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Corresponding Address
Current Perspectives on Medicinal and Aromatic Plants (CUPMAP),
Nazım Şekeroğlu
Gaziantep University, Faculty of Art and Sciences, 27310, Şehitkamil, Gaziantep - Türkiye
Phone: 0 342-317 19 22
Web: http://www.cupmap.org/
Contact: sekeroglunazim@gmail.com / editor@cupmap.org

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Web	http://www.cupmap.org/
Contact	Phone: 0 342-317 19 22/2960 E-mail:sekeroglunazim@gmail.com editor@cupmap.org

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Sincerely,
Prof. Dr. Nazım ŞEKEROĞLU
Editor-in-Chief
Current Perspectives on Medicinal and Aromatic Plants (CUPMAP)
Contact: sekeroglunazim@gmail.com / editor@cupmap.org

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RESEARCH ARTICLES

REVIEW ARTICLES

---------- Gaziantep University, Publishing House ----------
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Research article
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Antimicrobial Activities and Enzyme Inhibition Effects of *Nepeta* Species

Semiha YENIGUN[1], Yunus BASAR[2], Yaşar IPEK[3], Mesut GOK[4], Lutfi BEHCET[5], Ibrahim DEMİRTAS[2,6], Tevfik OZEN[1*]

[1] Department of Chemistry, Faculty of Science, Ondokuz Mayıs University, 55139, Samsun, Türkiye
[2] Research Laboratories Application and Research Center (ALUM), University of Igdir, 76000, Igdir, Türkiye
[3] Department of Biochemistry, Faculty of Science, University of Cankiri Karatekin, 18100, Cankiri, Türkiye
[4] Science and Technology Application and Research Center, University of Siirt, 56210, Siirt, Türkiye
[5] Department of Molecular Biology and Genetics, Faculty of Science and Art, University of Bingol, Bingol, Türkiye
[6] Department of Pharmacy Vocational Sciences, Faculty of Pharmacy, Ondokuz Mayıs University, Samsun, Türkiye

*Corresponding author: tevfikoz@omu.edu.tr

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Abstract

Various *Nepeta* species, widely used among the public, have valuable phytochemical contents and clinical and biological activities. For this reason, our study examined the enzyme inhibition and antibacterial properties of methanol: chloroform (1:1) extracts of six *Nepeta* species. *N. aristata* showed a higher inhibitory effect than the standard drug on seven of the eight enzymes studied. *N. baytopii* had a high inhibition effect on urease and lipase. It was determined that *N. italica* inhibited other enzymes except for urease, CA, and lipase. In addition, BChE is also the only effective plant. *N. nuda* subsp. *albiflora* has a high effect on inhibiting urease, AChE, and lipase. *N. stenantha* and *N. trachonitica* also showed inhibition effects on urease, AChE, and tyrosinase. In the disc diffusion method of antibacterial activity, extracts against *B. cereus* had antibacterial activity. The antimicrobial activity of *N. aristata* extract was effective against *P. aerugonisa* and *K. pneumoniae*. Additionally, when looking at the minimum inhibition concentration method of antibacterial activity, *Nepeta* extracts were effective against most bacteria. This research determined *Nepeta* extracts are effective natural products with antioxidant and enzyme inhibition activities.

Key Words: *Nepeta* species, bioactivity, enzyme inhibition activity, antibacterial activity

1. Introduction

The *Nepeta* genus, which has the largest place in the Lamiaceae family, contains three hundred species and is used by humans for medicinal purposes in many countries, including Turkey (Sharma et al., 2021). There are 40 different taxa in Turkey, and 19 of them are endemic. Most of *Nepeta* species in Turkey are used to treat many diseases such as stomach, bacteriostatic, diuretic, skin diseases, infusion, and nauseation (Baytop, 1999). Enzymes are among the biological macromolecules that are regarded as potential therapeutic targets. As of late, almost half of all pharmaceuticals utilized in clinical settings are enzyme inhibitors (Copeland, 2005). The inhibitions of certain

Nepeta species' extracts against various enzymes were investigated for this reason. *N. baytopii* (Zengin et al., 2021), *N. italica* (Acquaviva et al., 2023), *N. cadmea* and *N. nuda* subsp. *glandulifera* (Sarikurkcu et al., 2019), and so on are a few examples. Antimicrobial agents are substances, either manufactured or natural, that eliminate or stop germs' reproduction (Moreno et al., 2000). Numerous *Nepeta* species, including *N. trachonitica* (Köksal et al., 2017), *N. distans* (Alkahtani et al., 2022), etc. are among these plants.

A limited number of studies have been conducted on the antibacterial and enzyme inhibition activities of *N. baytopii* (Zengin et al., 2021), *N. italica* (Acquaviva et al., 2023), *N. stenantha* (Kazemi et al., 2016), and *N. trachonitica* (Köksal et al., 2017). Therefore, in this study, the enzyme inhibition effects and antibacterial activities of methanol:chloroform extracts of the previously unstudied *N. aristata*, and *N. nuda* subsp. *albiflora*, and the few studied.

N. baytopii, N. italica, N. stenantha, and *N. trachonitica* were investigated. Enzyme inhibitions of the extracts were determined using carbonic anhydrase (CA), urease, acetylcholinesterase (AChE), lipase, butyrylcholinesterase (BChE), α-amylase, tyrosinase and α-glucosidase enzymes. In addition, the antibacterial activities of these extracts were examined against six different bacteria by disk diffusion and minimum inhibition concentration methods. The enzyme inhibition effects and antibacterial activities of *Nepeta* plants, which have not been studied much, were evaluated by comparing them with each other, standard substances and drugs.

