleküllerin kan-beyin bariyeri (KBB) boyunca taşınımı, bu moleküllerin periferik düzeylerdeki değişimlerinden büyük ölçüde etkilendiğinden, periferik hiperinsülinemi beyin insülin seviyelerinde artışa yol açmaktadır. Dolayısıyla, periferik hiperinsülinemi, beyindeki insülin düzeylerinin yükselmesine neden olmaktadır [44,45].

İnsülin, insüline karşı daha yüksek bir afiniteye sahip olmasına rağmen aynı zamanda Aβ proteinini de parçalayan insülini parçalayan enzim tarafından parçalanmaktadır [37,45]. Sonuç olarak, hiperinsülinemi, Alzheimer hastalığının ayırt edici özelliklerinden biri olan Aβ protein birikimine ve biriken Aβ proteinleri nedeniyle insülin reseptörüne bağlanmanın engellenmesine yol açabilir [37,45,46]. Genetik polimorfizmlere veya tip 1 diyabet gibi genetik olmayan faktörlere bağlı olarak insülini parçalayan enzimin işlev bozukluğu Aβ birikimini daha da artırabilir [47-50]. Bu nedenle insülin eksikliği, tip 1 diyabette olduğu gibi bilişsel işlevlere zarar verebilir [51]. İnsülin direnci ve hiperglisemi, aynı zamanda hafıza performansını ve nöronal büyümeyi de etkileyerek Alzheimer hastalığının ilerlemesine katkıda bulunur [52]. Tip 2 diyabet hastalarında postprandiyal glukoz seviyeleri, Alzheimer hastalığının gelişiminin göstergeleri olan beyaz cevher hiperintensiteleri ve beyin atrofisi ile ilişkili bulunmuştur [8,20]. İnsülin direnci, nörotransmitter seviyelerini etkileyerek, özellikle asetilkolin seviyelerini düşürmek suretiyle Alzheimer hastalığının ilerlemesine yol açar [17,18,53]. İnsülin direnci, tau hiperfosforilasyonu ile ilişkilidir [54,55]. Tauopati, Alzheimer hastalığının bir diğer kritik özelliğidir. Hücre tabanlı yaklaşımlar kullanılarak yapılan çalışmalarda, insülin direncinin tau hiperfosforilasyonunu artırdığı bulunmuştur [54]. Ayrıca, tau proteinleri beyindeki insülin sinyal yoluna müdahale ederek beyin insülin direncine ve bilişsel gerilemeye neden olur [55]. Alzheimer hastalığı ile insülin direnci bozuklukları arasında başka yaygın patolojik yollar da vardır. Bu yollar, genetik faktörler, bozulmuş bağırsak mikrobiyotası, kronik inflamasyon ve oksidatif stres gibi yaygın patojenik faktörleri içerir [56-61]. Ayrıca, insülin direnci ile ilişkili kardiyovasküler hastalıklar da Alzheimer hastalığı ile bağlantılıdır [3,24,62-65]. Aşağıdaki bölümlerde, Alzheimer hastalığı ile insülin direnci bozuklukları arasındaki yaygın patojenik bağlantılardan bazıları tartışılacaktır. Şekil 1, Alzheimer hastalığı patogenezinde insülin direnci ↔ hiperinsülinemi kısır döngüsünün rolünü göstermektedir.



Şekil 1. Alzheimer Hastalığında insülin direncinin ve hiperinsülineminin rolü: İnsülin direnci ↔ hiperinsülinemi kısır döngüsü beyni çeşitli yollardan etkiler. Yüksek insülin seviyeleri, insülin parçalayan enzim (IPE) için Aβ proteini ile rekabet eder ve insülin parçalayan enzimin insüline karşı afinitesi Aβ'dan daha yüksektir ve bu da Aβ birikimine yol açar. Aβ proteini, insülinin insülin reseptörlerine bağlanmasını engelleyerek beyinde insülin direncine neden olur. Tau hiperfosforilasyonu insülin direnciyle de bağlantılıdır. Genetik polimorfizmler, Alzheimer hastalığının patofizyolojisine de katkıda bulunan (insülin direnci ↔ hiperinsülinemi) kısır döngüsüne katkıda bulunur. Kardiyovasküler hastalıklar (KVR), insülin direnci ve Alzheimer hastalığı ile yakından ilişkilidir. Benzer şekilde, bağırsak mikrobiyota (BM) bozuklukları da hem insülin direnci hem de Alzheimer hastalığı ile bağlantılıdır. Kısaltmalar, Aβ: amiloid beta, BM: bağırsak mikrobiyotasının, IPE: insülin parçalayan enzim, İD: insülin direnci, KVR: kardiyovasküler hastalıklar, Tau HF: tau hiperfosforilasyonu. Şekil şuradan değiştirilerek uyarlanmıştır [66]

Alzheimer Hastalığı ile İnsülin Direnci Bozuklukları Arasında Ortak Genetik Bağlantılar

İnsülin direnci ile Alzheimer hastalığı arasındaki ilişkinin genetik faktörlere bağlı olabileceği belirtilmektedir. Çeşitli çalışmalar, insülin direnci bozuklukları ile Alzheimer hastalığı arasındaki genetik bağlantıları ortaya koymuştur. Ho ve arkadaşları, obezite-beyin atrofisi ile genetik faktörler arasındaki ilişkiyi, özellikle de yağ kütlesi ve obezite ile ilişkili (FTO) genindeki obezite riski taşıyan tek nükleotid polimorfizmini (SNP) (*rs3751812*) araştırmıştır [34]. Çalışmaları, *rs3751812* obezite riski alelinin taşıyıcılarının beyin hacminde eksiklikler sergilediğini ortaya çıkarmıştır. Ek olarak, *rs17817449, rs9939609, rs8050136* ve *rs1421085* gibi diğer yağ kütlesi ve obezite ile ilişkili (FTO) gen tek nükleotid polimorfizmleri (SNPs), bilişsel değişiklikler ve beyin fonksiyonu değişikliği ile bağlantılıdır [34,36,67]. Örneğin, *rs1421085* tek nükleotid polimorfizmi (SNP), obezite ve azalmış medial prefrontal kortikal fonksiyon ile ilişkilendirilmiştir [67]. Bu yağ kütlesi ve obeziteyle ilişkili (FTO) gen tek nükleotid polimorfizmleri (SNPs), aynı zamanda metabolik sendrom ve tip 2 diyabet gibi diğer insülin direnci bozukluklarında da suçlanmıştır [68-71]. Yağ kütlesi ve obezite ile ilişkili (FTO) gen risk alelleri, iştah uyarıcı bir hormon olan plazma ghrelin ile bağlantılıdır ve bir tokluk hormonu olan leptin ile ters ilişkilidir, bu da doygunluğun bozulmasına ve kilo alımına yol açar [72]. Bu sonuçta hiperinsülinemiye ve insülin direncine katkıda bulunacaktır [73,74]. Yüksek ghrelin düzeyleri, bilişsel işlevlerde azalma ile ilişkilidir. Bu durum, potansiyel olarak ghrelin'in Alzheimer hastalığı patogenezinde rol oynadığını gösterir ve insülin direnci bozuklukları ile Alzheimer hastalığı arasındaki ilişkide yağ kütlesi ve obezite ile ilişkili (FTO)-ghrelin-insülin direnci yolağının bir rol vardır [73,75-77]. Wang ve Lin, Genom çapında ilişkilendirme çalışması (GWAS) veri setlerinin analizi yoluyla Avrupa popülasyonlarında hem tip 2 diyabet hem de Alzheimer hastalığı ile ilişkili sekiz yeni pleiotropik tek nükleotid polimorfizmi tanımladılar [34]. Tanımlanan tek nükleotid polimorfizmleri, oksidatif stres, mitokondriyal disfonksiyon, otofaji aktivasyonu, fosfoinositid-3-kinazın (PI3K) düzenlenmesi ve apolipoprotein E (ApoE) gibi yolaklarla bağlantılıdır [35,45,78]. Ayrıca bunların hepsi hem Alzheimer hastalığının hem de insülin direnci bozukluklarının patogenezinde rol oynamaktadır [35,45,78]. İnsülin parçalayan enzim geninin genetik değişkenliği, Alzheimer hastalığı-insülin direnci patojenik bağlantısında da önemli bir role sahiptir. Aslında hem Alzheimer hastalığı hem de tip 2 diyabetle ilişkili çeşitli insülin parçalayan enzim geni tek nükleotid polimorfizmleri vardır [47,79-81]. İnsülin parçalayan enzimin genindeki *rs1887922* tek nükleotid polimorfizmi (SNP), geç başlangıçlı Alzheimer hastalığı, açlık plazma glukozu, glikozillenmiş hemoglobin A1C (HbA1C), tip 2 diyabet riski, insülin direnci ve koroner arter hastalığı riski ile ilişkilidir [47,48,79-81]. Alzheimer hastalığı ve tip 2 diyabette *rs1887922* tek nükleotid polimorfizminin (SNP) rolüne ilişkin olası mekanizmalar arasında, azalmış insülin parçalayan enzim ekspresyonu veya artan Aβ birikimine yol açan metabolik aktivite ve erken insülin direncini gösteren hiperinsülinemi yer alır [47-49,81]. Alzheimer hastalığıyla ilişkili spesifik genetik varyantlar, diğer risk faktörleriyle birleştirildiğinde daha yüksek bir etkiye sahip olabilir. Örneğin Apolipoprotein E (ApoE) ε4 aleli, tip 2 diyabet, hipertansiyon, kronik düşük dereceli inflamasyon ve menopoz sonrası kadınlarda bulunan Alzheimer hastalığıyla bağlantılıdır [10,11,13,14,16,82]. Apolipoprotein E (ApoE) ε4, metabolik sendrom ve obezite ile ilişkilidir [12,83]. Benzer şekilde tip 2 diyabet, apolipoprotein J/Klusterin (APOJ/CLU) *rs11136000* varyantının C aleli ile Alzheimer hastalığı arasındaki ilişkiyi güçlendirir. Buna karşılık, aynı tek nükleotid polimorfizminin (SNP) T aleli, Alzheimer hastalığına karşı koruma sağlayabilir [15,84,85]. Klusterini kodlayan APOJ/CLU geni, özellikle eşzamanlı insülin direnci koşulları olan Alzheimer hastalarında Alzheimer hastalığının bir biyobelirtecidir [86-89]. Klusterin düzensizliğinin, Alzheimer, kardiyovasküler hastalıklar, kanser ve tip 2 diyabet mellitus gibi çeşitli hastalıklarla ilişkili olduğu düşünülmektedir [86-88,90-92]. Bu durum, klusterin'in Alzheimer Hastalığı ve İnsülin direnci arasındaki patojenik bağlantıya katkıda bulunduğunu göstermektedir. Alzheimer hastalığı ile *rs11136000* arasındaki ilişki etnik kökene göre değişmekle birlikte, 75 yaş üstü bireylerde daha tutarlı olma eğilimindedir [15,93]. Ayrıca hem Alzheimer hastalığı hem de insülin direnci bozuklukları ile ilişkili genetik varyantların birden fazla polimorfizmine sahip olmak Alzheimer hastalığı riskini artırmaktadır. Örneğin, hem FTO (*rs9939609*) hem de ApoE ε4 varyantlarının taşıyıcıları Alzheimer hastalığı açısından daha yüksek riske sahiptir [94]. Diğer bir örnek ise NDUFAF6 (*rs6982393*) taşıyıcılarıdır ve ApoE ε4 varyantlarının Alzheimer hastalığı riski daha

yüksektir [82]. Bu etkileşimler, insülin direnci bozuklukları ve Alzheimer hastalığı arasında çevresel faktörlerle birlikte gen duyarlılığının anlaşılmasının önemini vurgulamaktadır.

Alzheimer Hastalığı ile Kardiyovasküler Hastalıklar Arasındaki İlişkide İnsülin Direncinin Rolü

Alzheimer hastalığı, ateroskleroz, kardiyovasküler hastalıklar, serebrovasküler hastalıklar, karotis arter intima-media kalınlığı, artmış trombosit reaktivitesi ve endotel disfonksiyonu ile ilişkilidir [24,63-65]. Çalışmalar, Alzheimer hastalığı ile kardiyovasküler hastalıklar arasında, potansiyel olarak Aβ proteini aracılığıyla gerçekleşen çift yönlü bir ilişki olabileceğini öne sürmektedir [65,95]. Kardiyovasküler ve serebrovasküler hastalıklar, bilişsel işlev bozukluğu ve Alzheimer hastalığı ile ilişkili vasküler beyin hasarlarına yol açmaktadır [24,62-64]. İnsülin direnci, Alzheimer hastalığı ile kardiyovasküler hastalıklar arasındaki ilişkiyi açıklayabilir. İnsülin direnci, kardiyovasküler hastalıklarda önemli bir rol oynar ve kardiyovasküler hastalık patogenezinde yer alan (insülin direnci ↔ hiperinsülinemi) kısır döngüsüne yol açar [96-98]. Bu kısır döngü, felç ve diğer kardiyovasküler hastalıklar için önemli bir risk faktörü olan dislipidemi ile ilişkilidir [99-102]. Ayrıca, (insülin direnci ↔ hiperinsülinemi) kısır döngüsü, artmış trombosit reaktivitesi ve endotel disfonksiyonu ile bağlantılıdır [103,104]. Bu nedenle, kardiyovasküler hastalık riski, tip 2 diyabet ve obezite gibi insülin direnci bozuklukları olan bireylerde artmaktadır [103-106]. Çalışmalar, ortak karotis arter intima-media kalınlığının artmasının, insülin direnci, hiperinsülinemi ve yüksek kan şekeri seviyeleri ile ilişkili olduğunu göstermiştir [107-109]. Tip 2 diyabet, obezite ve metabolik sendrom gibi insülin direnci bozukluklarına sahip hastalarda kardiyovasküler hastalık riskinin artması nedeniyle, Alzheimer hastalığı riski de daha yüksektir [24,25,29,31-33].

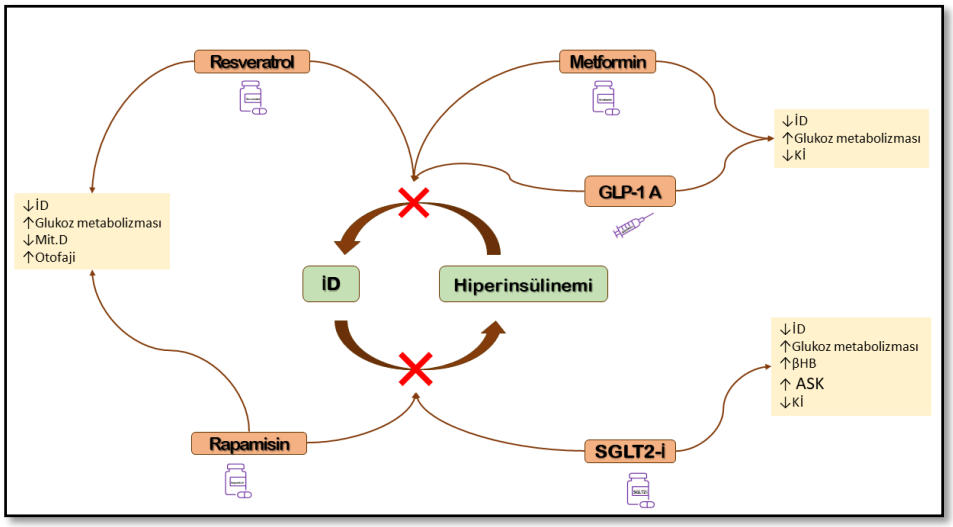
Alzheimer Hastalığı ile İnsülin Direnci Bozuklukları Arasındaki İlişkide Bağırsak Mikrobiyotasının Rolü

İşlevsiz bağırsak mikrobiyotası, Alzheimer hastalığı da dahil olmak üzere çeşitli nörodejeneratif hastalıklarla ilişkilendirilmiş olup, bu durum Alzheimer hastalığının bağırsakta ortaya çıkabileceği hipotezini gündeme getirmektedir [110,111]. Bağırsak mikrobiyotası bozukluğu, bağırsak-beyin eksenini bozarak, bu eksen üzerinden merkezi sinir sistemi ile bağırsak arasında çift yönlü biyokimyasal iletişimi etkilemektedir [110-114]. T Bağırsak-beyin ekseni, duygusal ve bilişsel beyin bölgeleri ile periferik bağırsak fonksiyonlarını etkileyerek bilişsel ve psikiyatrik işlev bozukluklarına yol açabilir [110,112]. Obezite ve tip 2 diyabet gibi insülin direnci bozuklukları ile bağırsak mikrobiyotası bozukluğu arasında bir ilişki olduğu gösterilmiştir [57,61]. Ayrıca, bozulmuş bağırsak mikrobiyotası, tip 2 diyabet ve obezite gibi insülin direnci bozukluklarında kronik inflamasyona neden olmaktadır [115]. Kronik inflamasyon ise Alzheimer hastalığının patogenezinde rol oynayan bir risk faktörüdür [25,28]. Bu nedenle, insülin direnci bozukluğu olan bireylerde bağırsak mikrobiyotasındaki bozulma, bilişsel işlev bozukluklarına yol açabilmektedir.

Farmakolojik Tedavi Yoluyla Alzheimer Hastalığı-İnsülin Direnci Patojenik Bağlantısını Hedeflemek

Alzheimer hastalığını yönetmek için çok sayıda farmasötik tedavi incelemeye tabi tutulmuş olsa da, mevcut farmakolojik müdahaleler hastalığın ilerlemesini durdurmamaktadır. Bunun yerine, bu tedaviler spesifik semptomları hafifletebilir [2,5]. Mevcut Alzheimer hastalığını ilaçlarının hiçbiri, Alzheimer hastalığındaki insülin direncinin patojenik yoluna değinmemektedir. ABD Gıda ve İlaç İdaresi (FDA), Alzheimer hastalığı semptomlarını yönetmek için iki temel yaklaşımı kullanan ilaçları onaylamıştır. İlk yaklaşım, asetilkolin nörotransmiterini parçalayan ve bu nörotransmitteri yükselten asetilkolinesteraz enziminin (AChE) inhibe edilmesini içerir [5,116]. Asetilkolin nörotransmitter düzeylerindeki bu artış donepezil, rivastigmin ve galantamin gibi ilaçlarla sağlanır. Diğer bir yaklaşım ise, FDA onaylı bir Alzheimer hastalığı ilacı olan Memantin ile N-metil D-aspartat (NMDA) reseptörünü antagonize etmektir [5]. Memantin, Alzheimer hastalığı olan bireylerde yüksek glutamatın uyarıcı etkilerini hafifletir ve tek başına veya donepezil ile kombinasyon halinde mevcuttur [116]. Yakın zamanda FDA tarafından onaylanan bir diğer ilaç ise, beyindeki Aβ'yı azaltmayı amaçlayan ve erken evre Alzheimer hastalığına yönelik bir anti-amiloid antikoru olan Aducanumab'dır [117,118]. Ancak, bu ilacın hızlandırılmış FDA onayı ve fiyatı konusunda uzmanların görüşleri tartışmalıdır ve bu durum

bugüne kadarki klinik kullanımını engellemektedir [119,120]. Alzheimer hastalığının farmakoterapisi için çeşitli tip 2 diyabet ilaçları önerilmiştir. Bunlar arasında metformin, sodyum glukoz ko-transporter 2 inhibitörleri (SGLT2-İ/gliflozinler) ve glukagon-benzeri peptid-1 (GLP-1) reseptör agonistleri yer almaktadır [53,121-124]. Bu ilaçların etkisi, kan glukoz seviyelerini düşürmek, insülin direncini hafifletmek, oksidatif stresi ve inflamasyonu azaltmak, kardiyovasküler olayların insidansını azaltmak ve asetilkolin ile keton seviyelerini artırmak gibi çeşitli mekanizmalar aracılığıyla elde edilmektedir [53,121,122]. Sonraki başlıklarda Alzheimer hastalığı-insülin direnci patojenik bağlantısına müdahale eden ilaçlar ele alınacaktır. Şekil 2, Alzheimer hastalığı ile insülin direnci arasındaki bağlantıyı hedef alan farmakolojik müdahaleleri göstermektedir.