2. Material and Methods

2.1. Chemicals

Urease from *Jack bean*, AChE from *Electrophorus electrius*, α-amylase from *Porcine pancreas*, BChE from *Horse serum*, lipase from *Porcine pancreas*, α-glucosidase from *Saccharomyces cerevisiae*, tyrosinase from mushroom, galantamine, CA from *Bovine erythrocytes*, 5,5′-dithiobis(2-nitrobenzoic acid), NaOCl, sodium nitroprusside, phenol, NaOH, urea, orlistat, starch, iodide, acetazolamide, *p*-nitrophenyl acetate, *p*-nitrophenyl octanoate, thiourea, amoxicillin, 3,4-dihydroxy-L-phenylalanine, tetracycline, $MgCl_2.6H_2O$ and $CaCl_2.2H_2O$, NaCl, Trisma, HCl, K_2HPO_4, KH_2PO_4, Na_2HPO_4 from Sigma-Aldrich; acarbose from TCI; Muller Hinton Agar (MHA), and Muller Hinton II Broth (MHB) from Himedia; kojic acid from Gelentham.

2.2. Extraction and Plant Materials

While five *Nepeta* L. species were gathered from the Bingöl province in Turkey's Eastern Anatolia Region, one was collected from the Ağrı province. These plant species were described in the 7th volume of the book "Flora of Turkey and the Eastern Aegean Islands" (Hedge & Lamond, 1982). The identification of plants Prof. Dr. It was made by Lütfi Behçet and kept in the Herbarium of Bingöl University, Department of Biology (Yenigün et al., 2023).

The aerial parts of *Nepeta* species were cleaned and dried in a calm and dark laboratory environment. Dried plant samples (624-1120 g) were ground to powder with a laboratory grinder. The high efficiency of the chloroform/methanol solvent mixture is widely used to extract of wide range of plants. The solvent mixture is used because it is the most suitable solvent mixture required for obtaining secondary metabolites in plants and for the fractionation process (Başar et al., 2023; Yenigun et al., 2024; Yenigün et al., 2023). Then, methanol: chloroform (1:1, 9-15 L) solvent mixture was added and kept closed. This process was repeated three times at one-week intervals. A rotary evaporator operating at +40 °C was used to extract the solvent mixture after the mixture had been filtered through Whatman no. 1 filter paper. In order to prepare the acquired

crude extracts for additional tests and analyses, they were dried using a lyophilizer device until they were transformed into a powder and then kept in a refrigerator at -20°C (Yenigün et al., 2023).

2.3. Enzyme Inhibition Activities

The possible inhibition activities of the *Nepeta* extracts (1024–0.5 µg/mL) against urease (Zhang et al., 2006) inhibitory activity were measured by previously described methods with slight modifications (Başar et al., 2023). In a 96-well plate, 10 µL of samples of different concentrations or thiourea as standard into each well, 25 µL of 1 U urease (in 100 mM pH 8.2 sodium-phosphate buffer) and 50 µL of 100 mM urea solution were mixed until homogeneous and incubated for 15 minutes at 30°C. It was kept waiting for minutes. 45 µL of phenol reagent [8% (w/v) phenol and 0.1% (w/v) sodium nitroprusside] and 70 µL of alkaline reagent [2.5% (w/v) NaOH and 4.7% NaOCl] solutions were added to the mixture in each well. The samples were kept at 30°C for 50 minutes. The absorbance values of each well were measured at 630 nm and IC_{50} (µg/mL) values were determined.

The possible inhibition activities of the *Nepeta* extracts (1024–0.5 µg/mL) against AChE and BChE (Ellman et al., 1961) inhibitory activity were measured by previously described methods with slight modifications (Başar et al., 2023). In a 96-well plate, 20 µL of samples of different concentrations or galantamine, 20 µL of 0.03 U AChE or BChE (in 100 mM pH 8.0 phosphate buffer), 20 µL of 3.3 mM DTNB, and 140 µL of 100 mM pH 8.0 phosphate buffer were added to each well and mixed until homogeneous. and left at room temperature for 15 minutes. 10 µL of 1 mM ATCh (acetylthiocholine iodide) or B solution was added to the mixture in each well. The absorbance values of each well were measured at 412 nm and IC_{50} (µg/mL) values were determined.

The possible inhibition activities of the *Nepeta* extracts (1024–0.5 µg/mL) against CA (Chanda et al., 2019) inhibitory activity were measured by previously described methods with slight modifications (Başar et al., 2023). In a 96-well plate, 60 µL of samples of different concentrations or acetazolamide and 90 µL of 115 U CA solution (in 0.05 M pH 7.4 Tris-SO4 buffer) were mixed into each well until homogeneous and left at room temperature for 15 minutes. 60 µL of 10 mM 4-nitrophenyl acetate solution was added to the mixture in each well and left at room temperature for 15 min. The absorbance values of each well were measured at 400 nm and IC_{50} (µg/mL) values were determined.

The possible inhibition activities of the *Nepeta* extracts (1024–0.5 µg/mL) against α-amylase (Yang et al., 2012) inhibitory activity were measured by previously described methods with slight modifications (Başar et al., 2023). In a 96-well plate, 82 µL of samples of different concentrations or acarbose and 10 µL of 1 U α-amylase (in 20 mM pH 6.9 potassium phosphate buffer) solution were mixed into each well until homogeneous and kept at 37°C for 10 min. 8 µL of 1% starch solution was added to the mixture in each well and kept at 37°C for 12 minutes. 50 µL 10% HCl and 15 µL iodine-KI (2.5 mM iodine+6.5 mM KI) solutions were added. The samples were kept in boiling water for 10 minutes and the absorbance values of each well were measured at 620 nm and IC_{50} (µg/mL) values were determined.