Şekil 2. Alzheimer hastalığını hedefleyen farmakolojik müdahaleler - insülin direnci bağlantısı ve etki mekanizmaları. Alzheimer hastalığını insülin direnci bozukluklarına bağlayan (İnsülin direnci ↔ hiperinsülinemi) kısır döngüsü, farmakolojik müdahalelerle hedeflenebilir. ↑: artırmayı veya iyileştirmeyi belirtir. ↓: azalmayı veya engellemeyi gösterir. Kısaltmalar; İD: insülin direnci, Kİ: kronik inflamasyon, Mit.D: mitokondriyal disfonksiyon, SGLT2-İ: sodyum glukoz ko-transporter 2 inhibitörleri, GLP-1A: glukagon-benzeri peptid-1 reseptör agonistleri, βHB: β-hidroksibütirat ve ASK: asetilkolin

Metformin

Metformin, tip 2 diyabetin tedavisinde birincil ilaç olarak kabul edilir çünkü tip 2 diyabetin altında yatan temel sorun olan insülin direnciyle mücadele eder ve böylece periferik hiperinsülinemiyi azaltır [125]. Metformin, Alzheimer hastalığı ve kardiyovasküler hastalıklar gibi diğer hastalıklar için potansiyel bir tedavi olarak önerilmiştir [122,126]. Aslında metformin, aterosklerozu ve trombosit reaktivitesini azaltarak kardiyovasküler ve serebrovasküler olay riskini düşürerek kardiyo-koruyucu özellikler sergiler [126-128]. Yukarıda sayılan tüm bu etkiler Alzheimer hastalığı ve ilerleme riskini azaltır. Araştırmalar metforminin farelerde yaşam süresini uzatabildiğini ve sağlıklı yaşlanmayı destekleyebildiğini göstermiştir [129]. Ayrıca hipokampus ve korteksteki inflamatuar hücreleri, reaktif astrositleri ve ölen nöronları azaltarak bilişsel performansı artırabilir [130]. Metforminin, tip 2 diyabetli yaşlı hastalarda demans riskini doza bağımlı bir şekilde azalttığı gösterilmiştir [131]. Metforminin Alzheimer hastalığına karşı koruyucu etkisi, insülin direncini, aterosklerozu, oksidatif stresi, inflamasyonu, mitokondriyal disfonksiyonu ve yaşa bağlı rahatsızlıkları hafifletme yeteneğine bağlanabilir [122,126,132-134]. Bugüne kadar metforminin Alzheimer hastalığının tedavisindeki rolünü araştıran klinik çalışmalar kısıtlıdır. Daha ileri klinik çalışmalara ihtiyaç vardır.

Sodyum Glukoz Ko-Transporter 2 İnhibitörleri (SGLT2-İ) (Gliflozinler)

Sodyum glukoz ko-transporter 2 inhibitörleri (SGLT2-İ) olarak bilinen empagliflozin, dapagliflozin ve kanagliflozin gibi ilaçlar, antidiyabetik etkilerini SGLT2 enzimini inhibe ederek gösterir [135]. Bu inhibisyon, renal tübüller tarafından glukozun yeniden emilmesini azaltır ve glukoz atılımını artırarak kan şekeri seviyelerinin ve insülin direncinin düşmesine yol açar [53,121]. SGLT2-İ, kan şekeri ve insülin direnci üzerindeki etkilerinin ötesinde, çeşitli mekanizmalar yoluyla Alzheimer hastalığı için potansiyel faydalar sunabilir [136,137]. İlk olarak, kan şekeri düzeylerini düşürerek Alzheimer hastalığı-İnsülin direnci bağlantısındaki zararlı bir yolu hedeflerler [135]. İkinci olarak, insülin direncini azaltarak Alzheimer hastalığı-insülin direnci bağlantısında temel bir unsur olan periferik hiperinsülinemiyi düşürürler [135]. Üçüncüsü, SGLT2-İ, beyindeki insülin direncinden etkilenen bölgeler için alternatif bir yakıt görevi görebilen β-hidroksibütirat (βHB) gibi keton cisimlerinin seviyelerini yükseltir [53,121,135]. Ayrıca βHB antioksidatif, antiinflamatuar ve damar genişletici etkiler sergileyerek Alzheimer hastalığı için önemli risk faktörleri olan kardiyovasküler ve serebrovasküler olay riskini azaltır [138,139]. Gliflozinler (SGLT2-İ), SGLT2 taşıyıcısı üzerindeki önleyici etkilerinin yanı sıra, asetilkolini parçalayan asetilkolin esteraz enzimini (AChE) de inhibe ederek beyindeki asetilkolin düzeylerinde artışa yol açar [123,137]. Beyin asetilkolin seviyelerindeki bu artış, Alzheimer hastalığına yönelik mevcut tedavi stratejileriyle uyumludur [5,116]. Genel olarak, SGLT2-İ'ler Alzheimer tedavisinde ümit verici görünmektedir, ancak etkinlikleri ve güvenlikleri konusunda daha fazla araştırmaya ihtiyaç vardır. İyi bilinen öglisemik ketoasidoz riskleri nedeniyle SGLT2 inhibitörlerinin dikkatli kullanılması gerektiğine dikkat etmek önemlidir [135,140]. Bu nedenle vücut keton düzeylerini yükseltebilecek düşük karbonhidratlı, yüksek yağlı diyet (ketojenik diyet) uygulayan bireylerde, ketojenik diyet SGLT2-İ kullanımıyla birleştirildiğinde ketoasidoz gelişimini önlemek için SGLT2-İ kullanımı önerilmez. [141]. Ayrıca, klinisyenlerin SGLT2-İ tedavisinin ilk haftalarında, özellikle yaşlı bireylerde kırık riskine dikkat etmeleri gerekmektedir, çünkü bu popülasyon düşme ve kırık açısından daha yüksek risk altındadır [142]. Sonuç olarak, SGLT2-İ Alzheimer hastalığının tedavisi için potansiyel taşırken, bunların etkinliğini ve güvenliğini doğrulamak için daha fazla klinik çalışmaya ihtiyaç vardır.

Glukagon Benzeri Peptid-1 (GLP-1) Reseptör Agonistleri

Glukagon benzeri peptid-1 (GLP-1) reseptör agonistleri, tip 2 diyabet tedavisinde kullanılan ilaçlar arasında yer almakta olup, son dönemde Alzheimer hastalığının tedavisinde de önerilmektedir [143]. Tip 2 diyabet ve obezite için yaygın olarak reçete edilen bir GLP-1 agonisti olan liraglutid, Alzheimer hastalığındaki potansiyel uygulaması nedeniyle dikkatleri üzerine çekmiştir [144]. Liraglutidin Alzheimer hastalığındaki rolü, glukoz metabolizmasını ve insülin sinyallemesini iyileştirme yeteneği ile ilişkilidir [145]. Ayrıca, insülin direncini azaltmada ve inflamasyonu hafifletmede önemli bir rol oynadığı bildirilmektedir [145]. Yakın zamanda yapılan çok merkezli, randomize, çift kör, plasebo kontrollü faz IIb klinik bir çalışma, liraglutidin Alzheimer hastalığı tedavisindeki rolünü değerlendirmiştir [146]. Sonuçlar, liraglutidin Alzheimer hastalığı ile tedavi edilen hastalarda bilişsel işlevi ve beyin hacminin manyetik rezonans (MR) görüntüleme taramasını plasebo ile tedavi edilen hastalara göre daha fazla artırabildiğini ortaya koymuştur. Liraglutide benzer şekilde simaglutid de daha fazla ilgi görmüştür [147]. Farelerde beyin glukoz kullanımını ve bilişsel işlevi iyileştirdiği bulunmuştur. Bu bulgular umut verici olsa da Alzheimer tedavisinde bu ilaç sınıfının onaylanması için bu tür insan çalışmalarının tekrarlanması gerekmektedir.

Resveratrol

Alzheimer hastalığında kapsamlı bir şekilde araştırılan önemli bir polifenol bileşiği olan resveratrol, zeytinyağı, üzüm, antep fıstığı, kakao ve meyveler gibi gıdalardan elde edilmektedir [148]. Antiinflamatuar, antioksidan, epigenetik ve mitokondriyal düzenleyici özelliklere sahiptir [148,149]. Çalışmalar resveratrolün insülin duyarlılığını artırma ve glukoz ve lipid metabolizmasını düzenleme yeteneğini göstermiştir [150]. Resveratrol, kalori kısıtlamasının olumlu etkilerini taklit eder, yaşa bağlı azalmayı hafifletir ve nitrik oksit üretimini artırarak mitokondriyal ve endotel fonksiyonunu iyileştirerek kardiyoprotektif bir rol oynar [149,151-153]. Azalan nitrik oksit seviyeleri, Alzheimer hastalığı

patolojisine katkıda bulunan endotel disfonksiyonu ve azalan serebral kan akışı ile ilişkilidir [154]. Ek olarak resveratrol, bağırsak mikrobiyotasını etkileyerek aterosklerozla ilişkili metabolit trimetilamin oksitin (TMAO) sentezini azaltır [155]. Resveratrol gibi diyet bileşenleri aracılığıyla TMAO üretiminin engellenmesi, Alzheimer hastalığı için bir risk faktörü olan aterosklerozu azaltır [155-157]. Ek olarak, resveratrol otofajiyi indükler, yaşlanma karşıtı özelliklere sahiptir ve bilişsel işlevi olumlu yönde etkiler [148,157]. Alzheimer hastalığı olan bireylerde beyin, omurilik ve plazma örneklerini retrospektif olarak inceleyen bir çalışmada, resveratrolün nörodejeneratif ve nöroinflamatuar biyobelirteçleri azalttığı bulunmuştur [158]. Yakın zamanda yapılan bir çalışmada resveratrol ile donepezil kombinasyonu 90 Alzheimer hastası üzerinde test edilmiştir [159]. Bu çalışmada, resveratrol ve donepezil kombinasyonunun inflamatuar göstergeleri azalttığı ve bilişsel işlevi iyileştirdiği bulunmuştur. Bu bulgular ışığında, resveratrolün Alzheimer hastalığındaki rolüne ilişkin daha fazla klinik araştırmalara ihtiyaç duyulmaktadır; böylece resveratrolün düzenli tedaviyle entegrasyonu mümkün olacaktır.

Rapamisin

Sirolimus olarak da bilinen rapamisin, rapamisin kinazın (mTOR) mekanik hedefini inhibe eden bir ilaçtır ve Alzheimer hastalığının tedavisi için önerilmiştir [160]. Rapamisin, mTOR'u hedef alarak yaşlanma sürecini etkiler, otofajiyi indükler ve mitokondriyal fonksiyonu modüle eder [161,162]. Resveratrol gibi rapamisin de kalori kısıtlamalı diyetlerin ve aralıklı orucun faydalı etkilerini taklit eder [163]. Çalışmalar rapamisinin tip 2 diyabetli sıçanlarda insülin direncini azalttığını ve yaşlı farelerde kalp fonksiyonunu iyileştirdiğini göstermiştir [164]. Bu nedenle rapamisinin Alzheimer hastalığına karşı umut verici bir etki yaratacağı öne sürülmektedir. Ancak Alzheimer hastalığının tedavisinde kullanımına ilişkin klinik çalışmalar sınırlıdır.

SONUÇ VE TARTIŞMA

Alzheimer hastalığı ile insülin direnci (IR) bozuklukları arasında güçlü bir ilişki bulunmaktadır. Bu bağlantı, hem genetik hem de çevresel faktörlerin etkileşimini içermektedir. Birden fazla faktörün varlığı, hastalığın ilerlemesini hızlandırarak yönetim stratejilerini zorlaştırmaktadır. Alzheimer hastalığı ile insülin direnci arasındaki bu bağlantının incelenmesi, Alzheimer hastalığının patogenezi hakkında bilgi ve farkındalığı artıracak, bu da yeni tedavi hedeflerinin belirlenmesine katkı sağlayacaktır. Bugüne kadar, Alzheimer hastalığının patogenezi üzerine odaklanan bir tedavi geliştirilmiş değildir. Ancak, Alzheimer hastalığı ile insülin direnci bozuklukları arasındaki patojenik ilişkiyi farmakolojik olarak hedeflemenin önemli bir potansiyele sahip olduğu öne sürülmektedir. Bu kapsamda, tip 2 diyabet ilaçları ve insülin direncini azaltan ajanlar dikkate alınmaktadır. Önerilen ilaçlar arasında metformin, sodyum-glikoz ko-taşıyıcı-2 inhibitörleri (SGLT2i), glukagon benzeri peptit-1 reseptörü (GLP-1) agonistleri, resveratrol ve rapamisin yer almaktadır. Bu ilaçlar, kan glukoz seviyelerini düşürmek, insülin direncini hafifletmek, oksidatif stresi ve inflamasyonu azaltmak, kardiyovasküler olayları önlemek ve asetilkolin ile keton seviyelerini artırmak gibi çeşitli mekanizmalar aracılığıyla Alzheimer hastalığı üzerinde etkili olabilmektedir. Bununla birlikte, Alzheimer hastalığında insülin direncini hedefleyen ilaçların etkinliğini değerlendiren klinik çalışmalar yetersizdir. Bu nedenle, bu ilaçları etkili bir tedavi planına entegre edebilmek için ek araştırmalara ihtiyaç duyulmaktadır. Ayrıca, Alzheimer hastalığı ile insülin direnci arasındaki patojenik bağlantıya katkıda bulunan tüm faktörleri inceleyen çalışmalara ihtiyaç vardır.

YAZAR KATKILARI

Kavram: A.M.A.; Tasarım: A.M.A.; Denetim: A.M.A.; Kaynaklar: A.M.A.; Malzemeler: A.MA.; Veri Toplama ve/veya İşleme: A.M.A.; Analiz ve/veya Yorumlama: A.M.A.; Literatür Taraması: A.M.A.; Makalenin Yazılması: A.M.A.; Kritik İnceleme: A.M.A.; Diğer: -

ÇIKAR ÇATIŞMASI BEYANI

Yazar bu makale için gerçek, potansiyel veya algılanan çıkar çatışması olmadığını beyan eder.

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J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 49(3): 981-998, 2025
Doi: 10.33483/jfpau.1590697

REVIEW ARTICLE / DERLEME MAKALE

SILVER NANOCOMPOSITES: A GLIMPSE INTO THEIR GAME-CHANGING ROLES IN ANTIBACTERIAL THERAPY

GÜMÜŞ NANOKOMPOZİTLER: ANTİBAKTERİYEL TERAPİDEKİ OYUN DEĞİŞTİRİCİ ROLLERİNE BİR BAKIŞ

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ABSTRACT

Objective: *The application of nanocomposites in biomedicine is a promising approach that scientists have discovered to eradicate infection-causing microorganisms properly and safely. Silver nanocomposites (AgNCs) can be recognized as hopeful antibacterial prospects that can fight both in vivo and in vitro infection-causing bacteria. The purpose of this review is to identify the variables that influence the antibacterial effect of AgNCs, given the pressing need for new, effective antibacterial agents.*
Result and Discussion: *In the literature, many reports indicate the capacity of AgNCs to combat both gram-positive and gram-negative bacteriomers, including those that are resistant to multiple drugs. This capacity is due to the multiple simultaneous modes of AgNCs action. This capacity also results in a synergistic impact on bacteria when mutually applied with natural or synthetic antibacterial medications. Because of their unique properties, AgNCs can be effectively used to manage or prevent infections in a variety of medical and healthcare products. The study focuses on the synthetic methodologies and antibacterial mechanisms of AgNCs. Furthermore, factors influencing the action of AgNCs against bacteriomers as well as the advantages of combining AgNCs with antibiotics to create novel antibacterial combinations were covered. The authors wanted to make it possible to reduce the dose required and prevent unfavorable off-target effects associated with both by providing access to the reviewed data.*
Keywords: *Antibacterial activity, antibiotic, silver nanocomposites, synergistic effect*

ÖZ

Amaç: *Nanokompozitlerin biyomedikal alanda uygulanması, bilim insanlarının enfeksiyona neden olan mikroorganizmaları uygun ve güvenli bir şekilde ortadan kaldırmak için keşfettiği umut verici bir yaklaşımdır. Gümüş nanokompozitler (AgNC'ler), hem in vivo hem de in vitro enfeksiyona neden olan bakterilerle savaşabilen umut verici antibakteriyel olasılıklar olarak kabul edilebilir. Bu incelemenin amacı, yeni ve etkili antibakteriyel ajanlara olan acil ihtiyaç göz önüne alındığında, AgNC'lerin antibakteriyel etkisini etkileyen değişkenleri belirlemektir.*
Sonuç ve Tartışma: *Literatürde, birçok rapor, AgNC'lerin çoklu ilaçlara dirençli olanlar da dahil olmak üzere hem gram pozitif hem de gram negatif bakteriyomerlerle savaşma kapasitesini göstermektedir. Bu kapasite, AgNC'lerin birden fazla eşzamanlı etki modundan kaynaklanmaktadır. Bu kapasite, doğal veya sentetik antibakteriyel ilaçlarla birlikte uygulandığında bakteriler üzerinde sinerjik bir etkiyle de sonuçlanmaktadır. Benzersiz özellikleri nedeniyle, AgNC'ler çeşitli tıbbi ve*

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Submitted / Gönderilme : 24.11.2024
Accepted / Kabul : 22.04.2025
Published / Yayınlanma : 19.09.2025

sağlık ürünlerindeki enfeksiyonları yönetmek veya önlemek için etkili bir şekilde kullanılabilir. Çalışma, AgNC'lerin sentetik metodolojilerine ve antibakteriyel mekanizmalarına odaklanmaktadır. Ayrıca, AgNC'lerin bakteriyomerlere karşı etkisini etkileyen faktörler ve AgNC'leri antibiyotiklerle birleştirerek yeni antibakteriyel kombinasyonlar oluşturmanın avantajları ele alınmıştır. Yazarlar, incelenen verilere erişim sağlayarak gereken dozu azaltmayı ve her ikisiyle ilişkili olumsuz hedef dışı etkileri önlemeyi mümkün kılmak istemişlerdir.
Anahtar Kelimeler: *Antibakteriyel aktivite, antibiyotik, gümüş nanokompozitler, sinerjik etki*

INTRODUCTION

Bacteria quickly create antibiotic resistance strategies because of their enormous populations and high growth rates. This may happen when a portion of the bacterial population is exposed to antibiotics continuously at low doses [1]. However, misuse and abuse of antibiotics have been considered one of the main reasons for the increase in infections caused by antibiotic-resistant bacteria in recent years [2]. As infections brought on by multi-resistant microorganisms continue to spread and claim lives all over the world, research into the creation of new antibiotics is currently a challenging process that takes years to complete and involves extensive time and resource consumption [3,4].

Nanotechnology is a field that works with substances providing at least one dimension varying between 1 and 100 nm with the goal of obtaining novel particles and materials. Nanocomposites are quite distinct from macrocomposites because the former composite type has special, superior, and indispensable characteristics compared to the latter type [5]. The distinct nanoscale structure of nanocomposites confers exceptional properties, setting them apart from macrocomposites. Nanocomposites have a significantly better total area-to-volume ratio than macrocomposites because they contain nanoparticles with at least one dimension in the nanometer range. The matrix and filler's interfacial contacts are strengthened as a result, improving electrical conductivity, toughness, and heat stability. In the medical, automotive, and aerospace industries, for example, nanocomposites are essential due to their improved barrier qualities, decreased weight, and increased flame retardancy [6,7]. Furthermore, they can be molecularly tuned to achieve multifunctionality, something that macrocomposites cannot do [8]. Nanocomposites are water-insoluble particles whose size is less than 100 nanometers, which makes them have a greater surface-to-volume ratio (per unit mass), which gives them more surface area for antibacterial action and enables them to interact more closely with the bacterial membrane. Historically, antimicrobial agents have been created using silver in all of its forms, either alone or in conjunction with other methods [9]. AgNCs improved antibacterial capacity at the nanoscale has proven particularly useful in the medical care and health services fields, where the integration of AgNCs into many different products—such as surgical instruments, cosmetics, dental products, and dressings—has been researched [10]. By combining silver, either as silver nitrate or silver sulfadiazine, in food containers in order to stop contamination, burn and ulcer treatment creams, and various industrial applications, researchers have explored how to best utilize this metal's ability to limit bacterial growth [11].

Since ancient times, the biological technique has used microbes or medicinal herbs to create nanocomposites [12]. AgNCs have demonstrated antimicrobial effectiveness against a range of virulent and infectious pathogens. This is due to their strong antimicrobial efficacy against a wide range of bacteria, viruses, and fungi, as well as their broad-spectrum antimicrobial properties [13]. AgNCs are being used increasingly in consumer products, such as nutritional supplements, apparel, and packaging, due to their antibacterial characteristics against antibiotic-resistant microorganisms [14]. The prospect for AgNCs to be more hazardous than equal quantities of dissolved silver has been demonstrated in laboratory tests with plants and microorganisms [15]. Due to its distinct qualities and potential uses, nanocomposite manufacturing has attracted a lot of interest lately. Whether they are simple or composite, nanocomposites have distinctive features and are increasingly being employed to create novel nanodevices for a variety of physical, biological, biomedical, and therapeutic purposes [16]. As a result, there may be an urgent need to find new medications to replace those that are now in use. Most nanocomposites have special qualities that are useful in many biotechnology applications, resulting in their use in the creation of incredibly efficient diagnostics and therapeutic tools [17].

Because of AgNCs' distinctive characteristics, which include biomedical traits like antibacterial, delivery of medication, cancer-fighting, and immune-modulating activities, scientists are interested in

using AgNCs in nanotechnology applications [18]. Several scientific domains were significantly impacted by the invention of AgNCs' synthesis. The nanocomposites have drawn a lot of attention because of the significant demand and expenditure in associated research. The market for AgNCs has been expanding steadily over the past fifteen years, and production of over five hundred tons of nanocomposites annually is estimated to be needed to meet the demands of various industries [19]. The investigation of the biological function and safety of AgNCs, as well as the clarification of their precise modes of action in microbial and human cells, have become pressing issues [20]. These are due to the global expansion of the nanocomposite market and the availability of goods containing them. This brief overview covers the use of AgNCs in medicine to inhibit the growth of or kill bacteriomers, which can be an alternative to or with antibiotics. The headings and subheadings of the subjects discussed in this paper are graphically represented in Figure 1.

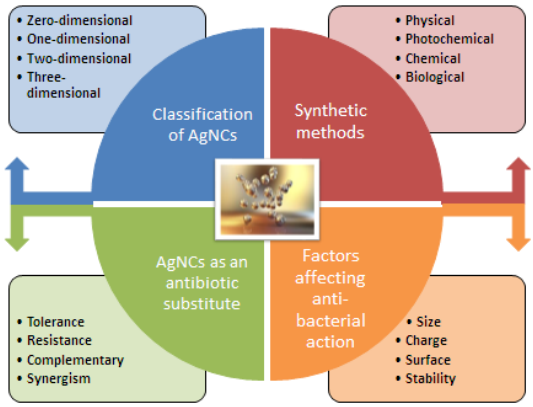


Figure 1. Graphical representation of the main subjects investigated in this review study

Classification of AgNCs

AgNCs can be roughly categorized into four distinct categories [19]: Zero-dimensional nanocomposites (Figure 2a), such as an aggregation or nanoshell, are measured in every dimension on the nanoscale. One-dimensional nanocomposites (Figure 2b) with a single layer that range in size from 1 to 100 nm. They are employed in many different fields, including solar cells, and they support a wide range of technological applications, including fiber-optic systems, natural sensors, and storage devices. Two-dimensional nanocomposites (Figure 2c), such as carbon nanotubes, and three-dimensional nanocomposites (Figure 2d), like quantum dots.

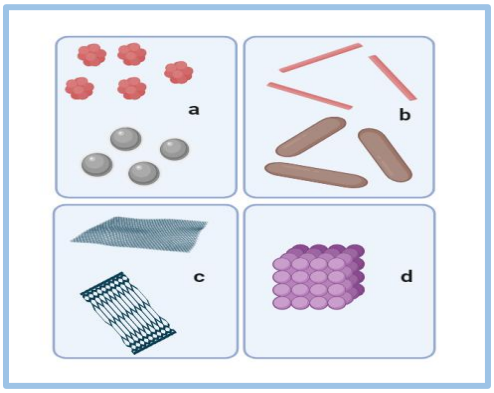


Figure 2. Various currently available categories of AgNCs

Methodologies for Creating AgNCs

The need for environmentally friendly, non-toxic nanocomposite production techniques that reduce the usage of hazardous chemicals as byproducts has spurred interest in biomedicine fields [21]. AgNCs have been considered a popular ingredient in many health products due to their exceptional ability to combat infectious disorders and stop the growth of bacteria, mold, and germs. They have unique chemical-, optical-, electrical-, magnetic-, and mechanical-based properties [18,22]. The main methodologies found in the literature for creating AgNCs are four, and they are explained below.

Physical Methodology

This method involves creating metal nanocomposites or wires through evaporation or intensification, which can be accomplished by keeping the furnace tube at the atmosphere's pressure. In the furnace, the materials inside a fixed vessel vaporize into the carrier gas. The vaporization process and intensification techniques were used to create nanosized particles of several materials, such as copper, silver, zinc, tin, and lead, as well as fullerene [23]. As a result, producing nanocomposites in a furnace has several drawbacks since the cylinder-shaped material requires a lot of energy, takes up a lot of space, raises the ambient temperature, and takes a while to achieve thermal consistency [24].

A unique cylinder-based furnace must heat for ten minutes to reach a steady temperature, using many kilowatts of energy. Moreover, laser ablation of the whole substance in a solution has been used to create AgNCs [19]. The advantage of the laser technique over the other approaches is that pure colloids may be manufactured and there is no need for a chemical reagent in solution, making it more beneficial in a variety of applications [25]. In general, AgNCs with full-size circulation are produced by physical methodology with the assistance of physically generated energy [26].

This type of method may effectively produce AgNCs in the form of ash while enabling the production of large quantities of product with a single methodology. Physical methodology can produce a huge amount of high-purity AgNCs. It produces stable AgNCs by synthesizing nanocomposites from solid or aerosolized metallic silver down to the nanoscale [27]. This group includes physical techniques including sputtering, laser ablation, and grinding balls. Unfortunately, high pressure and temperature conditions, costly instrumentation, and significant energy requirements are typically associated with these techniques [25,28].

Photochemical Methodology

Both Huang and Yang created AgNCs via the photoreduction of silver nitrate layers. This method is based on innocuous soil suspensions that serve as stabilizing oxidants to prevent particle aggregation. AgNCs were irradiated and split according to their distribution ratio until they reached a consistent size distribution ratio and absolute diameter. Nevertheless, there were some prerequisites, such as an expensive setup and an experimental setting [29].

Chemical Methodology

All options considered, the chemical methodology is the most effective, practical, and manageable. This process, which can be done relatively cheaply and with great productivity, is widely employed to produce AgNCs by chemical reduction. The chemical approaches of AgNCs in real solution usually involve three steps: Capping oxidizing agents, reducers, and metal-based precursors [30]. In addition, the utilization of capping agents in this method enables the regulation of metal-based nanocomposite growth, which is necessary to obtain tiny nanocomposite particle with a spherical form. To prepare a colloidal solution, the silver-natured salt reduction has two phases of development and nucleation. The shape of the nanocomposites depends on these two processes [29]. Nonetheless, the synthesis of particles with the same size and development rate is the prerequisite for the manufacture of single dispersed nanocomposites with regular sizes. By controlling the reaction utilizing various restrictions, such as precursors, pH, temperature, reducing substance, and capping material, the ensuing formation of primary nuclei may be detected [31].

Biological (Green Chemical) Methodology

The term "green chemistry" is another name for the biosynthetic technique that uses reducing agents such as biological microorganisms, polysaccharides, extracts from plants, bacteria, or fungi [32]. It is commonly known that bacteria can create inorganic substances both inside and outside of their cells. According to Yaqoob et al. (2019), they thus serve as more viable biofactories for the production of nanocomposites such as gold, copper, silver, etc. [19]. One of the acknowledged particles having biotic properties is the creation of AgNCs. Silver and gold nanocomposites can be created by Vilchis-Nestor et al. (2008) using an aqueous Camellia sinensis extract under ambient circumstances. This extract was employed as an agent for stabilization and reduction [33]. However, Kalishwaralal et al. (2008) also mentioned that AgNCs were produced by reducing their ions with Bacillus licheniformis supernatant [34]. This approach offers greater benefits than the others because non-pathogenic bacteria were employed, and AgNCs are incredibly stable. This method is both stable in terms of the environment and economical. However, the large-scale production of nanocomposites or wires via a biological technique is challenging [35]. Table 1 demonstrates the different approaches together with their methods and useful aspects.

Table 1. The various methodologies together with their techniques and practical qualities

Creating methodologies	Properties	Techniques	Basic requirements
Physical methodology	Produce homogeneous colloids solution in large quantities in a single operation.	Methods include chemical etching, ablation with lasers, and ball milling	Sophisticated, pricey equipment, and High atmospheric pressure, enormous energy
Photochemical methodology	Extremely sophisticated instruments, exact diameter, consistent size, and mean distribution ratio acquired	Photodissipation or photodegradation	Extended periods of time and high-priced equipment in an experimental setting
Chemical methodology	Successfully inexpensive, and easy to use a technique that produced spherical-shaped particles	Electrochemical technique, reduction of chemicals	Metal precursors and capping and reducing agents
Biological (green chemical) methodology	Cheap, stable in the environment, and intricate to operate	Technique mediated by plants algae-mediated approach, bacteria -and fungus-mediated approach	Algae, fungus, and bacteria that are Non-pathogenic, reducing agent

General Aspects of AgNCs Activity Against Pathogenic Bacteriomers

Since antibiotic-resistant bacterial infections are a major worldwide health concern, AgNCs present a great substitute. This is because they can be used to stop infections brought on by these bacteriomers, clean medical equipment, and even treat infections as they occur [36]. This substitution has received a lot of attention recently as an alternative to antibiotics, with the goal of creating new bactericidal items for infection therapies that take advantage of what is known about their effectiveness. Nanotechnology, the application of materials with atomic- or molecular-scale dimensions, has grown in popularity for medical uses and is highly sought-after as a method of eradicating or suppressing the activity of several pathogens [37]. Some naturally occurring antibacterial materials, like silver, exhibit enhanced antibacterial properties as they get smaller within the nanometer order (because of an increase in the surface-to-volume ratio for a given mass) [38]. However, different antibacterial mechanisms may be related to the physical composition of the nanocomposites and how they interact with and introduce

pathogens. To obtain nanocomposites with antibacterial properties, the shape and size of the nanocomposites are among the most important characteristics that are supposed to be present in order to achieve this effect. It should be 3–100 nanometers in size, but the shape varies: spherical, cubic, triangular, pentagonal, hexagonal, and nanowire [39].

All shapes were able to connect and eventually cause destruction of the bacterial cell's membrane, according to observations of the membrane structure. The most facets, however, are found in the truncated triangular shape, which promotes bacterial contact and increases surface binding, cell absorption, and bacterial killing [13]. AgNCs have the potential to be antibiotics because of their diverse modes of action, which allow them to kill different kinds of bacteria by attacking them simultaneously in several structures [40]. The antibacterial activity of AgNCs (5–9 nm) is inversely correlated with their size. Also, this activity has significantly increased, especially below 10 nm, according to Shim et al. [14]. Reactive oxygen species accumulation and making the bacterial membrane more invaded are both correlated with the smaller size of the nanocomposites used [39].

Different methodologies were used for checking the antibacterial effect of AgNCs; however, the disk and well diffusion approaches are the most common [41]. Minimum inhibitory and minimum bactericidal concentrations (MIC and MBC), respectively, were also used. MIC detects the concentration of AgNCs that can inhibit the visible growth of the bacterial population using the broth dilution method. To determine MBC, a fixed AgNCs concentration above the value of the MIC is added to nutritional media. This is in addition to increasing microbial inoculum, and bacterial growth is observed using UV-V spectroscopy or a plate analyzer for changes in the samples' optical densities [42]. The bacteriomer's shape and structure were also monitored before and after exposure to AgNCs using transmission electron microscopy and scan electron microscopy [43]. Combining AgNCs with antibiotics has been shown to boost their antibacterial efficacy, particularly against drug-resistant bacteriomers. This combination has been proposed as a possible way to overcome medication resistance in bacteria in the recent past. It was suggested that coupling different antibacterial agents would increase their mutual antibacterial effectiveness [44,45].

Table 2. Some bacteriomers that reported to be inhibited by AgNCs

Bacteriomer	**Reference**	**Bacteriomer**	**Reference**
Gram positive		**Gram negative**	
Lactobacillus fermentum	[43]	***E. coli***	[49]
Streptomyces sp.	[50]	***Escherichia fergusonii***	[51]
Streptococcus mutans	[51]	***Vibrio cholera***	[52]
Bacillus cereus	[51]	***Ureibacillus thermo sphaerius***	[53]
Bacillus anthracis	[46]	***Pseudomonas aeruginosa***	[54]
Brevibacterium casei	[55]	***Salmonella typhimurium***	[55]
Enterococcus faecalis	[5]	***Acinetobacter baumannii***	[56]
Staphylococcus aureus	[49]	***Shigella sp***	[57]
Staphylococcus epidermidis	[5]	***Enterobacter aerogenes***	[58]
Bacillus licheniromis	[40]	***Klebsiella pneumoniae***	[59]
Listeria monocytogenes	[5]	***Proteus mirabilis***	[46]

Against a variety of bacterial species, both gram-positive and gram-negative, AgNCs have demonstrated strong antibacterial activity [46]. The bacteriomers that are reportedly influenced by the use of AgNCs are listed in Table 2. The available experimental data supports a variety of ways that take into account the physical characteristics of AgNCs, such as their size and surface. These ways enable them to interact with intracellular components or even pass through membranes and cell walls. When exposed to the same quantity of silver ions, gram-positive (e.g., *Staphylococcus aureus*) and gram-negative (e.g., *E. coli*) bacteriomers have been shown to exhibit DNA condensation, cell membrane division from the bacterium wall, and cell wall destruction. Additionally, each species of bacterial

cytoplasm included silver ions. These results shed more light on the function of metal ions in silver's antibacterial properties [47]. All forms of AgNCs showed dose-dependent suppression of bacterial activity. It's possible that the creation of nanoplates added a positive charge to the outer layer of the small particles, enhancing the electrical interactions between particles and bacteriomers. In summary, the antibacterial activities of AgNCs were due to the influence of silver ions on DNA replication, respiration routes, and the cellular membrane's integrity [48].

Mechanisms of AgNCs Antibacterial Potential

Three mechanistic processes that have been identified as working together or independently to explain how AgNCs inhibit bacteriomers are the main ones supported by the literature [60]. According to the first mechanism, AgNCs function at the plasma membrane level because they can pass through the exterior membrane and accumulate in the interior membrane. The adherence of AgNCs to the cell causes it to become unstable and damaged, enhancing the permeability, causing cellular content to leak out, and ultimately causing the cell to die. Additionally, it has been demonstrated that AgNCs can bind with sulfur-containing proteins in bacterial cell walls, which could result in structural damage and cell wall rupture [61]. According to the second mechanism, AgNCs can enter cells and change their composition and permeability by breaking through the membrane of the cell. It has been proposed that the distinct characteristics of AgNCs will enable them to communicate with intracellular components, such as DNA and proteins, in order to change their structure and function by phosphorous or sulfur moieties. In a comparable way, AgNCs may modify the respiratory pathway of the interior membrane by engaging with the SH residues in the corresponding enzymes [62]. These events trigger the apoptotic process, generate harmful free radicals, and disrupt intracellular machinery. Simultaneously with the first two mechanisms, a third process is proposed: the AgNCs discharge silver ions. These positively charged ions may communicate with biological parts, altering pathways of metabolism, membranes, and even nucleic acids due to their size and charge [63]. Figure 3 depicts these three fundamental processes proposed for the antibacterial action of AgNCs.

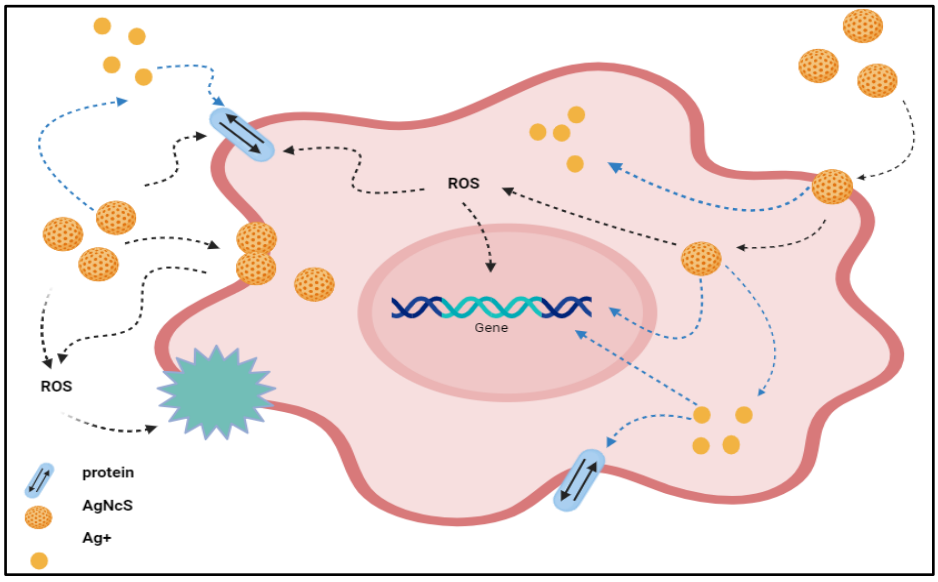


Figure 3. A diagram illustrating the pathways linked to AgNCs' antibacterial properties

Numerous renowned tactics that bacteriomers have evolved to make antibiotics useless. These tactics include obtaining drug-fighting genes from other species, growing biofilms, erecting obstacles to prevent antibiotics from penetrating, altering the drug's target, and removing the antibiotic from the intracellular medium by employing out-pump processes [64]. The fundamental mechanisms of resistance to antibiotics in bacteriomers involve drug inactivation, efflux pumps, entry barriers, and

structural alterations in response to drug targets. It also emphasizes the anti-bacterial mechanisms of AgNCs, including DNA damage, membrane accumulation, electron transport chain interference, efflux pump modification, and structural alterations. The foundation for these tactics is shown in Figure 4, which also shows that AgNCs may still affect bacteriomers regardless of drug-fighting mechanisms. AgNCs' antibacterial actions allow them to be used in combination with antibiotics or other lab-generated antibacterials as a supplement to their established capacity to inhibit biofilm growth [65].