The possible inhibition activities of the *Nepeta* extracts (1024–0.5 µg/mL) against lipase (Trentin et al., 2020) inhibitory activity were measured by previously described methods with slight modifications (Başar et al., 2023). In a 96-well plate, 20 µL of samples at different concentrations or orlistat, 200 µL of 100 mM

pH 8.2 Tris-HCl buffer and 20 µL of 1 mg/mL lipase (in 100 mM pH 8.2 Tris-HCl buffer) solution were added to each well and mixed until homogeneous. 20 µL of 5.1 mM p-nitrophenyl octanoate (100 mM pH 8.2 in Tris-HCl buffer) solution was added to the mixture in each well and kept at 37°C for 30 min. The absorbance values of each well were measured at 410 nm, and IC_{50} (µg/mL) values were determined. The possible inhibition activities of the *Nepeta* extracts (1024–0.5 µg/mL) against α-glucosidase (Mayur et al., 2010) inhibitory activity were measured by previously described methods with slight modifications (Başar et al., 2023). In a 96-well plate, 10 µL of samples of different concentrations or acarbose and 25 µL of 0.2 U α-glucosidase (in 20 mM pH 6.9 potassium phosphate buffer) solution were mixed into each well until homogeneous. 25 µL of 0.5 mM p-NPG and 20 µL of 20 mM pH 6.9 potassium phosphate buffer were added to the mixture in each well. The samples were kept at 37°C for 12 minutes. 100 µL of 0.2 M Na2CO3 solution was added to the mixture in each well and mixed until homogeneous. The absorbance values of each well were measured at 410 nm, and IC_{50} (µg/mL) values were determined.

The possible inhibition activities of the *Nepeta* extracts (1024–0.5 µg/mL) against tyrosinase (Addar et al., 2019) inhibitory activity were measured by previously described methods with slight modifications (Başar et al., 2023). In a 96-well plate, 10 µL of samples of different concentrations or kojic acid were into each well, 20 µL of 150 U tyrosinase (in 0.1 M pH 6.8 potassium phosphate buffer) and 20 µL of 0.1 M pH 6.8 potassium phosphate buffer were mixed until homogeneous and incubated at 37°C. It was kept for 10 minutes. 20 µL of 5 mM L-DOPA solution was added to the mixture in each 96-well. The absorbance values of each well were measured at 475 nm, and IC_{50} (µg/mL) values were determined.

2.4. Antibacterial Activity

Three gram-positive (*Bacillus cereus* CCM99, *Staphylococcus aureus* ATCC 25213, *Enterococcus faecalis* ATCC 29212) and three gram-negative (*Klebsiella pneumoniae* ATCC 10031, *Escherichia coli* ATCC 25922, *Psedomonas aeruginosa* ATCC 15442) bacteria were tested by the *Nepeta* extracts for antibacterial activity. In the Biochemistry Research Laboratory (Ondokuz Mayis University, Department of Chemistry), both newly developed and pre-existing microorganisms were cultivated.

2.4.1. Disc diffusion method (DDM)

The agar DDM was used with an MHA (Mueller Hinton Agar) medium and incorporated into the MHA for *Nepeta* extracts diffusion (Reller et al., 2009). The turbidity of the newly cultured bacteria was adjusted to 0.5 McFarland standards (10^8 CFU/mL). 0.5 McFarland gram-negative and positive bacteria were spread on MHA. After impregnating 6 mm sterile discs with 40 µL of extracts or antibiotics (amoxicillin and tetracycline), they incubated at 37 °C for 16-18 hours. The antimicrobial activity of the apparent transparent zone of inhibition diameter was measured around the wells and compared with antibiotic drugs to evaluate the sensitivity of the strains.

2.4.2. Minimum inhibition concentration (MIC) method

The antimicrobial activities of *Nepeta* extracts using the MIC method were performed for each bacterium (Andrews, 2001). The activity was performed using 96-well microplates in a cationic MHB medium containing $MgCl_2.6H_2O$ and $CaCl_2.2H_2O$. Before applying dilution, 100 µL of cationic MHB and 100 µL of extracts (or antibiotics) were combined evenly in the well. After adding 5 µL of bacterial solution (including cationic MHB and a McFarland value of 0.5) to each well, they were incubated for 16–18 hours at 37 °C after being held at +4 °C for two hours. The MIC was expressed as µg/mL. Solvents were utilized as the

negative control, and amoxicillin and tetracycline as the positive controls.

2.5. Statistical analysis

SPSS 20.0, an IBM statistical package for social studies, was used to examine the data. Multiple comparisons were conducted using One-Way ANOVA-Tukey HSD^{a,b} by the collected data. Statistical significance was determined by comparing the values' statistical importance to that of the activity analysis result group. A significant value of $p<0.05$ was agreed upon.

3. Results and Discussion

3.1. Enzyme Inhibitory Activities

The *Nepeta* species extracts were evaluated for inhibition activities, the results of which were depicted in Table 1. Thiourea, acetazolamide, kojic acid, galantamine, orlistat, and acarbose were used to compare the inhibitory enzyme potential of *Nepeta* extracts. In the present work, methanol-chloroform extracts of *N. trachonitica* presented considerable urease inhibition capacity with the IC_{50} of 1.51±0.28 µg/mL (Table 1). Fareed et al. (2013) found that the urease inhibition activities of chloroform (C), ethyl acetate (EA), methanol (M), water (W), and n-hexane (H) extracts of *N. praetervisa* were determined as 45.00, 68.00, 10.00, 25.00 and 30.00%, respectively. At the different concentrations of extracts and acetazolamide, the CA inhibition activity of *N. aristata* extract was determined to be the most effective (Table 1).