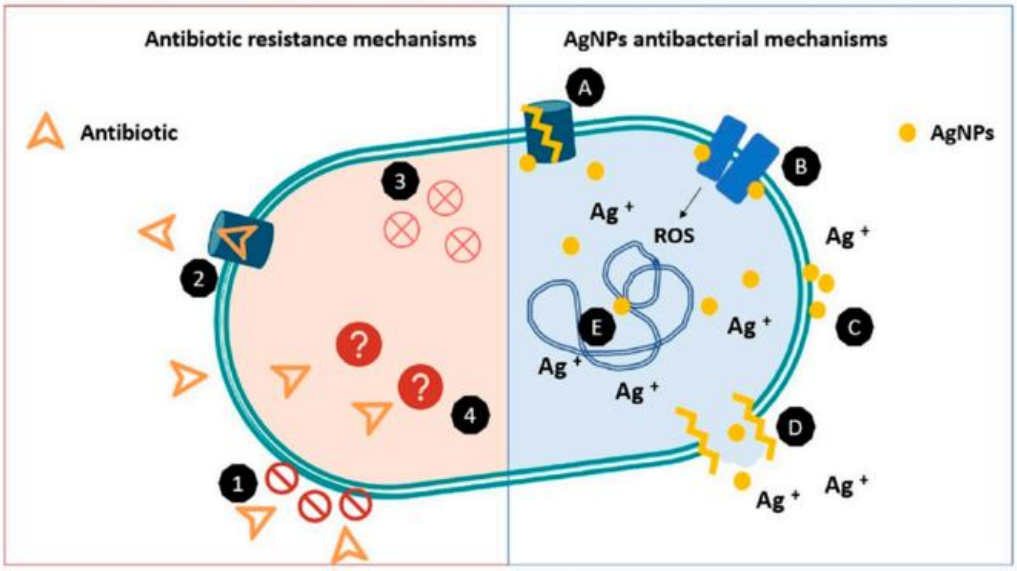


Figure 4. Comparison between the antibacterial mechanistic process of AgNCs and the resistance mechanisms of bacteriomers [9]. The following are the antibiotic- or synthetic antibacterial-resisting mechanisms: (1) entry hurdles; (2) outflow motors; (3) drug inactivation; and (4) structural modifications to drug targets (denoted as "?") that impede drug recognition. Conversely, AgNCs' antibacterial mechanisms involve the following: (A) modification of efflux pumps; (B) interference with electron transport chains and membrane proteins; (C) accumulation in the membrane that affects penetration; (D) change of the membrane structure and leakage of bacteriomers intracellular content; and (E) interaction and damage to DNA

The development of AgNCs was a logical and convincing step because silver has historically been used as an antibacterial agent. In the literature, there are many reports on how AgNCs work to kill bacteria [66]. The first thing that was believed to be impacted was membrane permeability due to the abundance of nanocomposites found inside the bacterium. The interaction of AgNCs with the bacterial membrane and proteins, especially those with sulfur, as well as DNA containing phosphorus, prevents cell division and results in cellular death [67]. The existence of biocidal ionized silver emitted from nanocomposite surfaces has also been confirmed by many published papers. Bacteria's DNA aggregation defense systems shield them from a hazardous environment when exposed to ionic silver, but this impairs the bacteria's capacity for replication. Ionic silver and its nanocomposites elicit different reactions, yet both are necessary to fully understand the antibacterial activity of AgNCs.

It is noteworthy that the use of AgNCs coupled with antibiotics for the medical treatment of bacterial infections has not been linked in the literature to the emergence of resistance. This finding may be connected to the mechanisms that govern the action of both of these agents [68,69]. AgNCs attack multiple cellular structures, whereas antibiotics only target one specific target in bacteriomers. AgNCs attack multiple cellular structures, whereas antibiotics only target one specific target in bacterial cells. As a result, when an antibiotic-AgNCs combination is used together, nanocomposites interact, act, and accumulate in bacterial walls and membranes. Since they can change and dissolve cellular barriers and membrane proteins, AgNCs allow antibiotics to work at the intracellular level. So, AgNCs are increasing

the concentration of antibiotics, debilitating bacteriomers, and even avoiding already-existing mechanisms of resistance [9].

Variables Impacting AgNCs Activity Against Bacteriomers

It has been shown through experiments that AgNCs' characteristics affect their antibacterial activity [60]. The ways in which AgNCs interact with bacteriomers are connected to how these properties affect their antibacterial potential. It has been found that the characteristics that increase antibacterial activity are also those that encourage interactions between bacteriomers and AgNCs. Accordingly, it is possible to manipulate and fabricate AgNCs in order to achieve the desired goals. This results in the emergence of an antimicrobial substance that can be prepared with optimized characteristics. In addition to the molecular components of AgNCs antibacterial action being clarified, it has been shown that many variables affect their antibacterial potential, which are listed below [9,44].

Composite Size

It was discovered that AgNCs prevented the development of gram-negative bacteriomers, especially *E. coli*, *Vibrio cholerae*, *Salmonella typhi*, and *Pseudomonas aeruginosa*, on agar plates with an average size of 21 nm and concentrations at or above 75 g/ml [70]. Lu et al. conducted a study to explore the effect of size on the antibacterial activity of AgNCs against the bacteriomers responsible for caries and periodontal diseases [71]. AgNCs were created in various sizes, and their antibacterial efficacy was assessed. It was discovered that smaller AgNCs had superior antibacterial activity compared to larger ones, with lower MIC limits [71]. In a subsequent study, the same agents and overall methodology were used to create AgNCs of various sizes. The variants are the pH levels and ratios of the stabilizing and reducing agents in the reactions that were altered. Then, against both gram-positive and gram-negative bacteriomers, the bacterial potential of AgNCs sizing 5-100 nm was assessed. The obtained MIC varied between small and large particle sizes, indicating lower concentrations in small particles relative to larger ones [72]. The increased surface area of the smaller nanocomposites, which is available for direct interaction with the bacterial cell, is thought to be the reason for the size dependence of the antibacterial activity, as demonstrated by the MIC data [73].

In further research, the efficacy of five different AgNCs to inhibit Pseudomonas aeruginosa and Escherichia coli was evaluated after they were created via chemical reduction. The results of the investigation showed that the AgNCs with the biggest diameter (30 to 200 nm) displayed the lowest activity with tiny inhibition halos. On the other hand, the smallest nanocomposites (15 to 50 nm) produced the most activity, with an inhibitory growth halo greater than that [74]. A more recent study assessed the antibacterial efficacy of laser-generated AgNCs in different sizes against *E. coli*. Antibacterial activity and AgNCs' size were similarly shown to be inversely correlated in this case; the most potent antibacterial activity was displayed by AgNCs with an average size of 19 nm. In this instance, the researchers demonstrated that smaller AgNCs would produce more reactive oxygen species and, as a result, be more potent against *Escherichia coli* [75].

Composite Charge and Surface

Regarding the charge's impact, it has been shown that particles with a positive charge exhibit stronger antibacterial activity [64]. Abbaszadegan et al. [76], stated that the electrostatic interaction between positively charged AgNCs and negatively charged bacteriomers is what drives AgNCs' antibacterial activity. So, the key factors are the charge of the AgNCs and that of microorganisms. Remarkably, the rate at which silver ions are released from the AgNCs is correlated with both size and surface features. While the size of the particles influences their contact area and interaction with the medium, the surface and charge of the AgNCs dictate their stability [77]. Accordingly, it has been noted that smaller AgNCs dissolve more quickly in various environments and release silver ions throughout the process, which may have a significant impact on their antibacterial activity [78].

Composite Stability

The AgNCs' stability is a crucial component influencing their ultimate antibacterial activity once the impact of size and charge on their survivability has been elucidated. The low stability of the

generated AgNCs will cause them to aggregate and form larger particles, and research has shown that larger AgNCs have less antibacterial action. The electrical charge and the covering are the main factors influencing AgNC's stability. Both the coating agent employed and the synthesis methodology may specify this value [79].

AgNCs as Antibiotic Substituents

AgNCs have emerged as a promising substitute for antibiotics in the battle against various pathogenic bacteria. In addition to their capacity to inhibit the growth [78] of multidrug-resistant strains, AgNCs have distinct properties, making them potentially useful against these pathogens [80]. First off, AgNCs are thought to act on the bacterial membrane, impact intracellular components, and modify the respiratory chain, among other methods, causing their antibacterial effects [81]. The second characteristic is that, among metallic nanocomposites, AgNCs are well-known for being the most powerful against bacteriomers and other pathogens. It is also biocompatible and simple to utilize for medical purposes [68]. The factors that have led to the promotion of AgNCs as an antibiotic substitute are discussed below.

Bacterial Tolerance

The use of nanocomposites against human pathogenic microorganisms has advanced rapidly in recent years, coinciding with observations of their positive consequences. This subject of bacterial tolerance to nanocomposites is still being researched to comprehend the mechanism underlying this learned tolerance, in addition to the precise mode of action of the nanocomposites [82].

Apart from bacterial resistance, Kaweeteerawat et al. [83] found that antibiotic efficacy could be lowered if bacteriomers had previously been treated with AgNCs. In this instance, isolates of *E. coli* and *Staphylococcus aureus* that had previously been treated with AgNCs showed resistance to ampicillin and other antibiotics, as evidenced by MIC values that were 2–8 times higher than those of the bacteriomers that had not previously had AgNCs' treatmentThe study found that bacteriomers infused with AgNCs acquired membrane-related alterations. These include decreased porosity and stiffer membranes, both of which can increase the organism's downstream antibiotic- or synthetic-antibacterial resistance.

The emergence of tolerance in bacteriomers that were progressively biocultured with different amounts of AgNCs was examined by Kūedziora et al. [84]. Strains of *E. coli, Klebsiella pneumoniae,* and *Staphylococcus aureus* have been repeatedly biocultured with AgNCs and studied. The results indicated a change in the antibacterial capacity of AgNCs, which resulted in mounting MIC values. This study also discovered that extended AgNC contact may induce a reduction in the desired effect.

Bacterial Resistance

Regarding this, a number of studies examining the potential consequences of using AgNCs as antibiotic substituents have been published recently. One of the main concerns is that AgNCs may cause the emergence of resistance strains. For instance, several studies showed that certain bacteriomers showed reduced susceptibility to AgNCs following repeated and continuous exposure [6]. Panáček A, et al. [85] studied this effect when AgNCs-exposed *E. coli* and *Pseudomonas aeruginosa* were multiplied. After multiple rounds of culture, the values of MIC for *E. coli* showed a rise from 3.38 mg/ml to 13.5 mg/ml, respectively. This indicated that both of the bacteriomers under study developed a tolerance to the AgNCs.

Sublethal dosages of AgNCs—which had previously been established in the same study—were given to *E. coli* and *Staphylococcus aureus* bacteriomers for a period of five days. *E. coli* and *Staphylococcus aureus* showed an increase in their IC50 values from 11.89 to 17.59 mg/ml and 6.98 to 18.09 mg/ml, respectively, suggesting that these bacteriomers are becoming more tolerant of AgNCs. This information indicates that, following successive applications, bacteriomers may have become resistant to a sublethal dose of AgNCs. Even though they were not connected to genetic changes, these reported examples of AgNCs resistance emphasize the importance of investigating doses and probable mechanisms of resistance or avoidance of resistance [86].

A recent relevant study also showed an effective alternative for treating multi-resistant

bacteriomers that doesn't result in the development of nanocomposite resistance. In this instance, ampicillin was used as a reducing and capping agent during the direct synthesis of the AgNCs. The bactericidal properties of ampicillin-AgNCs were evaluated using gram-positive and gram-negative bacteriomers that were both drug-resistant and drug-sensitive. The MIC values showed that the ampicillin-AgNCs were always more effective than ampicillin or chemically generated AgNCs [87]. An effective substitute for using AgNCs in conjunction with antibiotics is the structural alteration or coupling of AgNCs with other chemicals. This approach aimed to achieve high bactericidal activity while preventing the development of bacterial resistance [88].

Synergistic and Complementary Effects

In addition to studies that show how AgNCs induce resistant bacterial strains to emerge, other research has been published to explain how AgNCs can work in concert with other compounds. These include polymers, plant extracts, or antibiotics to create additive or synergistic effects. This could lead to the development of an alternative treatment for multidrug-resistant bacterial strains without running the risk of encouraging the emergence of new resistant strains or preventing bacteriomers from becoming resistant to AgNCs [19]. The two most researched bacteriomers are gram-positive *Staphylococcus aureus* and gram-negative *E. coli*. A number of other strains have been identified, including *Vibrio cholerae*, *Proteus mirabilis*, *Enterobacter*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Staphylococcus epidermidis*, and *Listeria monocytogenes*. Over time, bacteriomers have grown resistant to traditional antibiotics due to the overuse of antibiotics, which has resulted in the development of new antibiotic generations [89]. The antibacterial activity of metallic (silver, gold, and platinum) nanocomposites combined with a small amount of antibiotics is significantly increased. This is particularly true when fighting multi-strain-resistant bacteriomers, as shown in Figure 5. Because AgNCs exhibit significant antibacterial action against a variety of bacteriomers, these nanocomposites have garnered a lot of attention. When used at levels up to 350 µg per day, they have no toxic off-target effects on people. Various studies examining the antibacterial activity of AgNCs in combination with antibiotics have indicated the above fact [9].

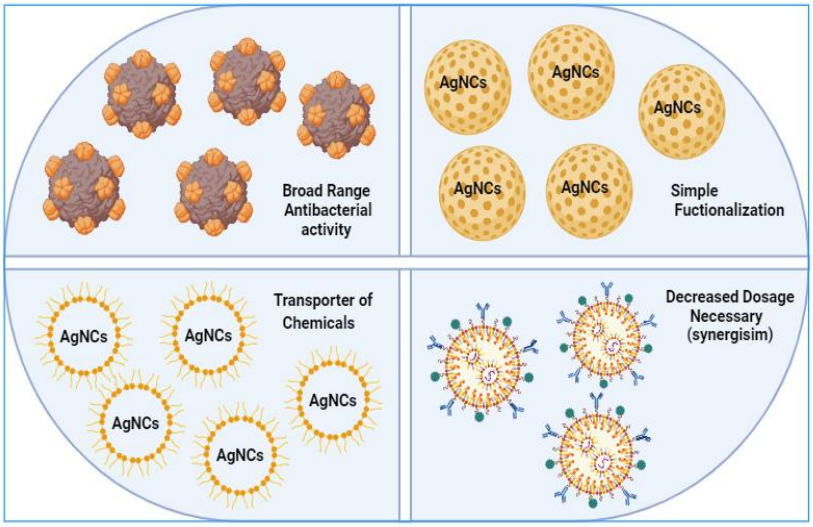


Figure 5. Advantages associated with employing AgNCs instead of or in addition to antibiotics

A recent study found that treating *E. coli*, *Salmonella typhimurium*, and *Staphylococcus aureus* with chloramphenicol-AgNCs could reduce the bacterial growth by up to 50%. However, the development of these bacteriomers was inhibited at a rate that approached 95% when kanamycin-AgNCs therapy was administered concurrently [90]. The mutual action is more effective because the AgNCs modify the integrity and action potential of the membrane, increasing cellular permeability and

making it easier for antibiotics to pass through. It should be noted that half the MIC of the antibiotic and sublethal amounts of AgNCs were used in these studies. The spleen of mice colonized with *Pseudomonas aeruginosa* was treated by the azlocillin-AgNCs conjugate, as shown by the study of Alizadeh, A. et al. [91]. The dosage of biocompatible AgNCs has shown synergistic effects with antibiotics of various classes, improving bacterial death, including both gram-positive and gram-negative bacteriomers [92].

AgNCs in combination with the antibiotics vancomycin and amikacin shown synergistic activity against gram-positive and gram-negative bacteria, respectively, in a separate study. The zone of inhibition test, which assessed antibacterial activity, showed that antibiotics and AgNCs might cooperate to accomplish this goal. The amount of bacterial inhibition produced by the combination of antibiotics and AgNCs was almost double that of the antibiotic used alone [93]. Green chemistry-prepared AgNCs using chitosan and brown marine algae extract are able to create stable AgNCs, with an average size of 12 nm. These nanocomposites have increased bactericidal efficacy towards infectious bacteriomers, including *Bacillus cereus* and *Salmonella enterica*. According to the results, AgNCs made with chitosan and algal extract had better bactericidal action than AgNCs or extract alone, with inhibition zones ranging between 6 and 12 mm for each of the bacteriomers under study [94].

The research shows that the AgNCs are positioned as an excellent supplement to or replacement for antibiotics. This shows that the antibacterial activity of AgNCs against human pathogenic bacteriomers is on par with or even greater than that of the antibiotics frequently used. Because of this, research has been done to assess any potential negative effects of exposure to AgNCs in addition to studies on their antibacterial capacity [95]. The goal is to determine the doses and characteristics of the nanocomposites that would be safe for use in humans.

Potential Cytotoxicity and Environmental Implications of AgNC Use

AgNPs, or silver nanocomposites, are widely employed in various applications, including wastewater treatment, food wrapping, and pharmaceuticals. because of their special physicochemical characteristics and antibacterial capabilities. However, there are serious environmental issues with their extensive use, especially in light of their ecotoxicity and future effects on ecosystems [7]. It has shown notable effectiveness in clinical settings in avoiding infections and accelerating wound healing. For example, its use in wound dressings has sped up tissue regeneration and decreased bacterial colonization. Likewise, their application in drug delivery systems has improved medicinal medicines' targeted administration and bioavailability. Notwithstanding these benefits, there is still worry about AgNPs' possible cytotoxicity to human cells and potential long-term repercussions on patients. To ensure its safe and efficient use in healthcare, these problems must be resolved [96]. However, their potential toxicity has raised significant environmental and human health concerns [97]. AgNCs can enter the human body through intravenous exposure, ingestion, inhalation, or skin contact. Once inside, they may accumulate in organs such as the kidneys, liver, lungs, and brain, leading to DNA damage, oxidative stress, and inflammation. Studies indicate that AgNPs can induce apoptosis, damage cellular membranes, and cause chronic toxicity after prolonged exposure. For instance, maternal exposure to AgNCs is linked to developmental toxicity, including neurobehavioral impairments and reproductive issues in children [96,97].

RESULT AND DISCUSSION

The evolution of resistant bacterial strains and the off-target effects associated with long-term usage constitute significant challenges to creating medications for the management of infections. Consequently, it takes interdisciplinary research projects to generate better antibacterials using different approaches. With the development of nanotechnology, it is now possible to manipulate the sizes of known antibacterial substances, like metals, to re-examine their biological properties and to accomplish delivery to particular targets and selectivity. AgNCs have shown great promise across a range of applications, including medical. They provide a new line of inquiry into a class of materials with potential uses in the biological, biomedical, and pharmaceutical fields. AgNCs have remarkable antibacterial efficacy when combined with modest amounts of antibiotics. This combination type has

been shown to produce outstanding results in vitro and has promise for effective bacterial eradication in vivo. Bacteriomers' exposure to sublethal concentrations of AgNCs must be prevented since this might promote the development of tolerance. Instead, the dosage of these agents needs to be monitored and managed. By using AgNCs, off-target effects are less likely to occur since fewer amounts of the antibiotic and AgNCs are needed to produce efficient antibacterial activity. In order to enhance their release and selectivity, AgNCs can form complexes to operate as drug or antibiotic carriers. Furthermore, AgNCs may be functionalized with a variety of substances to improve the antibacterial activity of the former particles against resistant strains and both gram-positive and gram-negative bacteriomers. According to these findings, AgNCs can be used as antibacterial agents; still, suitable usage strategies need to be investigated and created so as not to exacerbate the establishment of resistance strains. Because of their unique physicochemical features and antibacterial qualities, silver nanoparticles (AgNPs) are used extensively in industries like water treatment, agriculture, and medicine. But their widespread use has raised questions about their influence on the natural world, particularly their ecological toxicity and possible long-term repercussions on ecosystems.In order to improve AgNPs' biocompatibility and lower their toxicity, future studies should concentrate on improving their production. Green synthesis techniques, which make use of biological agents or plant extracts, present a viable way to create less hazardous and environmentally friendly nanoparticles. In order to lessen negative consequences, further research is also required to comprehend the processes of AgNP interacting with biological components, especially at molecular scale.

ACKNOWLEDGEMENTS

The University of Mosul's College of Pharmacy supplied the tools needed to complete this project, making the study feasible.

AUTHOR CONTRIBUTIONS

Concept: M.Q.Y., R.R.K., E.T.M.; Design: M.Q.Y., E.T.M.; Control: - ; Sources: M.Q.Y., R.R.K., E.T.M.; Materials: M.Q.Y., R.R.K.; Data Collection and/or Processing: M.Q.Y., R.R.K., E.T.M.; Analysis and/or Interpretation: E.T.M., R.R.K.; Literature Review: M.Q.Y., R.R.K., E.T.M.; Manuscript Writing: M.Q.Y., R.R.K., E.T.M.; Critical Review: M.Q.Y., R.R.K., E.T.M.; Other: -

CONFLICT OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

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ÖZGÜN MAKALE / ORIGINAL ARTICLE

Düzeltme Yazısı / Erratum

2024 yılı 48. Cilt 1. Sayıda 21 numarada yayınlanan; "Piyasadan Temin Edilen Nioli Uçucu Yağı Örneklerinin Farmakope Analizi" başlıklı makale ile ilgili yapılan düzeltmeler aşağıdaki gibidir.