Table 1. Enzyme inhibition results of methanol-chloroform extracts of six *Nepeta* species

Sample	Inhibition, IC_{50} (µg/mL)							
	Urease	AChE	BChE	CA	α-Amylase	α-Glucosidase	Lipase	Tyrosinase
N. aristata	1.65±0.34^{a}	33.96±1.20^{d}	-	5.29±0.51^{b}	3.48±0.51^{a}	11.89±0.42^{a}	4.84±0.21^{a}	6.23±0.25^{b}
N. baytopii	4.95±0.00^{c}	-	-	12.50±0.40^{c}	101.39±0.45^{f}	41.30±0.43^{e}	8.15±0.28^{bc}	29.96±0.00^{f}
N. italica	-	25.94±1.11^{c}	9.35±0.88^{a}	19.35±0.27^{d}	27.71±0.38^{b}	14.59±0.00^{b}	16.48±0.51^{d}	13.20±0.86^{c}
N. nuda	5.29±0.00^{d}	4.06±0.19^{a}	-	148.46±0.94^{f}	67.17±0.69^{e}	158.58±0.52^{f}	4.24±0.33^{a}	69.21±0.14^{g}
N. stenantha	2.79±0.30^{b}	13.92±0.33^{b}	-	12.63±0.04^{c}	63.70±0.75^{d}	33.25±0.81^{d}	41.83±0.10^{e}	22.57±0.53^{d}
N. trachonitica	1.51±0.28^{a}	3.18±0.09^{a}	-	33.85±0.17^{e}	39.30±0.46^{c}	21.02±0.89^{c}	10.16±0.38^{c}	2.05±0.02^{a}
Standards	9.97±0.00^{e} (Thiourea)	38.47±0.00^{e} (Galantamine)	22.20±0.00^{b} (Galantamine)	2.35±0.06^{a} (Acetazolamide)	25.93±0.16^{b} (Acarbose)	13.50±0.04^{ab} (Acarbose)	6.30±1.01^{ab} (Orlistat)	25.83±1.58^{e} (Kojic Acid)

Data are means of three repetitions ± standard deviation (SD), Different superscripts (a–e) in the same column indicate significant differences between the tested extracts ($p<0.05$, as determined by ANOVA).
Abbreviations: AChE: Acetylcholinesterase; BChE: butyrylcholinesterase; CA: Carbonic anhydrase.

In this work, the AChE inhibitory effect of *N. trachonitica* was determined in the highest inhibition and exhibited in Table 1. Zengin et al. (2021) determined the AChE inhibitions of H, EA, M, and water/methanol (W/M) extracts of *N. baytopii* as 3.97±0.32, 4.57±0.06, 3.65±0.11 and 2.68±0.07 mg galantamine equivalent (GALAE)/g. The previous study conducted with *N. baytopii* determined that AChE inhibition of methanol and water/methanol extracts was low. However, in our study, no inhibition was observed by the methanol-chloroform extract of the same plant. The reason for observing this result may be due to the phenolic compounds, fatty acids, or volatile compounds contained in this extract. Acquaviva et al. (2023) obtained the AChE inhibitions of H, dichloromethane (DCM), EA, ethanol (E), ethanol-water (E-W), and water (W) extracts of *N. italica* as 3.02±0.47, 2.93±0.01, 2.69±0.17, 2.88±0.03, 2.80±0.02 and 0.04±0.01 mg GALAE/g. The previous study conducted with *N. italica* determined that AChE inhibition of different extracts was low. Nonetheless, in our study, low inhibition was also observed by the methanol-chloroform extract of the same

plant. This may be due to the compounds contained in this extract. Sarikurkcu et al. (2019) found that AChE inhibition of *N. nuda* and *N. cadmea* methanol extracts were determined as 1.26±0.01 and 1.35±0.02 mg GALAE/g extract. In the previous study with *N. nuda*, it was found that the AChE inhibition of the methanol extract was low. However, our study noted that the methanol-chloroform extract of a different species of *N. nuda* had high inhibition. This may be because both plants are high in and their species are different, or the components they contain may be different due to the different extracts.

BChE inhibition potential activities were not observed in all *Nepeta* extracts. In the *N. italica* extract, the IC_{50} value of the inhibition effect of BChE is higher than in galantamine, but the BChE inhibition effect is in no other extract (Table 1). Zengin et al. (2021) determined the BChE inhibitions of H, EA, and M extracts of *N. baytopii* as 6.93±1.14, 10.85±0.73, and 2.98±0.46 mg GALAE/g. The previous study with *N. baytopii* determined low BChE inhibition of methanol and water/methanol extracts. However, the identical plant's methanol-chloroform extract showed no inhibition in our investigation. The reason for observing this result may be due to the phenolic compounds, fatty acids, or volatile compounds contained in this extract. Acquaviva et al. (2023) obtained the BChE inhibitions of H, DCM, EA, E, and E-W extracts of *N. italica* as 1.88±0.20, 2.40±0.37, 1.79±0.40, 4.01±0.28, and 1.24±0.07 mg GALAE/g. The previous study conducted with *N. italica* determined that BChE inhibition of different extracts was low. However, in our study, high inhibition was observed by the methanol-chloroform extract of the same plant. This may be due to the compounds contained in this extract. Akdeniz et al. (2020) found that BChE inhibition of root, stem, leaf, flower, and the mixture of ethanol extracts of *N. heliotropifolia* was between 14.58±0.87 to 54.79±0.77%. Also, *N. congesta* was determined as between 4.50±0.81 and 48.35±0.77%.

The extract of *Nepeta* species exhibited a remarkable α-amylase inhibition activity and found the highest inhibition in *N. aristata* (Table 1). Zengin et al. (2021) determined the α-amylase inhibitions of H, EA, M, W/M, and W extracts of *N. baytopii* as 0.66±0.01, 0.84±0.02, 0.67±0.02, 0.50±0.01, and 0.10±0.01 mmol acarbose equivalent (ACAE)/g. The previous study conducted with *N. baytopii* determined that α-amylase inhibition of methanol and water/methanol extracts was moderate. However, in our study, low inhibition was observed by the methanol-chloroform extract of the same plant. The reason for observing this result may be due to the phenolic compounds, fatty acids, or volatile compounds contained in this extract. Acquaviva et al. (2023) obtained the α-amylase inhibitions of H, DCM, EA, E, E-W, and W extracts of *N. italica* as 0.37±0.02, 0.58±0.02, 0.51±0.01, 0.33±0.01, 0.25±0.01 and 0.05±0.01 mmol ACAE/g. The previous study conducted with *N. italica* determined that the α-amylase inhibition of different extracts was good. However, in our investigation, the same plant's methanol-chloroform extract also showed comparable inhibition when used with the usual medication. This may be due to the compounds contained in this extract. Malik, Roy [12] found that α-amylase inhibition of ethanol, methanol, and water extracts of *N. cataria* were 29.37±1.45, 52.03±0.71, and 16.59±1.79%, respectively. In reference, Sarikurkcu et al. (2019) showed that α-amylase inhibition of *N. nuda* and *N. cadmea* methanol extracts were 0.36±0.01 and 0.24±0.01 mg ACAE/g extract. In the previous study with *N. nuda*, it was found that the α-amylase inhibition of the methanol extract was high. However, our study noted that the methanol-chloroform extract of a different species of *N. nuda* had low inhibition. This may be because both plant species are different, or

the components they contain may be different due to the different extracts.

The α-glucosidase inhibition potency of *N. aristata* was determined to be the most effective at 11.89±0.42 µg/mL (Table 1). Zengin et al. (2021) determined the α-glucosidase inhibitions of H, EA, M, W/M, and W extracts of *N. baytopii* as 7.87±0.02, 7.76±0.01, 8.15±0.08, 0.61±0.04, and 1.06±0.09 mmol ACAE/g. In the previous study conducted with *N. baytopii*, it was obtained that α-glucosidase inhibition of methanol extract was high. However, the methanol-chloroform extract of the same plant showed considerable inhibition in our investigation. The reason for observing this result may be due to the phenolic compounds, fatty acids, or volatile compounds contained in this extract. Acquaviva et al. (2023) obtained the α-glucosidase inhibitions of H, DCM, EA, E, E-W, and W extracts of *N. italica* as 4.91±0.01, 0.14±0.01, 0.53±0.07, 5.38±0.01, 5.60±0.01 and 0.94±0.04 mmol ACAE/g. The previous study conducted with *N. italica* determined a low α-glucosidase inhibition of different extracts. However, the methanol-chloroform extract of the same plant also showed significant inhibition in our investigation. This may be due to the compounds contained in this extract. Sarikurkcu et al. (2019) found that α-glucosidase inhibition of *N. nuda* and *N. cadmea* methanol extracts were determined as 3.67±0.02 and 2.02±0.01 mg ACAE/g extract. In the previous study with *N. nuda*, it was found that the α-glucosidase inhibition of the methanol extract was high. However, our study noted that the methanol: chloroform extract of a different species of *N. nuda* had low inhibition. This may be because both plant species are different, or their components may differ due to the different extracts.

In our study, the inhibition effect of lipase was observed to be the most effective in the *N. nuda* extract (Table 1). Roh and Jung (2012) found that lipase inhibition of ethanol extract of *N. japonica* was determined as 37.3±2.5%.

In our work, *N. trachonitica* extract exhibited an effective tyrosinase inhibition activity with the IC_{50} of 2.05±0.02 µg/mL (Table 1). Zengin et al. (2021) determined the tyrosinase inhibitions of H, EA, M, W/M, and W extracts of *N. baytopii* as 77.84±1.83, 78.60±1.58, 96.06±0.70, 95.31±1.77, and 6.15±1.02 mg kojic acid equivalent (KAE)/g. The previous study conducted with *N. baytopii* determined that tyrosinase inhibition of methanol extract was high. Nevertheless, our investigation found that the methanol-chloroform extracts of six *Nepeta* species inhibited the medicine in a similar way to the conventional treatment. The reason for observing this result may be due to the phenolic compounds, fatty acids, or volatile compounds contained in this extract. Acquaviva et al. (2023) obtained the tyrosinase inhibitions of H, DCM, EA, E, E-W, and W extracts of *N. italica* as 72.12±2.44, 49.91±1.32, 56.29±7.29, 64.61±0.94, 59.52±1.31 and 15.04±0.22 mg KAE/g. The previous study conducted with *N. italica* determined that tyrosinase inhibition of different extracts was high. Yet in our investigation, the methanol-chloroform extract of six *Nepeta* species also showed significant inhibition. This may be due to the compounds contained in this extract. Akdeniz et al. (2020) found tyrosinase inhibition capacity of ethanol extracts of *N. heliotropifolia* root, stem, and leaf between 51.78±0.82 to 22.04±1.12%, and also *N. congesta* extracts exhibited between 26.25±1.51 and 16.27±1.14%.