Düzeltme Açıklaması: 2024 yılı 48. Cilt 1. Sayıda yayımlanan; "Ergene, B., & Öztürk, G. (2024). Piyasadan Temin Edilen Nioli Uçucu Yağı Örneklerinin Farmakope Analizi. Journal of Faculty of Pharmacy of Ankara University, 48(1), 215-225" referanslı makalede *Melaleuca quinquenervia* veya *M. alternifolia* bitkisinin uçucu yağı olarak piyasada satılan 5 farklı markanın analizleri yapılmıştır. Makale başlığında ve tüm metinde yer alan "Nioli uçucu yağı" ifadesi bu uçucu yağın özellikle piyasa ve halk arasında tanındığı ismi olması nedeniyle kullanılmıştır. Ancak başlıkta ve girişten itibaren tüm metinde geçen "Nioli, Nioli Yağı ve Nioli Uçucu Yağı" ifadelerinin Türk Patent tarafından marka olarak tescillenmiş olması nedeniyle, söz konusu ifadenin Türk Farmakopesi'nde geçtiği haliyle "Nioauli Yağı" olarak düzeltilmesi uygun görülmüştür."

Yayım Koşulları

1. Ankara Üniversitesi Eczacılık Fakültesi Dergisi (Ankara Ecz. Fak. Derg. – J. Fac. Pharm. Ankara), açık erişimli, hakemli bir dergi olup yılda üç kez (Ocak-Mayıs-Eylül) yayımlanır.

2. Dergiye Eczacılığın her alanında daha önce hiç bir yerde yayınlanmamış, Türkçe veya İngilizce olarak hazırlanmış makaleler kabul edilir. Deneylerde, insan için "the Declaration of Helsinki" ve hayvan için "European Community Guidlines"'a bağlı kalınmalıdır. Etik Kurul Onayının zorunlu olduğu çalışmalarda, etik kurul onayı alınan kurumun adı ve etik kurul onay numarası, gereç ve yöntem bölümünde ve Etik Kurul Onay bölümünde belirtilmeli ve ilgili belge makale gönderim sırasında yüklenmelidir.

3. Yayın Komisyonuna gelen makaleler en az 2 danışmana gönderilir. Ankara Üniversitesi Eczacılık Fakültesi Dergisi'nin makale değerlendirme süreci çift taraflı kör hakemlik ilkesiyle yürütülür.

4. Makaleler yayına kabul ediliş sırasına göre yayınlanır.

5. Danışmanlar tarafından önerilen düzeltmelerin yapılması için yazar/ yazarlara geri gönderilen makaleler, düzeltilip yayınlanmak üzere 3 ay içinde tekrar yayın kuruluna gönderilmezse, yeni başvuru olarak işlem görür. Makale yayımlanmadan önce yazarların yayımcıya makalenin "Copyright Transfer Form'unu doldurarak telif hakkını göndermesi gerekmektedir.

6. Yayımlarda intihal olup olmadığı kontrol edilmelidir. Ankara Üniversitesi Eczacılık Fakültesi Dergisi'ne yayımlanmak üzere gönderilen makaleler intihal tarama programları (iThenticate) ile taranmalı ve çevrim içi makale gönderim sırasında makalelerin intihal içermediğine dair rapor yüklenmelidir.

7. Ankara Üniversitesi Eczacılık Fakültesi Dergisi'nin makale yayın ücreti (APC) veya abonelik ücreti yoktur.

8. Ankara Üniversitesi Eczacılık Fakültesi Dergisi'ne aşağıdaki makale türleri kabul edilir:

    a) **Özgün makaleler**: Türkçe veya İngilizce hazırlanmış, şekiller ve tablolar dahil tamamı en çok 25 A4 kağıdı sayfası olan, orjinal araştırmaların bulgu ve sonuçlarını açıklayan makalelerdir. Araştırma makalelerinin yenilikçi ve bilime katkı sağlayan çalışmalar olması beklenir. Makaleler, yazım kurallarında belirtilen ana başlıkları taşımalı ve Windows uyumlu bir program kullanılarak hazırlanmalıdır.
    b) **Derleme makaleler:** Türkçe veya İngilizce hazırlanmış, şekil ve tablolar dahil tamamı en çok 30 A4 kağıdı sayfası olan, yeterli sayıda bilimsel makale taranarak, o güne kadarki gelişmeleri özetleyerek ortaya koyan ve sonuçlarını yorumlayarak değerlendiren makalelerdir. Makaleler, yazım kurallarında belirtilen ana başlıkları taşımalı ve Windows uyumlu bir program kullanılarak hazırlanmalıdır.
    c) **Kısa bildiriler:** Devam etmekte olan bir çalışmanın bulgularını zaman kaybetmeden duyurmak için Türkçe veya İngilizce yazılan en çok 5 A4 kağıdı sayfası olan makalelerdir. Makaleler, yazım kurallarında belirtilen ana başlıkları taşımalı ve Windows uyumlu bir program kullanılarak hazırlanmalıdır.

Yazım Kuralları

1. Metinler, A4 normunda (21 x 29.7 cm) yazılmış olmalıdır.
2. Metinler A4 normundaki sayfanın sağ ve sol tarafından 2.5 cm., üst ve alt kenarlarından 3 cm. boşluk bırakılarak 1 satır aralıkla yazılmalıdır. Yayımı kabul edilen makaleler doğrudan "Microsoft Word" dosyası halinde çevrim içi olarak sisteme yüklenecektir (online submission). Ana metin yazı karakteri "**Times New Roman**" ve **11 punto** olmalıdır.
3. Sayfa numaraları makalede **belirtilmemelidir**.
4. Paragraf başları **1 cm içeriden** başlamalıdır. Paragraflar arası ilave boşluk bırakılmamalıdır.
5. Başlık sayfasında yayın adı, yazar/yazarların adları, ORCID noları ve yazışma yapılacak yazarın açık adresi, telefon ve e-mail adresi belirtilmeli ve ortalı yazılmalıdır. İlk sayfada başlıktan önce yukarıdan 3 satır aralığı bırakılmalıdır. Başlık ile Öz/Abstract arası 1 satır aralıkla yazılmalıdır. Sorumlu yazarın soyadının üstüne (*) işareti konularak belirtilmelidir. Bu kişinin Adı Soyadı, açık adresi, telefon numarası ve e-mail adresi başlık sayfasının en altında belirtilmelidir.
6. **Yazar Adı** (ilk harfi büyük diğerleri **küçük harf**) ve **SOYADI** (tamamı **büyük harf**) **koyu** olarak başlığın altına bir satır aralık verildikten sonra altına unvan belirtmeden yazılmalıdır. Birden çok yazar varsa virgülle ayrılıp bir boşluk bırakılarak yazılmalıdır. Yazarların soyadları üzerine konulacak rakamlarla hemen isimlerin altındaki satıra kurum adları ve posta adresleri (Örneğin: Ankara Üniversitesi Eczacılık Fakültesi, Farmasötik Kimya Anabilim Dalı, 06560, Ankara, Türkiye) açıkça yazılmalıdır.
    - **Tüm yazarlar için ORCID numarası** mutlaka beyan edilmelidir. Yazarların ORCID ID'leri ilgili logoya köprü oluşturularak URL linklerinin eklenmesiyle gerçekleştirilmelidir.
7. Uluslararası kısaltmalar kullanılabilir. Metin içinde mililitre için ml; dakika için dak. olarak belirtilen şekliyle yazılmalıdır.
8. Birimler metrik sistemi kullanılarak ifade edilmelidir.
9. Bütün tablo ve şekiller metin içindeki yerlerine yazım alanından taşmadan yerleştirilmiş olmalıdır.
10. Tablolar üstlerine, şekiller (formül, grafik, şema, spektrum, kromatogram, fotoğraf vb.) de altlarına arabik rakamlarla (**Şekil 1**., **Tablo 2**.,) numaralandırılmalı ve metin içinde yer verilmelidir. "Tablo", "Şekil" sözcükleri ile bunlara ait numaralar koyu yazılmalı ve 11 punto olmalıdır. Şekil/Resim (**JPEG formatında**) makale içinde yerleşmiş ve **resimler 300 dpi veya daha yüksek çözünürlükte** olmalıdır. Üzerinde oynanmış (parlaklık, kontrast, gama ayarı vb.) şekillerde şekil altı metninde yapılan ayarlar belirtilmelidir. **Yazarlar, önceki makalelerinden alıntılanmış olsalar bile, diğer kaynaklardan herhangi bir görüntüyü çoğaltmak için ilgili yayıncılardan yazılı izin almalıdır**.
11. **Tablo** başlıkları Tabloların üstüne ve iki yana yaslı ve bunların genişliğini aşmayacak şekilde 11 punto ve bir satır aralıkta yazılmalıdır. Tabloya ait açıklama varsa tablonun altına 9 punto ile yazılmalıdır. Tablo içindeki metin 8-11 punto arasında yazılabilir. **Şekil** başlıkları ise şekillerin altına birer satır aralıkla ortalı ve 11 punto yazılmalıdır. Şekil başlığı ve şekil arasında 6 nk aralık olmalıdır. Tablo ve Şekiller metin içine yerleştirilirken metin ile aralarında 18 nk aralık olmalıdır.

    Örnek tablolar için bakınız.
    - Tüm satır ve sütun çizgileri yer almalı.
    - Tablo tasarımı tüm makalede tek tip ve düz olmalı, herhangi bir renklendirme/gölgelendirme kullanılmamalıdır.
    - Tablo içinde yer alan başlıklar **bold/koyu** renkte yazılmalıdır. Tablo başlığı ve tablo arasında 6 nk aralık olmalıdır.

Tablo 1. Türlere ait morfolojik özellikler

Bitki kısmı*	***C. nummularia***	***C. integerrimus***
Yaprak	Genişçe eliptik-orbikular, 0.9-2.5-(4) x 0.5-2.5-(3-5) cm	Orbikulardan ovata kadar farklı şekillerde, 1.2-(4-5) x 0.9-3 cm
Tohum	3.5-4 x 1-2 mm, koyu kahverengi	3-4 x 1.5-2 mm, açık kahverengi

*Açıklama: 9 punto, 1 aralık olmalı.

Tablo 2. Hastaların özellikleri

Demografik bilgiler	**A grubu***	**B grubu**	**C grubu**
Erkek cinsiyet	10 (%30)	20 (%60)	10 (% 30)
Sigara kullanımı	20 (%60)	10 (%30)	20 (%60)

*Açıklama: 9 punto yazılmalıdır.

Örnek şekil;



Şekil 1. *C. nummularia*'nın genel görünüşü (Yazı karakteri "Times New Roman" ve 11 punto, "1" aralık, ortalı)

12. Makalelerin bölümleri **BAŞLIK** (Türkçe ve İngilizce), **ÖZ**, **ABSTRACT**, **GİRİŞ, GEREÇ VE YÖNTEM, SONUÇ VE TARTIŞMA, TEŞEKKÜR** (varsa eklenmeli), **YAZAR KATKILARI**, **ÇIKAR ÇATIŞMASI**, **ETİK KURUL ONAYI** (varsa eklenmeli) ve **KAYNAKLAR** sırasına uygun olarak hazırlanmalıdır. Bu bölümleri ifade eden başlıklar (Makalenin ilk başlığı hariç) **12 punto ile koyu olarak büyük harflerle ve sayfanın solundan başlanarak** yazılmalıdır. **GİRİŞ**'ten önce ve sonra sırasıyla 18 nk ve 6 nk aralık bırakılmalıdır. Diğer ana başlıklardan önce ve sonra sırasıyla 12 nk ve 6 nk aralık olmalıdır. Bölüm başlıkları ile metin arasında belirtilenin dışında ayrıca aralık **bırakılmamalıdır.**

- **BAŞLIK:** Türkçe ve İngilizce olarak büyük harf ve **ilk başlık** (Türkçe makalelerde Türkçe başlık, İngilizce makalelerde İngilizce başlık ilk başlıktır) **14 punto, koyu** ve ikinci başlık 12 punto, *italik* olarak yazılmalıdır. Başlık metine uygun, kısa, çalışmayı tanıtıcı ve açık ifadeli olmalıdır.

- **ÖZ** ve **ABSTRACT:** Türkçe (**ÖZ**) ve İngilizce (**ABSTRACT**) olarak makalelerin başında **200**'er kelimeyi geçmeyecek şekilde 10 punto ile *italik* olarak yazılmalıdır. Yabancı dilde yazılmış makalelerde önce **ABSTRACT** daha sonra mutlaka Türkçe olarak **ÖZ** bulunmalıdır. **ÖZ** ve **ABSTRACT** başlıkları 12 punto ve koyu yazılıp kendi içlerinde alt başlıklar (aşağıda görüldüğü gibi) halinde makalenin özeti sunulmalıdır. Her bir alt başlık 10 punto, koyu, normal yazılmalıdır. Alt başlıkların içeriğindeki metinler *italik* yazılmalıdır. **ÖZ ve ABSTRACT metni blok halinde sağdan ve soldan 1 cm boşluk bırakılarak yazılmalıdır.**

Özgün makalelerde;

ÖZ için kullanılacak alt başlıklar:
Amaç: *Metin italik yazılmalıdır.*
Gereç ve Yöntem: *Metin italik yazılmalıdır.*
Sonuç ve Tartışma: *Metin italik yazılmalıdır.*
Anahtar Kelimeler: *Metin italik yazılmalıdır, alfabetik sıralama gözetilmelidir*

ABSTRACT için kullanılacak alt başlıklar:
Objective: *Metin italik yazılmalıdır.*
Material and Method: *Metin italik yazılmalıdır.*
Result and Discussion: *Metin italik yazılmalıdır.*
Keywords: *Metin italik yazılmalıdır, alfabetik sıralama gözetilmelidir*

Derleme makalelerde;

ÖZ için kullanılacak alt başlıklar:
Amaç: *Metin italik yazılmalıdır.*
Sonuç ve Tartışma: *Metin italik yazılmalıdır.*
Anahtar Kelimeler: *Metin italik yazılmalıdır, alfabetik sıralama gözetilmelidir*

ABSTRACT için kullanılacak alt başlıklar:
Objective: *Metin italik yazılmalıdır.*
Result and Discussion: *Metin italik yazılmalıdır.*
Keywords: *Metin italik yazılmalıdır, alfabetik sıralama gözetilmelidir*

- **Anahtar Kelimeler (Keywords):** En az 3 sözcükten oluşmalı, ilgili dilde alfabetik, *italik* olarak, yalnızca ilk anahtar sözcüğün ilk harfi büyük olacak şekilde (büyük harf kullanılarak yapılan kısaltmalar hariç) aralara virgül konularak yazılmalı son anahtar sözcükten sonra ise bir imla işareti **kullanılmamalıdır.**

- **METİN:** Orijinal Türkçe makalede metin kısmı **GİRİŞ, GEREÇ VE YÖNTEM, SONUÇ VE TARTIŞMA** olmak üzere 3 ana başlıktan oluşmalıdır. Bu ana başlıkların tamamı 12 punto, **büyük harflerle** ve koyu olacak şekilde yazılmalıdır. Derleme makalelerde ise **GİRİŞ** ile **SONUÇ VE TARTIŞMA ana başlıkları olmalı,** diğer başlıklar yazarın belirleyeceği şekilde **her kelimenin ilk harfi büyük diğerleri küçük ve koyu** olacak şekilde yazılmalıdır. Alt başlıklar 11 punto, 1satır aralık, **bold/koyu** yazılmalı ve sola dayalı olmalıdır Alt başlıklarda numaralandırma sistemi **kullanılmamalıdır**. Alt başlıklardan önce ve sonra 6 nk aralık olmalıdır.

- **GİRİŞ:** Araştırmanın amacı ve konuyla ilgili çalışmaların yer aldığı bölüm olmalıdır.

- **GEREÇ VE YÖNTEM**: Kullanılan gereç belirtilerek, uygulanan yöntem hakkında gerekli bilgiler açıkça ifade edilmelidir. **Bileşiklerin karakterizasyonu** ayrı bir paragraf ile gösterilmeli ve yeni bileşiklerin saflıkları ve yapı aydınlatılmaları sağlanmalıdır. Eğer çalışmada hayvan ya da insan örnekleri/gönüllüler kullanılıyorsa, araştırıcılar tüm işlemlerin ilgili kanun ve kurumsal kılavuzlara uygun şekilde gerçekleştirildiğine ve uygun idari kurul tarafından bu işlemlerin onaylandığına ve Etik Kurul onayı alındığına dair ifadenin çalışma içinde yer almasını sağlamalıdırlar. Etik Kurul onayının zorunlu olduğu çalışmalarda, etik kurul onayı alınan kurumun adı ve etik kurul onay numarası, gereç ve yöntem kısmında belirtilmelidir. Ayrıca, kullanılan protokol ve prosedürlerin etik olarak gözden geçirildiği ve onaylandığı, makalenin gereç ve yöntem bölümüne eklenmelidir. Detaylı bilgi için lütfen http://journal.pharmacy.ankara.edu.tr/en/ethical-principles-and-publication-policy/ web sayfasını ziyaret ediniz.

- **SONUÇ VE TARTIŞMA:** Bulguların verilerek değerlendirildiği bölümdür.
  - Dileyen yazar, RESULT AND DISCUSSION bölümünün son paragrafı olarak "Conclusion" başlığı oluşturabilir. Ancak 11 punto Times New Roman karakterinde İlk harfi büyük diğer harfleri küçük olmalıdır.

- **TEŞEKKÜR:** Varsa araştırmayı destekleyen kuruluşa ve katkısı olan kişilere Yazarların Katkısından önce yer alan bu bölümde kısaca teşekkür edilebilir.

- **YAZAR KATKILARI:** Makalede yer alan yazarların katkısı yazarlar tarafından imzalanan Telif Hakkı Devir Sözleşmesi (*Copyright Transfer Agreement*) uyarınca, çıkar çatışması bildiriminden hemen önce, makalede yer alan isim sırası gözetilerek yazılmalıdır. Lütfen bu bildirim için açık ad ve soyad yerine aşağıdaki örnekte olduğu gibi yazarların baş harflerini kullanınız. Yazar katkısı belirtilmeyecek alanlar için "-" işareti konulmalıdır.

  Örnek:

  YAZAR KATKILARI

  Kavram: İ.Y., M.M.H., C.H., K.B.; Tasarım: İ.Y., C.H., I.Ö.G., Ö.Ü.; Denetim: C.H., I.Ö.G., M.M.H., K.B.; Kaynaklar: Ö.Ü., Z.K., K.B., M.M.H., A.K., İ.A., G.A.G., B.G., B.K.; Malzemeler: I.Ö.G., B.E., G.A.G., B.K., D.Ç.P.; Veri Toplama ve/veya İşleme: A.K., Ö.Ü., M.K., A.S., D.Ç.P., T.C.Ş.T.; Analiz ve/veya Yorumlama: Ö.Ü., B.G., T.C.Ş.T., E.K.S.; Literatür Taraması: B.K., D.Ç.P, B.G., B.E.; Makalenin Yazılması: A.K., İ.A., T.C.Ş.T.; Kritik İnceleme: İ.Y., B.G., Ö.Ü., İ.A.; Diğer: -

- **ÇIKAR ÇATIŞMASI BEYANI**

  Çıkar çatışması varsa ne şekilde olduğu açıkça beyan edilmelidir. Eğer yok ise "Yazarlar bu makale için gerçek, potansiyel veya algılanan çıkar çatışması olmadığını beyan ederler." ifadesini kullanmalıdırlar.

- **ETİK KURUL ONAYI**

  Çalışmanın sonunda kaynaklardan önce etik kurul onayı alınmışsa hangi kurumdan ve ne zaman alındığı onay numarası ile mutlaka belirtilmeli ve Etik Kurul Onayını makale gönderim sırasında yüklemelidir. Etik kurul onayına gerek olmayan çalışmalarda aşağıdaki cümle yazılmalıdır.

  "Yazarlar bu çalışma için etik kurul onayının zorunlu olmadığını beyan etmektedir."

- **KAYNAKLAR:** Kaynak yazım stili Amerikan Psikoloji Derneği'ne (APA) göredir. Yazı karakteri "Times New Roman" ve 10 punto, "1" aralık, iki yana yaslı. Metinde, geçiş sırasına göre köşeli parantez içinde, örneğin: [1,6,9], [5-7] gibi numaralandırılmalı ve metin sonunda bu numaralara göre sıralanmalıdır. Alt başlıkların yanına kaynak belirtilmemelidir. Tablo içinde kaynak bildirilmesi gerekiyorsa metin içinde verildiği gibi belirtilmelidir.