3.2. Antibacterial Activities

The antibacterial activity of six *Nepeta* species extracts against three-gram negative bacteria and three-gram positive bacteria was investigated, and different *Nepeta* extracts varied in antibacterial potential. The antimicrobial activities of six *Nepeta* species extracts were illustrated as

the mm and µg/mL values in Table 2. In this work, *N. aristata* extract showed effective activity against *P. aeruginosa* and *K. pneumoniae* bacteria while not showing antibacterial activity on *E. coli*, *E. faecalis*, and *S. aureus,* according to DDM. However, other *Nepeta* extracts did not show antibacterial activity on *P. aeruginosa*, *E. coli*, *K. pneumoniae*, *E. faecalis*, and *S. aureus*. In addition, *Nepeta* extracts had a strong against *B. cereus* bacteria. In a previous research, Köksal et al. (2017) showed that the antimicrobial activities against *E. coli*, and *P. aeruginosa* in DDM, measured by absorbing 90 µL of *N. trachonitica* ethanol extract at a concentration of 20 mg/mL onto the disc, was 12.00±1.24, and 9.00±0.00 mm, respectively. A previous study showed that *N. trachonitica* has antibacterial properties on the bacteria we used. However, our study determined that it had no antibacterial effect on solid media. The reason for this is that the extract solvent is different because the secondary components in the plant allow different components to pass into the extract in different solvents. In reference, Ahmad et al. (2020) showed that antimicrobial activities in DDM of extracts of *N. deflersiana* prepared in different polarities were determined the ethanol extract against *P. aeruginosa* as 14.00±0.47 mm, the ethanol, acetone, and ethyl acetate extract against *K. pneumoniae* as 11.00±0.65, 13.00±0.82, and 13.00±0.70 mm, respectively, and the ethyl acetate extract against *E. coli* as 16.00±0.22 mm. A previous study showed that solvent extracts of *N. deflersiana* of different polarities had antibacterial properties on the bacteria we used in our study. However, we found that the plants we used in our study did not have an antibacterial effect on solid media. The reason for this is the difference in the components in the extract when a single solvent is used and the components in the extract when a solvent mixture is used.

Table 2. Antimicrobial activity results of methanol-chloroform extracts of six *Nepeta* species

Antibacterial properties	Samples	Gram-negative bacteria			Gram-positive bacteria		
		E. coli	*P. aeruginosa*	*K. pneumoniae*	*E. faecalis*	*B. cereus*	*S. aureus*
DDM, mm	*N. aristata*	-	28.00±2.83	14.00±2.83	-	17.00±0.00	-
	N. baytopii	-	-	-	-	14.00±1.41	-
	N. italica	-	-	-	-	12.00±4.24	-
	N. nuda	-	-	-	-	10.00±1.41	-
	N. stenantha	-	-	-	-	10.00±0.50	-
	N. trachonitica	-	-	-	-	28.00±0.00	-
	Amoxicillin	21.00±5.66	-	-	-	27.00±0.00	8.00±0.00
	Tetracycline	31.00±0.00	32.00±0.00	31.00±0.00	32.00±0.00	37.00±0.00	31.00±0.00
MIC, µg/mL	*N. aristata*	256	128	256	64	512	256
	N. baytopii	128	256	128	128	1024	512
	N. italica	512	512	1024	512	256	1024
	N. nuda	256	128	256	128	1024	512
	N. stenantha	64	128	256	128	512	256
	N. trachonitica	128	128	256	64	1024	512
	Amoxicillin	1024	1024	1024	1024	<1	1024
	Tetracycline	8	<2	8	8	<0.5	8

Abbreviations: DDM: Disc diffusion method; **MIC:** Minimum inhibition concentration

The following six *Nepeta* extracts exhibited effective antimicrobial activity against all six bacteria used, which are *N. aristata* (64 to 512 µg/mL), *N. baytopii* (128 to 1024 µg/mL); *N. italica* (256 to 1024 µg/mL), *N. stenantha* (64 to 512 µg/mL), *N. nuda* (128 to 1024 µg/mL) (64 to 1024 µg/mL). These results prove bacteria were resistant to methanol: chloroform extracts were used. All six *Nepeta* extracts for which the MIC test was applied had antimicrobial properties, and these results were consistent with previous reports. In reference, Ahmad et al. (2020) showed that antimicrobial activities in MIC of *N. deflersiana* prepared in ethanol extract were determined as 250 µg/mL

against *P. aeruginosa*, and *K. pneumoniae*.

A previous study showed that solvent extracts of *N. deflersiana* of different polarities had antibacterial properties on the bacteria we used in our study. However, we determined that the plants we used in our study had a higher antibacterial effect against *P. aeruginosa* bacteria and antibacterial activity against *K. pneumoniae* bacteria, similar to the *N. deflersiana*. This is because of the difference in the components in the extract when a single solvent is used and the components in the extract when a solvent mixture is used.

4. Conclusion

Since the enzyme inhibition and antibacterial activities of *N. aristata*, *N. stenantha*, and the enzyme inhibition of *N. trachonitica* have not been investigated, this study was the first of its kind. *N. aristata*; urease (1.65±0.34 µg/mL), AChE (33.96±1.20 µg/mL), α-amylase (3.48±0.51 µg/mL), α-glucosidase (11.89±0.42 µg/mL), lipase (4.84±0.21 µg/mL), tyrosinase (6.23±0.25 µg/mL), *N. baytopii*; urease (4.95±0.00 µg/mL), *N. italica*; AChE (25.94±1.11 µg/mL), BChE (9.35±0.88 µg/mL), tyrosinase (13.20±0.86 µg/mL), *N. nuda* ; urease (5.29±0.00 µg/mL), AChE (4.06±0.19 µg/mL), lipase (4.24±0.33 19 µg/mL), *N. stenantha*; urease (2.79±0.30 µg/mL), AChE (13.92±0.33 µg/mL), tyrosinase (22.57±0.53 µg/mL) and *N. trachonitica*; urease (1.51±0.28 µg/mL), AChE (3.18±0.09 µg/mL), tyrosinase (2.05±0.02 µg/mL) showed the highest enzyme inhibition effect than standards. *N. aristata* showed the highest effect in enzyme inhibition. Additionally, antibacterial activity in DDM showed an effect against *N. aristata* as *P. aeruginosa*, *K. pneumoniae*, *B. cereus* bacteria, while other plants showed an effect only against *B. cereus* bacteria. The MIC method determined that almost all plants were effective against all bacteria. For this reason, it was predicted that plants would lead to further studies.

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Author Contribution

Semiha Yenigun, Yunus Basar, Yasar Ipek, Mesut Gok: Writing–Review, Visualization & Editing. Semiha Yenigun: Antibacterial and Enzyme Inhibitor Activities, Writing–Review. Tevfik Ozen: Biologic Studies, Writing–Review, Supervision. Lutfi Behcet: Sourcing plants. Ibrahim Demirtas: Writing–Review, Supervision.

Conflicts of Interest

The authors declare no conflict of interest.

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Antioxidant and Antimicrobial Properties of Different Silver Nanoparticles Produced by Green Synthesis

Leyla ERCAN[1*], **Ümmügülsüm TÜKENMEZ EMRE**[2]

[1] Mardin Artuklu University, Department of Health, Culture and Sports, 47000, Mardin, Turkiye
[2]Mardin Artuklu University, Vocational School of Health Services,
Department of Medical Services and Techniques, Mardin, Turkiye
*Corresponding author: leylaercan@artuklu.edu.tr

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Abstract

Nanoparticles are materials that can be used in a wide range from medicine to industry. In recent years, especially the fruits, flowers, leaves, and roots of plants have come to the fore in nanoparticle synthesis because they are environmentally friendly and economical. *Rosa damascena* is a plant that is used both in foods such as jams, desserts, and beverages, and in many cosmetic products such as perfumes, creams, and lotions due to its pleasant smell and taste. In addition to its pleasant aroma, valuable bioactive components are among the main uses of these flowers. *Berberis crataegina* fruit is a wild shrub fruit that can be consumed by humans but is unknown to many. This study aims to examine the antibacterial and antioxidant properties of silver nanoparticles produced from *Rosa damascena* flowers and *Berberis crataegina* fruits, both of which are rich in anthocyanins. For this, first of all, the produced silver nanoparticles were evaluated using SEM and SEM EDX. In addition, the size and properties of the nanoparticle were defined by performing XRD and FTIR analyses. Furthermore, these nanoparticles were subjected to antioxidant and antibacterial analyses. As a result, two different silver nanoparticles with high antioxidant properties were synthesized from both. However, nanoparticles synthesized from *R. damascena* flowers showed more antimicrobial activity than nanoparticles synthesized from *Berberis crataegina* fruits.

Key Words: Antimicrobial activity, Antioxidant capacity, *Berberis crataegina, R. damascena*

1. Introduction

Nanotechnology has significantly impacted the therapeutic, diagnostic, biomedical, industrial, environmental protection, and scientific research fields throughout recent decades (Farokhzad & Langer, 2009; Guerra et al., 2018). Nanoparticles (NP) are particles that make up nanomaterials and have diameters between 1 and 100 nm (Algotiml et al., 2022). For a wide range of biological applications, NP provides a very appealing platform (Jain et al., 2021).

There are many different kinds of metal NP, such as iron, platinum, gold, thallium, silver, titanium, cerium, and others (Piñón-Segundo et al., 2013). Silver nanoparticles (AgNPs) are one of the most important and intriguing nanomaterials among the several metallic NPs used in biological applications (Karmous et al., 2020; Yesilot & Aydin, 2019).

Numerous methods, including physical, chemical, and biological ones, can be used to produce nanoparticles (Chen et al., 2008). Various AgNP kinds, forms, sizes, and crystal materials have been created using various physicochemical techniques as a result of research and applications. These physicochemical techniques, however, have major drawbacks, including being costly, costing a lot of time and energy, and also producing extremely substantial chemical by-products (Bagheri & Banihashemi, 2015; Ueno et al., 2015). The biological synthesis of the nanoparticle, or "green synthesis," of AgNPs, on the other hand, is a natural, affordable, and eco-friendly procedure (Ali et al., 2015; Maddinedi et al., 2017). Furthermore, green-produced nanoparticles have great yields, solubility, and stability. AgNPs may create well-defined sizes and shapes under ideal circumstances for traditional study using biological techniques, which are straightforward, quick, non-toxic, dependable, and environmentally friendly (Rai et al., 2016; Yesilot & Aydin, 2019).

Recently, green biosynthesis techniques have become quite popular for creating nanoparticles employing a variety of biological systems, including yeast, fungus, bacteria, and plant extracts (Khan et al., 2017; Kowshik et al., 2002; Shahverdi et al., 2007). The most well-known of them is the manufacture of controlled-physicochemical AgNPs based on plant extracts (Dipankar & Murugan, 2012; Keshari et al., 2020).

The potential for natural antioxidants and antibacterial agents found in plants makes them very interesting (Stanković et al., 2016). Customers and companies desire to switch from synthetic antioxidants to natural ones due to their potential harm (Branen, 1975; Pokorný, 1991).