  - **Makale için:** Yazarın soyadı, adının baş harfleri (Birden fazla adı olan yazarın her bir isminin baş harfinden sonra nokta konmalı ve arada boşluk bırakılmamalıdır. Birden fazla yazarların arasında virgül yer almalıdır. **Son yazar ile bir önceki yazar arasında "ve" kelimesi veya "&" sembolü <u>kullanılmamalıdır</u>**.), makalenin tam başlığı, derginin adı, cilt no, varsa sayı no (parantez içinde), başlangıç ve bitiş sayfa numarası (veya makale numarası), yıl yazar isimlerinden sonra (parantez içinde) yazılmalıdır. **Birden fazla yazar varsa hepsi yazılmalıdır.** Makalenin adı yazılırken ilk kelimenin ilk harfi büyük diğer kelimelerin ilk harfi küçük yazılmalıdır. Kaynaklarda verilen **dergi adları kısaltma yapılmadan açık olarak yazılmalıdır.**

Her bir referansın sonuna [CrossRef] **ekleyerek aşağıdaki formatta DOI numarasını köprü olarak giriniz. Lütfen https://www.crossref.org/'da yer almayan makaleleri** [CrossRef] şeklinde **belirtmeyiniz.**
https://doi.org/10.1016/0006-2952(89)90403-6

Örnekler:

1. Martinez, M.J.A., Del Olmo, L.M.B., Benito, P.B. (2005). Antiviral activities of polysaccharides from natural sources. Studies in Natural Products Chemistry, 30, 393-418. [CrossRef]
2. Bahiense, J.B., Marques, F.M., Figueira, M.M., Vargasa, T.S., Kondratyuk, T.P., Endringer, D.C., Scherer, R., Fronzaa, M. (2017). Potential anti-inflammatory, antioxidant and antimicrobial activities of *Sambucus australis*. Pharmaceutical Biology, 55(1), 991-997. [CrossRef]

- **Elektronik Makale için:**

Örnek:
Perneger, T.V., Giner, F. (1998). Randomized trial of heroin maintenance programme for adults who fail in convential drug treatments. British Medical Journal, 317, from http://www.bmj.com/cgi/content/full/317/7150/ Erişim tarihi: 14.03.2021

- **Web sitesi için:**

Örnek:
Clinical Pharmacology Web site. (2001). Erişim adresi http://cpip.gsm.com/ Erişim tarihi: 14.03.2021.

- **Kitap için:** Yazarın soyadı, adının baş harfleri, kitabın adı, cilt no (varsa), kitabevi, yayınlandığı şehir, sayfa no, basıldığı yıl (parantez içinde) yazılmalıdır.

Örnek:
Franke, R. (1984). Theoretical Drug Design Methods, Elsevier, Amsterdam, p.130.

- **Kitap bölümü için:** Yazarın soyadı, adının baş harfleri, bölümün başlığı, editör/editörlerin soyadı, adının baş harfleri, (Ed./Eds.) ibaresi, kitabın adı, varsa cilt no, kitabevi, yayınlandığı şehir, sayfa no, basıldığı yıl (parantez içinde) yazılmalıdır.

Örnek:
Weinberg, E.D. (1979). Antifungal Agents. In: M.E. Wolff and S.E. Smith (Eds.), Burger's Medicinal Chemistry, (pp. 531-537). New York: John Wiley and Sons.

- **Tez için:** Yazarın soyadı, adının baş harfleri, yıl yazar isimlerinden sonra (parantez içinde) yazılıp nokta işareti konmalıdır. Ne tür tez olduğu belirtildikten sonra tezin başlığı, nerde yapıldığı yazılmalıdır.

Örnek:
Ahmed, J. (2008). PhD Thesis. Pharmaceutical Botany investigations on *Prangos* Lindl. (Umbelliferae) growing in Konya province. Department of Pharmaceutical Botany, Faculty of Pharmacy, Ankara University, Ankara, Turkey.

- **Patent için:** Yazarın soyadı, adının baş harfleri, yıl yazar isimlerinden sonra (parantez içinde) yazılıp nokta işareti konmalıdır. Patent başlığı ve patent numarası yazılmalıdır.

Örnek:
Mahoney, S., Molz, L., Narayan, S., Saiah, E. (2018). Heteroaryl RHEB Inhibitors and Uses Thereof. WO 2018/191146 A1.

ETİK İLKELER VE YAYIN POLİTİKASI

Ankara Üniversitesi Eczacılık Fakültesi Dergisi, açık erişimli, hakemli bir dergi olup Türkçe veya İngilizce olarak farmasötik bilimler alanındaki önemli gelişmeleri içeren orijinal araştırmalar, derlemeler ve kısa bildiriler için bir yayım ortamıdır. Ankara Üniversitesi Eczacılık Fakültesi Dergisi'nin makale yayın ücreti (APC) veya abonelik ücreti yoktur.

Yayın kurulu olarak dergi kapsamında önemli katkı sağlayan kaliteli yeni çalışmaların yayınlanması amaçlanmaktadır. Bu amaca ulaşmak için gönderilen makaleler, dergide yayınlanmak için bilimsel ve biçimsel gerekli kriterleri karşıladıklarından emin olmak adına baş editör ve/veya editör yardımcıları tarafından ilk değerlendirmeye tabi tutulur. Yalnızca bu ön değerlendirme sürecini geçen çalışmalar, daha ileri değerlendirme için diğer aşamalara devam ettirilir.

Ön Değerlendirme

- Çalışmanın bilimsel kalitesi ve yeniliği dergide yayınlanmak için yeterli olmalıdır.
- Dergiye gönderilen çalışmalar derginin amaç ve kapsamına uygun olmalıdır.
- Metin İngilizce veya Türkçe olarak dilbilgisi kurallarına uygun ve bilimsel olarak iyi yazılmış olmalıdır.
- Dergiye gönderilen çalışmaların benzerlik oranı %20'i geçmemelidir.
- Çalışmalar derginin yazım kurallarına ve şablonuna uygun olacak şekilde düzenlenmelidir.
- Telif hakkı devir formu, etik kurul onay belgesi, yazar katkı formu mutlaka yüklenmeli ve imzalı olmalıdır.
- Çalışmalar elektronik online başvuru sistemi aracılığı ile dergiye gönderilmiş olmalıdır.

Bu yeterlikleri taşımayan çalışmaların ileri değerlendirme süreci başlatılamaz.

Dergi yayınlanma sürecinde dergi editörleri, hakemler ve yazarlara bazı sorumluluklar düşmektedir. Bu sorumluluklar aşağıdaki şekilde açıklanmıştır.

1. Editörün Görevleri ve Etik Sorumlulukları

Editör, dergiye gönderilen makalelerden hangilerinin yayınlanması gerektiğine bağımsız olarak tek başına karar verebileceği gibi editör kurulunun üyelerine veya hakemlere de danışabilir. Derginin etik ilkeleri ve yayın politikası çerçevesinde, çalışmaların ön değerlendirme, hakem değerlendirmesi ve yayınlanma aşamalarının tarafsız, denetlenebilir, adil, çıkar ilişkisinden bağımsız ve gizlilik ilkelerine uygun şekilde yürütülmesinden sorumludur. Yayın politikası ve etik ilkeleri açısından ihlal yoksa derginin amacına ve kapsamına uygun çalışmaları, ön değerlendirme aşamasına almalıdır.

Baş editörün, editör yardımcılarının, alan editörlerinin ve editoryal danışma kurulunun görevleri ve tanımları aşağıdaki gibidir:

Baş Editör: Dergi içeriğinin yayınlanması konusunda tam yetkiye sahip kişidir. Editör yardımcıları, alan editörleri ve editöryal danışma kurulu ile birlikte çalışır.
Editör Yardımcıları: Dergi ilgili sorulara cevap vermek, dergi hakem ve kuruluna önerilerde bulunmak, makale yayın sürecinde baş editöre yardımcı olan kişilerdir.
Alan Editörleri: Çift kör hakem atamalarının gerçekleşmesi ve dergi ile ilgili sorulara cevap vermek konusunda yazarlara yardımcı olan kişilerdir.
Editoryal Danışma Kurulu: Editoryal Danışma Kurulu, Ankara Üniversitesi Eczacılık Fakültesi Dergisinin, amacına uygun ve kaliteli yayın üretilmesine ilişkin konularda Baş Editör ve Editör Yardımcılarına kılavuzluk eder.

1.1. Yayın Politikası

- Baş editör, dergiye gönderilen makalelerden hangilerinin yayımlanması gerektiği kararından tek başına sorumludur. Editörün kararı, derginin editör kurulunun prensipleri doğrultusunda olabileceği gibi, onur kırıcı yayım yapmak, telif hakkı ihlali ve intihal gibi konularla ilgili olarak yürürlükte olan yasal gereklilikler ile sınırlandırılmıştır.
- Baş editör, makale yayımlanmadan önce yazarların yayımcıya makalenin “Copyright Transfer Form” unu, doldurarak telif hakkını gönderdiğinden emin olmaktadır.
- Baş editör, yazarların makale yayımlanmadan önce "Conflict of Interest Form”unu ve “Author Contribution Form” unu doldurduğundan emin olmaktadır.
- Baş Editör, dergiye gönderilen makalelerin biçimsel olarak incelenmesi için editör yardımcılarını görevlendirmektedir. Ankara Üniversitesi Eczacılık Fakültesi Dergisinin kurallarını sağlamayan makaleler kesinlikle değerlendirmeye alınmadan reddedilmektedir.

1.2. Yayın Değerlendirmesi

- Baş editör, yayın değerlendirme sürecinin adil, tarafsız ve zamanına uygun şekilde gerçekleşmesini sağlamaktan sorumludur.
- Editör, tüm makaleleri genel olarak dışardan ve bağımsız en az iki hakem ile değerlendirilmesini sağlamaktadır. Gerek olması durumunda editör üçüncü bir hakemden ek görüş istemektedir.
- Editör, hakem seçimini makale kapsamına uygun olan uzmanları değerlendirerek yapar.
- Editör, olası çıkar çatışmaları için yapılan açıklamaları, hakemler tarafından yapılan “self-citation” önerilerini ve herhangi bir taraflılık olasılığını değerlendirmek ve karar vermek için dikkatli bir şekilde yayın sürecini gözden geçirmektedir.
- Baş editör/editörler, hakem değerlendirmesi veya değerlendirme/yayım sürecinin herhangi bir noktasında bir benzerlik tespit yazılımı (iThenticate) tarafından taratılmasını yazardan istemektedir veya kendisi yapmaktadır. Bu anlamda ifadelerin veya cümlelerin yazarın/yazarların kendileri olsa dahi metin daha önce yayınlanmış verilerle kabul edilemez bir benzerliğe sahip olmamalıdır.
- Baş editör, bir makaledeki hataları yayımlanmadan önce tespit ederse düzeltmektedir. Eğer daha sonra tespit ederse bu durumda düzeltmeleri yayımlamak zorundadır. Tüm düzeltme veya geri çekme bildirimlerini dergide belirgin bir şekilde yayımlamalıdır. Ayrıca içindekiler sayfasında listelemelidir.
- Ankara Üniversitesi Eczacılık Fakültesi Dergisinin editörleri, Yayın Etiği Komitesi (Committee on Publication Ethics (COPE)) tarafından yayınlanan "COPE Code of Conduct and Best Practice Guidelines for Journal Editors" ve "COPE Best Practice Guidelines for Journal Editors" kılavuzlarına uyarak çalışmalarını sürdürür.

1.3. Adil Değerlendirme

- Baş editör/editörler, makaleleri yazarların ırk, cinsiyet, cinsel eğilim, inanç, etnik köken, vatandaşlık ya da politik görüşlerine bakmaksızın bilimsel içeriklerine göre değerlendirmektedir. Derginin editoryal prensipleri şeffaf ve tümüyle dürüst değerlendirmeyi desteklemektedir.
- Editör, hakemlerin ve yazarların kendilerinden bekleneni tam olarak anladıklarından emin olmalıdır.
- Editör, dergi ile ilgili tüm iletişimini derginin elektronik başvuru sisteminden yapar ve kararlarında itirazlar olması halinde şeffaf ve hakkaniyetli bir yol izler.

1.4. Gizlilik İlkesi

- Baş editör/editör, dergiye yapılan başvurudaki tüm materyallerin ve hakemlerle yapılan tüm iletişimin gizliliğini (ilgili yazar ve hakemlerle aksi onaylanmadığı sürece) korumakla yükümlüdür.

- Baş editör/editör, hakemlerin isimlerinin açıklanmasını kabul etmediği sürece, hakemlerin kimliklerini ve haklarını korumakla sorumludur.
- Başvurusu tamamlanmış bir makaleye ait basılmamış materyaller, yazarın yazılı onayı alınmadan editörün kendi çalışmaları/araştırmaları için kullanılmamalıdır.
- Baş editör/editör, makale değerlendirme sürecinde edinilen tüm bilgileri veya fikirleri gizli tutmalı ve kişisel amaçlar için kullanmamalıdır.

2. Hakemlerin Görevleri ve Etik Sorumlulukları

Ankara Üniversitesi Eczacılık Fakültesi Dergisi'nin makale değerlendirme süreci çift taraflı kör hakemlik ilkesiyle yürütülmektedir. Dolayısıyla hakemler yazar/yazarlarla iletişim kuramazlar, değerlendirmeler dergipark yönetim sistemi üzerinden paylaşılır. Değerlendirme sürecinde tam metinlere ilişkin değerlendirme formları hakem yorumları editör aracılığı ile sorumlu yazara iletilir. Hakemler, değerlendirme süreci boyunca tarafsızlık, gizlilik, nesnellik, bilimsel yönden inceleme ilkelerine uygun hareket etmelidir. İlgili alanda uzman ve yetkinliğe sahip olmalıdır. Değerlendirmesine sunulan çalışmaya ilişkin raporunu belirtilen zaman aralığı içinde bitirmelidir. Zamanında sunulamayacak raporlar için gecikmeden editör ile iletişime geçilmelidir. Etik ilkeleri, telif hakkı ihlali, olası çıkar çatışması ve intihal yapıldığının fark edilmesi durumlarında editör kurulunu bilgilendirmelidir.

Ankara Üniversitesi Eczacılık Fakültesi Dergisi için makaleleri değerlendiren hakemlerin aşağıda belirtilen görevlere ve etik sorumluluklara uyması beklenmektedir.

2.1. Editöryal Kararlara Katkı

- Hakemler, yazarların sundukları çalışmaları yapıcı ve uygun şekilde değerlendirmelidirler.
- Hakemler, makalede yer alan araştırmayı değerlendirmeye yetkin olmadığını düşünüyorsa veya yeterli sürede tamamlayamayacaksa editöre durumu bildirmelidirler.
- Hakemler, yazarlara yönelik sert ve kişisel eleştirilerde bulunmamalıdırlar.
- Hakemler, makale değerlendirmesi için davet aldığında eğer kendilerini makalede çalışılan konu hakkında yetersiz hissederlerse makaleyi değerlendirmeyi reddetmelidirler.
- Hakemler, makale değerlendirmesini verilen süre içinde yapmalıdırlar.
- Hakemler, sadece çalışmanın içeriğine ilişkin değerlendirmeyi objektif olarak yapmalıdırlar.

2.2. Gizlilik

- Hakemler, değerlendirmeyi tarafsızlık ve gizlilik içerisinde yapmalıdırlar.
- Hakemler, makale hakkındaki değerlendirmelerini ya da bilgilerini üçüncü kişilerle paylaşmamalıdırlar.
- Hakemler, makale değerlendirme sürecinde edinilen bilgileri, fikirleri ve basılmamış materyal veya çalışmaları gizli tutmalı ve kişisel amaçlar için kullanmamalıdırlar.
- Hakemler, makalenin bir kopyasını elinde bulundurmamalı veya çoğaltmamalıdırlar.

2.3. Etik Sorunları Fark Etme

- Hakemler, makalede yer alan etik sorunları fark etmeli ve editörün dikkatine sunmalıdırlar.
- Hakemler, makalenin daha önce başka bir yerde basıldığını veya basılmış önceki bir makale ile önemli ölçüde benzerlik ya da örtüşme tespit ederse editöre bildirmelidirler. Daha önce yayımlanmış olan herhangi bir gözlem ve/veya argüman, ilgili referans ile birlikte verilmelidir.

2.4. Tarafsızlık ve Rekabet Standartları

- Hakemler, tarafsız olarak değerlendirmelerini yapmalı ve önyargıdan uzak şekilde değerlendirmelidirler. Yazarın kişi olarak eleştirilmesi uygun değildir. Hakemler, görüşlerini destekleyici argümanlarla ifade etmelidirler.

- Hakemler, makale değerlendirmeyi kabul etmeden önce olası çıkar çatışmasını kontrol etmelidirler. Eğer çıkar çatışmasıyla karşı karşıya olduğunu düşünüyorsa makaleyi incelemeyi reddetmeli ve editörü bilgilendirmelidirler.
- Hakemler, yazar tarafından hakemin (ya da hakemle çalışan kişilerin) çalışmalarının kaynak olarak alındığını ileri sürerse, gerçek bilimsel gerekçeler sunmalılar, bu durumun hakemin kaynak gösterilme sayısını ya da çalışmalarının görünürlüğünü artırmaya yönelik bir girişim olmamasına özen göstermelidirler.
- Hakemler, değerlendirmelerini yaparken bilimsel gerçeklikten uzaklaşmamalı ve gerekirse kaynak gösterme yoluna başvurmalıdırlar.

3. Yazarların Görevleri ve Etik Sorumlulukları

Ankara Üniversitesi Eczacılık Fakültesi Dergisi'ne gönderilen makaleler, daha önce herhangi bir yayın organında yayımlanmamış olmalıdır veya yayımlanmak üzere aynı zaman diliminde başka bir yayın organına gönderilmiş olmamalıdır. Çalışmalarda yararlanılan araştırmaların ve yayınların, alıntılarının veya atıflarının bilimsel araştırma ilkelerine uygun olarak eksiksiz yapılması ve kaynakların belirtilmesi zorunludur. Çalışmada yer alan yazar sayısı birden fazla ise, yazarların çalışmaya bilimsel ve akademik olarak somut ve yeterli düzeyde katkı sağlaması beklenir. Çalışmaya ait tüm finansal destek kaynakları açıklamalıdır. Olası çıkar çatışması durumlarını yayın kuruluna bildirmelidir.

Ankara Üniversitesi Eczacılık Fakültesi Dergisi'ne makale gönderen yazar/yazarların aşağıda belirtilen görevlere ve etik sorumluluklara uymalıdır.

3.1. Bildirim Standartları

- Yazar(lar)ın gönderdiği makale (araştırma, derleme veya kısa bildiri) özgün olmalıdır.
- Yazar(lar), çalışmanın önemine ilişkin tarafsız bir tartışma ile gerçekleştirilen araştırmayı net bir şekilde sunmalıdır.
- Yazar(lar), makalede verileri açık bir şekilde sunmalıdır.
- Yazar(lar)ın başka çalışmalardan faydalanması halinde tam ve doğru bir şekilde alıntı yapmalıdır.
- Makale, diğer araştırmacıların çalışmayı tekrar edebilmesine olanak verecek şekilde yeterli detay ve kaynak içermelidir.
- Yazar(lar), etik dışı davranarak yanıltıcı ya da net olmayan ifadeleri makalelerinde kullanmamalıdır.
- Yazar(lar), dergi kurallarına uymadıkları ve belirtilen sürede aksiyon almadıkları sürece makalelerinin dergi tarafından yayımlanmayacağını bilerek hareket etmelidir.

3.2. Veri Ulaşımı ve Saklama

- Yazarlardan editöryal değerlendirme için makalelerini destekleyici araştırma verisi istenebilir.
- Yazarlar, değerlendirme sürecinde makalelerine ilişkin ham verilerin veya makalelerini destekleyecek verilerin talep edilmesi durumunda belirtilen verileri yayın kuruluna sunmaya hazır bulunmalıdırlar.

3.3. Orijinallik, İntihal ve Kaynakların Belirtilmesi

- İntihal, yazarın başka bir makaleyi kendi çalışması olarak göstermesi, kaynak göstermeden başka birine ait çalışmanın belli bölümlerinin kopyalanması ya da başka sözcüklerle anlatılması veya başkaları tarafından yapılan çalışmanın sonuçlarının alınarak sunulması şeklinde olabilir. İntihalin her biçimi etik olmayan davranıştır ve kesinlikle kabul edilmemektedir. Yazarlar intihalden uzak durmalıdır. İntihal tanımı için buraya bakınız.
- Yazarlar çalışmalarının tümüyle orijinal olduğunu garanti etmelidirler. Yazarlar, başkalarının fikirlerini veya metinlerini kullanıyorlarsa mutlaka uygun şekilde kaynak ya da alıntı

göstermeliler ve gerekliyse izin almalıdırlar.

- Yazarlar kendilerine ait olan çalışmayı etkileyen ve çalışmaya ait uygun içeriğin oluşturulmasında katkısı olan tüm yayınları veya eserleri kaynak olarak göstermelidirler. Özel olarak (görüşme, yazışma ya da üçüncü taraflar ile tartışma) ile elde edilen bilgiler kullanılmamalı ya da kullanılacaksa izin alınarak bildirilmelidir.
- Yazarlar, Ankara Üniversitesi Eczacılık Fakültesi Dergisi'ne yayımlanmak üzere gönderdikleri makalelerini intihal tarama programları (iThenticate) ile taramalı ve dergipark sisteminde çevrim içi makale gönderim sırasında makalelerinin intihal içermediğine dair raporu yüklemek zorundadırlar.

3.4. Çoklu, Gereksiz ve Tekrar Yayınlama

- Aynı makale ile birden fazla dergiye başvuruda bulunmak etik olmayan bir davranıştır ve asla kabul edilmemektedir. Genel olarak, yazar daha önce basılmış bir yayını, özet formunda ya da yayınlanmış bir ders, akademik tez ya da elektronik ön baskının bir parçası olması dışında, değerlendirme için başka bir dergiye göndermemelidir.
- Yazarlar başvuru sırasında makaleyi başka bir dergiye daha aynı anda göndermediklerini garanti etmelidirler.
- Yazarlar, gönderilen yazının değerlendirme aşamasında olmadığını veya başka bir yerde yayımlanmak üzere kabul edilmediğini ve eğer kabul edilirse, aynı biçimde, başka bir dilde, elektronik ortam da dahil olmak üzere, yazarın yazılı izni olmaksızın başka bir yerde yayımlanmayacağını garanti etmelidir.

3.5. Yazar Katkıları

- Yazar katkıları, çalışmanın konseptine, tasarımına, gerçekleştirilmesine ya da yorumlanmasına önemli katkı sağlayan kişiler ile sınırlandırılmalıdır.
- Yazarlar, çalışmaya katkı veren yazarların listesini dikkatli bir şekilde hazırlamalıdır. Bazı durumlar eşyazar (co-author) olmayı bazı durumlar ise çalışmanın "Teşekkür" (Acknowledgement) bölümünde yer almasını hak edebilir.
- Sorumlu yazar, tüm eşyazarların çalışmada uygun şekilde yer aldığına, tüm eşyazarların çalışmayı görüp onayladıklarına ve yayınlanmak üzere başvuru yapılmasına dair verdikleri onaya ilişkin sorumluluğu üstlenmelidir.
- Sorumlu yazar, makaledeki tüm yazarların yazar sıralaması, çalışmanın kesinliği ve bütünlüğü gibi konularda fikir birliğinin sağlanmasından sorumludur ve orijinal başvuru sırasında kesin bir yazar listesi sunmalıdır.
- Çalışmanın başvurusu tamamlandıktan sonra, sadece istisna durumlarda, editör yazar listesinde ekleme, silme ya da yeniden düzenleme yapabilir. Tüm yazarlar bu şekilde yapılacak ekleme, silme ve yeniden düzenleme konusunda fikir birliği içinde olmalıdırlar. Tüm yazarlar çalışmanın ortak sorumluluğunu aldıklarını kabul ederler. Her yazar, uygun şekilde araştırılan ve karara bağlanan çalışmanın kesinliği ve bütünlüğü ile ilişkili sorulardan sorumludur.
- Sorumlu yazar, editör ile iletişime geçen kişi olarak Ankara Üniversitesi Eczacılık Fakültesi Dergisi'ne makale ile birlikte "Yazar Katkı Formu"nun da doldurulup gönderilmesinden sorumludur.

3.6. Çıkar Çatışması Beyanı

- Yazarlar, çalışmalarını uygunsuz bir şekilde etkileyebilecek olarak gördükleri diğer kişi veya organizasyonlarla çıkar çatışması oluşturabilecek her türlü durum ve ilişkileri beyan etmelidirler.
- Sorumlu yazar, editör ile iletişime geçen kişi olarak Ankara Üniversitesi Eczacılık Fakültesi Dergisi'ne makale ile birlikte "Çıkar Çatışması Beyanı Formu"nun da doldurulup gönderilmesinden sorumludur.

- Yazarlar çıkar çatışmalarının olduğu durumları mutlaka açıklamalıdırlar.

3.7. Temel Hataların Bildirimi

- Yazarlar, yayımlanmış, erken görünüm veya değerlendirme sürecinde olan bir çalışmasında önemli bir hata ya da eksiklik fark ettiğinde, acil olarak dergi baş editörüne/yayınevine veya ilgili editöre bildirmek ve editör tarafından gerekli görülmesi durumunda makaleyi geri çekmek veya düzeltmek için editörle işbirliği yapmak ile yükümlüdür.
- Editör/yayınevi yayımlanmış olan makalenin bir hata içerdiğini üçüncü bir taraftan öğrenirse, editör ile işbirliği yapmak ve gerektiğinde destekleyici kanıt sağlamak yazarın yükümlülüğüdür.

3.8. Olası Riskler ve İnsan veya Hayvan Konuları

- Yazarlar, kullanımları sırasında olağan dışı risk yaratan kimyasallar, işlemler ya da malzemeler ile çalışmışlarsa açıkça belirtmelidirler.
- Eğer çalışmada hayvan ya da insan örnekleri/gönüllüler kullanılıyorsa, araştırmacılar tüm işlemlerin ilgili kanun ve kurumsal kılavuzlara uygun şekilde gerçekleştirildiğine ve uygun idari kurul tarafından bu işlemlerin onaylandığına ve Etik Kurul Onayı alındığına dair ifadenin makale içinde yer alması sağlamalıdırlar.
- Yazarlar, Etik Kurul Onayının zorunlu olduğu çalışmalarda, etik kurul onayı alınan kurumun adı ve etik kurul onay numarasını, gereç ve yöntem kısmında ve Etik Kurul Onay bölümünde belirtmelidirler. Ayrıca, kullanılan protokol ve prosedürlerin etik olarak gözden geçirildiğini ve onaylandığını, makalenin gereç ve yöntem bölümüne eklemelidirler.
- Etik kurul raporu alınması gerektiği halde, etik kurul raporu olmayan çalışmalar reddedilecektir.
- İnsanlar veya insandan elde edilen örnekler üzerinde yapılan klinik araştırmalarda bilgilendirilmiş onam formu mutlaka alınmış olmalıdır ve gereç ve yöntem kısmında belirtilmelidir. İnsan gönüllüleri ile yapılan araştırmalar için araştırma protokolüne uygun olarak hazırlanmış yazılı bilgilendirilmiş gönüllü onam formu alınmalıdır.
- Yazarlar, çalışmalarında, hayvan ya da insan örnekleri/gönüllüler kullanmışsa gerekli etik kurul izinlerini aldığından emin olmalıdır. Etik kurul izin ifadesini makalede mutlaka belirtmelidir.
- Bu anlamda yazarlar aşağıda sıralanmış olan kılavuzlara uyarak çalışmalarını gerçekleştirmiş olmalıdırlar:
  İnsanlar üzerinde gerçekleştirilen tüm araştırmalar Helsinki Bildirgesi ilkelerine göre yapılmalıdır (World Medical Association (WMA) Helsinki Declaration for Medical Research in Human Subject). İnsan gönüllülerinden bilgilendirilmiş onam formu alınmış olmalıdır. Tüm hayvan çalışmaları ARRIVE kılavuzuna uygun olmalı (Animal Research: Reporting of In Vivo Experiments (ARRIVE) Guidelines) ve "Bilimsel Amaçlı Kullanılan Hayvanların Korunmasına İlişkin Konsey Direktifi"ne (EU Directive 2010/63/EU for animal experiments), "Birleşik Krallık Hayvan Yasası"na (The U.K. Animals (Scientific Procedures) Act 1986) ve/veya "U.S. İnsan Bakımı ve Laboratuvar Hayvanlarının Kullanımına İlişkin Halk Sağlığı Hizmeti Politikası" rehberine (U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals) uygun şekilde yürütülmelidir. Bitkiler ile ilgili tüm deneysel araştırmalar, uluslararası yönergelere uygun olmalıdır.

4. Ücret Politikası

- Hiçbir ad altında yazar veya kurumundan ücret alınmaz.
- Dergi ile işleme ve yayınlama ücretsizdir. Gönderilen veya kabul edilen makaleler için makale işleme ücreti veya gönderim ücreti yoktur.

Publication Terms

1. The Journal of Faculty of Pharmacy of Ankara University (J. Fac. Pharm. Ankara) is an open-access, peer reviewed journal and is published three times (January-May-September) a year.

2. The Journal of Faculty of Pharmacy of Ankara University publishes articles in every field of Pharmaceutical Sciences. The manuscript to the journal should not be published previously as a whole or in part and not be submitted elsewhere. Manuscript should be written in Turkish or in English. The experiments used have to be adhered to the Declaration of Helsinki for humans and European Community Guidlines for animals. In studies where Ethics Committee Approval is mandatory, the name of the institution from which ethics committee approval was obtained and the ethics committee approval number should be stated in the material and method section and the Ethics Committee Approval section, and the relevant document should be uploaded during article submission.

3. All manuscripts will be submitted to a review process by the editors and by qualified at least 2 outside reviewers. The article evaluation process of Journal of Faculty of Pharmacy of Ankara University is carried out on the principle of double-blind refereeing.

4. Manuscripts are published in order of final acceptance after review and revision.

5. If a manuscript returned to the authors for revision is not received back to the editor within 3 months it will be treated as a new article. When the article is published, authors must send the copyright of the article to the Publisher by filling out the "Copyright Transfer Form".

6. Manuscript will be controlled using plagiarism checker. Articles sent to Journal of Faculty of Pharmacy of Ankara University for publication must be scanned with plagiarism scanning programs (iThenticate) and a report stating that the articles do not contain plagiarism must be uploaded during online article submission.

7. Journal of Faculty of Pharmacy of Ankara University does not have an article publication fee (APC) or subscription fee.

8. The following types of articles are accepted in the Journal Faculty of Pharmacy of Ankara University:

    a) **Original articles**: Articles written in English or Turkish in scientific format presenting original research. Articles should be printed on A4 size papers not exceeding 25 pages (including tables and figures). Research articles are expected to be innovative and contributing to science. Articles must have the main headings specified in the writing rules and must be prepared using a Windows compatible program.
    b) **Review articles:** An updated comprehensive review of scientific works on a particular subject. Articles written in English or Turkish should be printed on A4 size papers not exceeding 30 pages (including tables and figures). Articles must have the main headings specified in the writing rules and must be prepared using a Windows compatible program.
    c) **Short communications:** Rapid announcement of the results of a continuing research written in English or Turkish, no longer than 5, A4 size pages. Articles must have the main headings specified in the writing rules and must be prepared using a Windows compatible program.

Preparation of Manuscript

1. Texts must be written in A4 norm (21 x 29.7 cm).
2. Texts should be written with 1 line spacing, with 2.5 cm margins on the left and right sides of the A4 norm page, 3 cm margins each from the top and bottom edges (3 line spacing from the top on the first page). Articles accepted for publication will be directly uploaded to the system as a "Microsoft Word" file (online submission). The main text font should be **"Times New Roman"** and **11 pt**.
3. Page numbers **should not be specified** in the article.
4. Paragraph headings must **begin 1 cm inside**. Additional spaces should not be left between paragraphs.
5. On the title page, the title of the manuscript the name/s, the full address/es and ORCID no of the author/s, and the full address, telephone number, e-mail address of the corresponding author should be written and all should be centered in the text. It should be indicated by placing (*) above the surname of the corresponding author. Name, surname, full address, telephone number and e-mail address of this person should be specified at the bottom of the title page.
6. **Author's Name** (**first letter capital, others lowercase**) and **SURNAME** (**all capital letters**) should be written in bold, three lines spaced under the title, and without a title underneath. If there is more than one author, they should be written by separating them with a comma and leaving a space. The numbers to be placed on the surnames of the authors and the institution names and postal addresses (For example: Ankara University Faculty of Pharmacy, Department of Pharmaceutical Chemistry, 06560, Ankara, Turkey) should be clearly written on the line just below the names.
   - **ORCID ID number must be declared for all authors**. ORCID IDs of the authors should be created by creating a hyperlink to the relevant logo and adding URL links.
7. International abbreviations may be used. ml for milliliter in the text; min. for minutes It should be written as specified.
8. Units should be expressed using the metric system.
9. All tables and figures should be placed in their places in the text without exceeding the writing area.
10. Tables should be numbered on the top, figures (formula, graph, chart, spectrum, chromatogram, photograph, etc.) should be numbered below with Arabic numbers (**Figure 1., Table 2.)** and should be included in the text. The words "Table", "Figure" and their numbers should be written in bold and in 11 pt. Figure/Picture (**in JPEG format**) must be placed in the article and pictures must be at least **300 dpi or in higher resolution**. Authors must obtain written permission to reproduce any images from other sources.
11. **Table** titles should be written in 11 font size justified on the top of the tables and not exceeding their width. If there is an explanation for the table, it should be written in 9 font size at the bottom of the table. The text in the table can be written between 8-11 points. **Figure titles** should be written at the bottom of the figures with a line spacing, centered and 11 pt. There must be **6 nk** space between the figure and figure title. There should be **18 nk** space between the text and title of figure and/or table.

    See for below examples for tables:
    - All row and column lines should be included.
    - Table design should be uniform and straight throughout the article, no coloring / shading should be used.
    - Headings in the table should be written in **bold**. There must be **6 nk** space between the table and table title.

Table 1. Morphological characteristics of the species

Plant part*	***C. nummularia***	***C. integerrimus***
Leaf	Broadly elliptical-orbicular, 0.9-2.5-(4) x 0.5-2.5-(3-5) cm	From orbicular to ovate, 1.2-(4-5) x 0.9-3 cm,
Seed	3.5-4 x 1-2 mm, dark brown	3-4 x 1.5-2 mm, light brown

* Explanation should be 9 font size, 1 range.

Table 2. Patient demographics

Demographics	**Group A***	**Group B**	**Group C**
Male gender	10 (%30)	20 (%60)	10 (% 30)
Cigarette consumption	20 (%60)	10 (%30)	20 (%60)

* Explanation should be 9 font size, 1 range.

Example for figure:



Figure 1. General view of *C. Nummularia* (The font size must be 11 pt with 1 line spacing and "Times New Roman" font, and must be centered in the text)

12. The sections of the articles should be prepared in accordance with the **TITLE** (Turkish and English), **ABSTRACT, INTRODUCTION, MATERIAL AND METHOD, RESULT AND DISCUSSION, ACKNOWLEDGEMENTS** (if available), **AUTHOR CONTRIBUTIONS, CONFLICT OF INTEREST, ETHICS COMMITTEE APPROVAL** (if available) and **REFERENCES**. Titles expressing these sections (except the first title of the article) should be written in **12 pt**, **bold capital letters and starting from the left of the page**. **There should be 18 nk space before and 6 nk space after the INTRODUCTION.** For, there should be 12 nk space before and 6 nk space after the other titles. Between the chapter titles and the text, a separate space **should not be left** other than the specified in this document.

- **TITLE:** Capital letters and **first title** in Turkish and English (Turkish title is the first title in Turkish articles, English title is the first title in English articles), **14 pt, bold** and the second title should be written in 12 pt, *italic*. The title should be appropriate to the text, short, introducing the work and clearly worded.

- **ABSTRACT** and **ÖZ:** It should be written in English (**ABSTRACT**) and Turkish (**ÖZ**) at the beginning of the articles, not exceeding 200 words, 10 pt, *italic* and within a frame. In articles written in a foreign language, first **ABSTRACT** and then **ÖZ** in Turkish. **ABSTRACT** and **ÖZ** titles should be written in 12 pt. And bold and the summary of the article should be presented as subheadings. Each subtitle should be written in 10 pt, bold, normal and 1 cm indented. **ABSTRACT** and **ÖZ** should be written in blocks with 1 cm margins from the right and left.

For original articles;

Subheadings to be used for **ABSTRACT**:

Objective: *Text should be written in italic.*
Material and Method: *Text should be written in italic.*
Result and Discussion: *Text should be written in italic.*
Keywords:

Subheadings to be used for **ÖZ**:

Amaç: *Text should be written in italic.*
Gereç ve Yöntem: *Text should be written in italic.*
Sonuç ve Tartışma: *Text should be written in italic.*
Anahtar Kelimeler: *Text should be written in italic.*

For review articles;

Subheadings to be used for **ABSTRACT**:

Objective: *Text should be written in italic.*
Result and Discussion: *Text should be written in italic.*
Keywords:

Subheadings to be used for **ÖZ**:

Amaç: *Text should be written in italic.*
Sonuç ve Tartışma: *Text should be written in italic.*
Anahtar Kelimeler:

- **Keywords (Anahtar Kelimeler)**: It should consist of a minimum of 3 words, should be written alphabetically, italic in the relevant language, with only the first letter of the first keyword capitalized (except for abbreviations using capital letters) with commas between them and a spelling mark **should not be** used after the last keyword.

- **TEXT:** The text part of the original Turkish article should consist of 3 main headings: **INTRODUCTION, MATERIAL AND METHOD, RESULT AND DISCUSSION**. All of these main headings should be written in 12 pt, **capital letters** and bold. In review articles, there should be the main headings of **INTRODUCTION** and **RESULT AND DISCUSSION**, other titles should be written with the first letter of each word capital, the others in lowercase and bold, as determined by the author. Subheadings should be written in 11 font size, 1.5 line spacing, **bold** and aligned to the left. Numbering system **should not be** used in subheadings.

- **INTRODUCTION:** There should be a section containing the purpose of the research and studies on the subject.

- **MATERIAL AND METHOD:** Required information about the method should be clearly stated by indicating the material used. **Characterization of compounds** should be shown in a separate paragraph and clarification of the purity and structure of the new compounds should be provided. If animal or human samples/volunteers are used in the study, researchers should ensure that a statement stating that all procedures are carried out in accordance with the relevant laws and institutional guidelines and that these procedures have been approved by the appropriate administrative committee and that the approval of the Ethics Committee is included in the study. In studies for which Ethics Committee approval is mandatory, the name of the institution for which the ethics committee approval was obtained and the ethics committee approval number should be specified in the materials and methods section. It should also be included in the materials and methods section of the article that the protocols and procedures used are ethically reviewed and approved. For detailed information, please visit http://journal.pharmacy.ankara.edu.tr/en/ethical-principles-and-publication-policy/ web page.

- **RESULT AND DISCUSSION:** This is the section where findings are given and evaluated.
  - If the author wishes, "Conclusion" can be added as the last paragraph of the RESULT AND DISCUSSION section. The font size must be 11 pt with 1 line spacing and “Times New Roman” font and the first letter must be uppercase and the other letters must be lowercase.

- **ACKNOWLEDGMENTS**: If any, the organization supporting the research and the people who contributed can be acknowledged briefly in this section prior to the Authors' Contribution.

- **AUTHOR CONTRIBUTIONS:** Contribution of the authors in the article should be written just before the conflict of interest notification, in accordance with the *Copyright Transfer Agreement* signed by the authors. Please use the initials of the authors for this notice instead of the full name and surname as in the example below. If there is not any author contribution for the specified sections, “-” should be added. Please see below example for writing author contributions.

  Example:

  AUTHOR CONTRIBUTIONS

  Concept: İ.Y., M.M.H., C.H., K.B.; Design: İ.Y., C.H., I.Ö.G., Ö.Ü.; Control: C.H., I.Ö.G., M.M.H., K.B.; Sources: Ö.Ü., Z.K., K.B., M.M.H., A.K., İ.A., G.A.G., B.G., B.K.; Materials: I.Ö.G., B.E., G.A.G., B.K., D.Ç.P.; Data Collection and/or Processing: A.K., Ö.Ü., M.K., A.S., D.Ç.P., T.C.Ş.T.; Analysis and/or Interpretation: Ö.Ü., B.G., T.C.Ş.T., E.K.S.; Literature Review: B.K., D.Ç.P, B.G., B.E.; Manuscript Writing: A.K., İ.A., T.C.Ş.T.; Critical Review: İ.Y., B.G., Ö.Ü., İ.A.; Other: -

- **CONFLICT OF INTEREST**

  If there is a conflict of interest, it should be clearly declared in what form it is. If not, "The authors declare that there is no real, potential, or perceived conflict of interest for this article." They should use the expression.

- **ETHICS COMMITTEE APPROVAL**

  If the ethics committee approval is obtained before the sources at the end of the study, the approval number must be specified from which institution and when it was obtained. Approval from the ethics committee should be uploaded during the manuscript submission. In studies that do not require ethics committee approval, the following sentence should be written.

  "The authors declare that the ethics committee approval is not required for this study".

- **REFERENCES:** Bibliography style is according to the American Psychological Association (APA). Typeface "Times New Roman" and 10 font size, "1" spacing, justified. In the text, it should be numbered in square brackets according to the order of appearance, such as: [1,6,9], [5-7] and listed according to these numbers at the end of the text. Reference should not be given next to the subtitles. If it is necessary to provide a source in the table, it should be specified as given in the text. References should be written in accordance with the examples below.

  - **For the article:** Author's surname, the initials of the name (There should be a period after the initial letter of each name of the author with more than one name, and there should not be a space in between. **There <u>should not be</u> “and” between the last author and the previous author. The “&” symbol <u>should not be</u> used**. The full title of the article should be written as the name of the journal, volume number, if available, the number (in parentheses), the beginning and ending page number (or article id), the year after the author names (in parentheses). **If there is more than one author, all of them should be written**. While writing the name of the article, the first letter of the first word should be capitalized

and the first letter of the other words should be written in lowercase. Journal names given in references should be written clearly without abbreviation.
Add the [CrossRef] **sign at the end of each reference and enter the DOI number as a hyperlink with the right click in the format below. Please do not add CrossRef hyperlink if the article is not listed at https://www.crossref.org/.**
https://doi.org/10.1016/0006-2952(89)90403-6

Examples:

1. Martinez, M.J.A., Del Olmo, L.M.B., Benito, P.B. (2005). Antiviral activities of polysaccharides from natural sources. Studies in Natural Products Chemistry, 30, 393-418. [CrossRef]
2. Bahiense, J.B., Marques, F.M., Figueira, M.M., Vargasa, T.S., Kondratyuk, T.P., Endringer, D.C., Scherer, R., Fronzaa, M. (2017). Potential anti-inflammatory, antioxidant and antimicrobial activities of *Sambucus australis*. Pharmaceutical Biology, 55(1), 991-997. [CrossRef]

- **Online articles:**

Example:
Perneger, T.V., Giner, F. (1998). Randomized trial of heroin maintenance programme for adults who fail in convential drug treatments. British Medical Journal, 317. Retrieved August 12, 2005, from ttp://www.bmj.com/cgi/content/full/317/7150/

- **Web sites:**

Example:

Clinical Pharmacology Web site. (2001). Retrieved June 16, 2004, from http://cpip.gsm.com/. Accessed date: 14.03.2021.

- **Books:** The surname of the author, the initials of the name, the name of the book, volume number (if any), the bookstore, the city where it was published, the page number, the year it was published (in parentheses) should be written.

Example:

Franke, R. (1984). Theoretical Drug Design Methods, Elsevier, Amsterdam, p.130.

- **Book chapters:** Author's surname, initials of the name, the title of the section, the editor / editors' surname, the initials of the name, the phrase (Ed./Eds.), The title of the book, if any, the book house, the city where it was published, the page number, the year it was published (in parentheses) should be written.

Example:

Weinberg, E.D. (1979). Antifungal Agents. In: M.E. Wolff and S.E. Smith (Eds.), Burger's Medicinal Chemistry, (pp. 531-537). New York: John Wiley and Sons.

- **For the thesis**: The surname of the author, the initials of the name, the year should be written (in parentheses) after the author's names and a full stop. After specifying the type of thesis, the title of the thesis and where it was made should be written.

Example:

Ahmed, J. (2008). PhD Thesis. Pharmaceutical Botany investigations on Prangos Lindl. (Umbelliferae) growing in Konya province. Department of Pharmaceutical Botany, Faculty of Pharmacy, Ankara University, Ankara, Turkey.

- **For patent:** The surname of the author, the initials of the name, the year should be written (in parentheses) after the author's names and a full stop. The title and number of the patent should

be indicated.

Example:

Mahoney, S., Molz, L., Narayan, S., Saiah, E. (2018). Heteroaryl RHEB Inhibitors and Uses Thereof. WO 2018/191146 A1.

ETHICAL PRINCIPLES AND PUBLICATION POLICY

Journal of Faculty of Pharmacy of Ankara University is an open-access, peer-reviewed journal and a publishing medium for original research, reviews and short communications covering important developments in the field of pharmaceutical sciences in Turkish or English. Journal of Faculty of Pharmacy of Ankara University does not have an article publication fee (APC) or subscription fee.

As the editorial board, it is aimed to publish high-quality new studies that make a significant contribution to the scope of the journal. To achieve this goal, articles submitted are subject to initial evaluation by the editor-in-chief and/or assistant editors to ensure that they meet the scientific and formal criteria to be published in the journal. Only studies that pass this preliminary evaluation process are continued to other stages for further evaluation.

Preliminary Assessment

- The scientific quality and novelty of the study must be sufficient to be published in the journal.
- Studies submitted to the journal must comply with the purpose and scope of the journal.
- The text must be written in English or Turkish, grammatically and scientifically well-written.
- The similarity rate of studies submitted to the journal should not exceed 20%.
- Studies should be arranged in accordance with the journal's writing rules and template.
- Copyright transfer form, ethics committee approval document and author contribution form must be uploaded and signed.
- Studies must be sent to the journal via the electronic online application system.

The further evaluation process of studies that do not meet these qualifications cannot be initiated.

Journal editors, reviewers and authors have certain responsibilities during the journal publication process. These responsibilities are explained below.

1. Editor's Duties and Ethical Responsibilities

The editor can independently decide which of the articles sent to the journal should be published, or can also consult with members of the editorial board or reviewers. Within the framework of the journal's ethical principles and publication policy, it is responsible for carrying out the preliminary evaluation, peer review and publication stages of the studies in an impartial, auditable, fair, independent of conflict of interest and in accordance with confidentiality principles. If there is no violation in terms of publication policy and ethical principles, studies that comply with the purpose and scope of the journal should be taken to the preliminary evaluation stage.

The duties and descriptions of the editor-in-chief, associate editors, section editors and editorial advisory board are as follows:

Editor-in-Chief: Editor in chief has full authority over the publication of the journal content. Editor in chief works with Associate Editors, Section Editors and the Editorial Advisory Board.
Associate Editors: Associate Editors are primarily responsible for answering questions about the journal, making suggestions to the journal reviewers and board, and assisting the Editor-in-Chief during the article publication process.
Section Editors: Section Editors assist authors in assigning double-blind referees and answering questions about the journal.
Editorial Advisory Board: The Editorial Advisory Board guides the Editor-in-Chief and Associate Editors on issues related to the production of quality publications that are appropriate for the purpose of Journal of Faculty of Pharmacy of Ankara University.

1.1. Publication Policy

- The editor-in-chief is solely responsible for deciding which articles sent to the journal should be published. The editor's decision may be in line with the principles of the journal's editorial

board or is limited by applicable legal requirements regarding issues such as defamatory publication, copyright infringement and plagiarism.

- The editor-in-chief ensures that the authors fill out the "Copyright Transfer Form" and send the copyright of the article to the publisher before the article is published.
- The editor-in-chief ensures that the authors fill out the "Conflict of Interest Form" and the "Author Contribution Form" before the article is published.
- The editor-in-chief assigns associate editors to formally review the articles sent to the journal. Articles that do not comply with the rules of the Journal of Faculty of Pharmacy of Ankara University are rejected without being evaluated.

1.2. Publication Review

- The editor-in-chief is responsible for ensuring that the publication evaluation process is fair, impartial and timely.
- The editor generally ensures that all articles are evaluated by at least two external and independent reviewers. If necessary, the editor requests additional opinion from a third reviewer.
- The editor selects the reviewers by evaluating experts who are suitable for the scope of the article.
- The editor carefully reviews the publication process to evaluate and decide on disclosures made for possible conflicts of interest, “self-citation” suggestions made by reviewers, and any possibility of bias.
- The editor-in-chief/other editors requests for the article to be scanned by a similarity detection software (iThenticate) at any point during the peer review or evaluation/publication process, or they do it themselves. In this sense, even if the expressions or sentences are the author(s) themselves, the text should not have an unacceptable similarity to previously published data.
- If the editor-in-chief detects errors in an article before it is published, he/she corrects them. If he/she detects it later, then he/she has to publish the corrections. All corrections or retraction notices must be prominently published in the journal. It should also be listed on the contents page.
- Journal of Faculty of Pharmacy of Ankara University’s editors follow the "COPE Code of Conduct and Best Practice Guidelines for Journal Editors" and "COPE Best Practice Guidelines for Journal Editors" guidelines published by the Committee on Publication Ethics (COPE).

1.3. Fair Evaluation

- Editor-in-Chief/other editors evaluates articles according to their scientific content, regardless of the authors' race, gender, sexual orientation, belief, ethnicity, citizenship or political views. The journal’s editorial principles support transparent and completely honest review.
- The editor must ensure that reviewers and authors fully understand what is expected of them.
- The editor makes all his communication regarding the journal through the journal’s electronic application system and follows a transparent and fair manner in case of objections to his decisions.

1.4. Privacy Policy

- The chief editor/other editors is obliged to maintain the confidentiality of all materials in the application to the journal and all communication with the reviewers (unless otherwise approved by the relevant authors and reviewers).
- The chief editor/other editors is responsible for protecting the identities and rights of the reviewers, unless the reviewers agree to their names being disclosed.
- Unpublished materials belonging to a submitted article should not be used for the editor's own studies/research without the written consent of the author.
- The chief editor/other editors must keep all information or ideas obtained during the article evaluation process confidential and should not use them for personal purposes.

2. Duties and Ethical Responsibilities of Referees

The article evaluation process of Journal of Faculty of Pharmacy of Ankara University is carried out on the principle of double-blind review. Therefore, reviewer cannot communicate with the author(s), evaluations are shared through the Dergipark management system. During the evaluation process, evaluation forms and reviewers' comments regarding the manuscripts are forwarded to the corresponding author through the editor. Reviewers must act in accordance with the principles of impartiality, confidentiality, objectivity and scientific review throughout the evaluation process. They must be an expert and competent in the relevant field. They must complete their report on the work submitted for evaluation within the specified time period. For reports that cannot be submitted on time, the editor should be contacted without delay. The editorial board should be informed in cases of copyright and/or ethical infringement, possible conflict of interest and plagiarism.

Reviewers who evaluate the manuscript of the Journal of Faculty of Pharmacy of Ankara University are expected to comply with the stated duties and ethical responsibilities describe below:

2.1. Contribution to Editorial Decisions

- Reviewers must evaluate the work submitted by authors constructively and appropriately.
- If the reviewers think that they are not competent to evaluate the research in the article or cannot complete it in sufficient time, they must notify the editor.
- Reviewers should not make harsh and personal criticisms towards the authors.
- When reviewers receive an invitation to evaluate an article, they should refuse to evaluate the article if they feel inadequate about the subject studied in the article.
- Reviewers must evaluate the article within the given time.
- Reviewers should only objectively evaluate the content of the study.

2.2. Privacy

- Reviewers must make the evaluation impartially and confidentially.
- Reviewers should not share their evaluations or information about the article with third parties.
- Reviewers must keep confidential the information, ideas and unpublished materials or studies obtained during the article evaluation process and must not use them for personal purposes.
- Reviewers should not retain or reproduce a copy of the article.

2.3. Detecting Ethical Issues

- Reviewers should notice the ethical problems in the article and bring them to the attention of the editor.
- If the reviewers detect that the article has been previously published elsewhere or that there is a significant similarity or overlap with a previously published article, they must notify the editor. Any previously published observations and/or arguments should be accompanied by the relevant reference.

2.4. Impartiality and Competition Standards

- Reviewers must make their evaluations impartially and free from bias. It is not appropriate to criticize the author as a person. Reviewers must express their opinions with supporting arguments.
- Reviewers must check for possible conflict of interest before agreeing to evaluate the article. If they feel they face a conflict of interest, they should refuse to review the manuscript and inform the editor.
- If reviewers claim that the reviewers' (or people working with the reviewers) work has been taken as a source by the author, they must provide real scientific justifications and be careful that this is not an attempt to increase the reviewers' number of references or the visibility of their work.

- Reviewers should not stay away from scientific reality when making their evaluations and should resort to citing sources if necessary.

3. Authors' Duties and Ethical Responsibilities

Articles submitted to Journal of Faculty of Pharmacy of Ankara University must not have been previously published in elsewhere or should not have been sent to another publication within the same time period for publication. It is mandatory that the quotations or citations of the research and publications used in the studies are made completely in accordance with the principles of scientific research and the sources are stated. If the number of authors in the study is more than one, the authors are expected to make a concrete and sufficient scientific and academic contribution to the study. All sources of financial support for the study must be disclosed. Authors must report possible conflict of interest situations to the editorial board.

The author(s) who sent articles to Journal of Faculty of Pharmacy of Ankara University, must comply with duties and ethical responsibilities listed below:

3.1. Notification Standards

- The article (research, review or short communication) sent by the author(s) must be original.
- The author(s) should clearly present the research performed with an unbiased discussion of the significance of the study.
- The author(s) must present the data clearly in the article.
- If the author(s) uses other works, they must cite them fully and accurately.
- The article must contain sufficient detail and sources to enable other researchers to replicate the study.
- Author(s) should not act unethically and use misleading or unclear expressions in their articles.
- Authors act with the knowledge that their articles will not be published by the journal unless they comply with the journal rules and take action within the specified time.

3.2. Data Transportation and Storage

- Authors may be asked for research data supporting their articles for editorial evaluation.
- Authors must be ready to submit the specified data to the editorial board in case raw data regarding their articles or data to support their articles are requested during the evaluation process.

3.3. Originality, Plagiarism and Citation of Sources

- Plagiarism may occur in the form of the author representing another article as his own work, copying or paraphrasing certain parts of someone else's work without citing the source, or presenting the results of work done by others. Any form of plagiarism is unethical behavior and is completely unacceptable. Authors should avoid plagiarism. Please click here for the definition of plagiarism.
- Authors must guarantee that their work is completely original. If authors use others' ideas or texts, they must indicate appropriate sources or citations and obtain permission if necessary.
- Authors must cite as references all publications or works that influence their work and contribute to the creation of appropriate content for the work. Information obtained privately (interview, correspondence or discussion with third parties) should not be used or, if used, should be reported with permission.
- Authors must scan the articles that they send to Journal of Faculty of Pharmacy of Ankara University for publication with plagiarism scanning programs (iThenticate) and upload a report stating that their articles do not contain plagiarism during online article submission in the Dergipark system.

3.4. Multiple, Redundant and Republishing

- Submitting to more than one journal with the same article is unethical behavior and is never accepted. In general, the authors should not submit a previously published publication to another journal for review, except in abstract form or as part of a published lecture, academic thesis, or electronic preprint.
- Authors must ensure that they do not submit the article to another journal at the same time during the submission.
- Authors must guarantee that the submitted manuscript is not under evaluation or has been accepted for publication elsewhere, and if accepted, it will not be published elsewhere in the same format, in another language, including electronic media, without the written permission of the author.

3.5. Author Contributions

- Author contributions should be limited to individuals who have made significant contributions to the concept, design, implementation, or interpretation of the work.
- Authors should carefully prepare the list of authors who contributed to the study. In some cases, the work may deserve to be a co-author, and in some cases, the work may deserve to be included in the "Acknowledgment" section.
- The corresponding author must take responsibility for ensuring that all co-authors are properly included in the work, that all co-authors have seen and approved the work, and that they have approved the submission for publication.
- The corresponding author is responsible for ensuring that all authors on the manuscript agree on issues such as author order, accuracy and integrity of the work, and must submit a definitive author list at the time of the original submission.
- After the application of the study is completed, the editor can add, delete or rearrange the author list only in exceptional cases. All authors must agree on such additions, deletions and rearrangements. All authors acknowledge shared responsibility for the work. Each author is responsible for questions relating to the accuracy and integrity of the work that have been properly researched and adjudicated.
- The corresponding author, as the person who contacts the editor, is responsible for filling out and sending the "Author Contribution Form" along with the article to Journal of Faculty of Pharmacy of Ankara University.

3.6. Conflict of Interest Declaration

- Authors must declare any situations or relationships that may create a conflict of interest with other individuals or organizations that they deem to be inappropriately influencing their work.
- The corresponding author, as the person who contacts the editor, is responsible for filling out and sending the "Conflict of Interest Declaration Form" along with the article to Journal of Faculty of Pharmacy of Ankara University.
- Authors must disclose situations where they have conflicts of interest.

3.7. Reporting Basic Errors

- When authors notice a significant error or omission in a published work that is in the early review or evaluation process, they are obliged to immediately notify the journal editor-in-chief / publisher or the relevant editor and, if deemed necessary by the editor, cooperate with the editor to withdraw or correct the article.
- If the editor/publisher learns from a third party that the published article contains an error, it is the author's obligation to cooperate with the editor and provide supporting evidence where necessary.

3.8. Potential Risks and Human or Animal Issues

- Authors should clearly state if they have worked with chemicals, processes, or materials that pose unusual risks when used.
- If animal or human samples/volunteers are used in the study, researchers should ensure that a statement is included in the article that all procedures were carried out in accordance with the relevant laws and institutional guidelines and that these procedures were approved by the appropriate administrative board and Ethics Committee Approval was obtained.
- In studies where Ethics Committee Approval is mandatory, authors must indicate the name of the institution from which ethics committee approval was obtained and the ethics committee approval number in the materials and methods section and the Ethics Committee Approval section. They should also include in the materials and methods section of the manuscript that the protocols and procedures used have been ethically reviewed and approved.
- Although an ethics committee report is required, studies without an ethics committee report will be rejected.
- In clinical research conducted on humans or samples obtained from humans, an informed consent form must be obtained and must be stated in the materials and methods section. For research conducted with human volunteers, a written informed consent form prepared in accordance with the research protocol must be obtained.
- Authors must ensure that they obtain the necessary ethics committee permissions if they use animal or human samples/volunteers in their studies. Ethics committee permission must be stated in the article.
- In this sense, authors must have carried out their work by following the guidelines listed below: All research conducted on humans should be conducted in accordance with the principles of the Declaration of Helsinki (World Medical Association (WMA) Helsinki Declaration for Medical Research in Human Subject). Informed consent must have been obtained from human volunteers. All animal studies must comply with the ARRIVE guideline (Animal Research: Reporting of In Vivo Experiments (ARRIVE) Guidelines) and the "Council Directive on the Protection of Animals Used for Scientific Purposes" (EU Directive 2010/63/EU for animal experiments), "United Kingdom Animal (The U.K. Animals (Scientific Procedures) Act 1986) and/or "U.S. It must be conducted in accordance with the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals. All experimental research on plants must comply with international guidelines.

4. Fee Policy

- No fee is charged from the author or his institution under any name.
- Processing and publishing with the journal is free. There are no article processing fees or submission fees for submitted or accepted articles.

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eISSN: 2564-6524
ISSN: 1015-3918 (1971-2010)



ANKARA ÜNİVERSİTESİ ECZACILIK FAKÜLTESİ DERGİSİ

(Ankara Ecz. Fak. Derg.)

JOURNAL OF FACULTY OF PHARMACY OF ANKARA UNIVERSITY

(J. Fac. Pharm. Ankara)

Cilt / Vol: 49
Sayı / Issue: 3
Yıl / Year: 2025

Ücretsizdir