In the past, there has been a great deal of interest in the plant extracts that were used as medicines by ancient civilizations (Grabley & Thiericke, 1999; Uzun et al., 2004), and antimicrobial compounds derived from plants are still a valuable resource in the battle against infectious diseases today. Various studies have shown a significant increase in the incidence of bacterial resistance to many antibiotics (Finch, 1998; Kunin, 1993). Therefore, it has become necessary to investigate the chemical compounds found in plants (Nascimento et al., 2000; Phillipson, 1991; Xu & Lee, 2001).

Rosa damascena is a member of the Rosaceae family and is widely distributed in Turkey, Bulgaria, Spain, France, Syria, India, Morocco, Tunisia, Saudi Arabia, and China (Chevallier, 1996; Commission, 2015; Liu et al., 2020; Takahashi et al., 2019). *R. damascena* is mostly used as decoration, fragrance, food additive, and medicinal treatment such as constipation, antistress activity, stomach ulcer, and cardiovascular diseases (Akram et al., 2020; Naveena & Thamaraiselvi, 2020). *R. damascena* flower essential oil is valuable, and the main producers of rose essential oil in the world are Bulgaria, Turkey, and Morocco (Mahboubi, 2015; Tosun et al., 2002). Additionally, Rosa *damascena* exhibits antibacterial, antioxidant, antitussive, anti-HIV, antidepressant, hypoglycaemic, anti-inflammatory, and analgesic properties (Akram et al., 2020; Labban & Thallaj, 2020; Naveena & Thamaraiselvi, 2020). Various useful compounds, including vitamins, flavonoids, carotenes, carbohydrates, organic acids, trace elements, phenolic and aromatic compounds are found in roses, according to research in the literature. Most of these compounds have been reported to have valuable properties such as antimutagenic, antioxidant, antimicrobial, anticancer, and anti-inflammatory, retarding or inhibiting oxidation processes (Bitis et al., 2017; Crespo et al., 1999; Jabłońska-Ryś et al., 2009; Kähkönen et al., 1999; Kaisoon et al., 2011; Krishnaiah et al., 2011; Kumar et al., 2009; Lamien-Meda et al., 2008; Mlcek & Rop, 2011; Nishihara & Nakatsuka, 2011; Yang & Shin, 2017).

Berberis crataegina is from the Berberidaceae family and has small oval leaves and yellow flowers. The fruit ripens in late summer and takes on a dark purple-to-black color (Baytop, 1963; Işikli & Yilmaz, 2014). In Turkey, it is called 'karamuk' or 'kadın tuzluğu' by the people (Baytop, 1994; Işikli & Yilmaz, 2014). *B. crataegina* fruit, which grows wild in Asia and Europe, has strong antioxidant properties. It is also rich in phenolic compounds such as chlorogenic acid, gallic acid, vanillic acid, p-coumaric acid, 4-hydroxybenzoic acid, syringic acid, trans-ferulic acid, caffeic acid, and sinapic acid (Eroğlu et al., 2020).

Particularly in the last decade, interest in phyto-nanotechnology has increased tremendously (Mehata, 2015). Plants can be easily processed as they are non-toxic compared to other biological sources and are therefore suitable sources for the synthesis of AgNPs. Many plants have been used to synthesize nanomaterials (Gardea-Torresdey et al., 2003). In this work, AgNP was produced using green biosynthesis using *B. crataegina* fruit and *R. damascena* flowers. The current study has aimed to characterize the physicochemical properties of synthesized *R. damascena* AgNPs (RAgN) and *B. crataegina* AgNPs (BAgN) and evaluate their antioxidant, and antimicrobial activities.

2. Material and Methods

2.1. Nanoparticle synthesis

40 grams of dried *R. damascena* samples were combined with 600 ml of distilled water. It was mixed on a magnetic stirrer at 60°C at 800 rpm for 4 hours. 600 ml of a 1 mM $AgNO_3$ solution was combined with 300 ml of extract that had been filtered via filter paper. A brown discoloration similar to mud color was observed after 24 hours of incubation at room temperature. The range of 300 to 600 nm was utilized to measure absorbance. It was then centrifuged at 5000 rpm for 20 minutes. The precipitate was separated, and it was then dried for six hours at 80°C in an oven.

B. crataegina fruit was collected from Kayseri at the end of summer. To 50 grams of dried *B. crataegina* fruit, 750 ml of distilled water was added. It was stirred for 4 hours at 650 rpm at 60°C on a magnetic stirrer. 400 ml of extract filtered through filter paper and 800 ml of 1 mM $AgNO_3$ solution were mixed. The solution was left at room temperature for 24 hours. Absorbance was measured between 300 - 600 nm. The solution was centrifuged at 5000 rpm for 20 minutes. The precipitate was separated, and it was then dried for six hours at 80°C in an oven.

2.2. Characterization of nanoparticles

SEM and EDX analyses were carried out with a JEOL JSM 6510 Scanning Electron Microscope to determine the elemental composition and shape of the nanoparticles. XRD analyses were carried out with the RIGAKU ULTIMA IV X-Ray Diffraction Spectrometer. FTIR analyses were performed with a Perkin Elmer Spectrum 100 FT-IR Spectrophotometer.

2.3. Antioxidant capacity assays

Three distinct techniques were used to conduct *in vitro* antioxidant capacity testing (DPPH, CUPRAC, and ABTS). Antioxidant capacity analyses were made by modifying the DPPH method of Makhlouf-Gafsi et al. (Makhlouf-Gafsi et al., 2018). For this, methanol was used to produce a 100µM DPPH radical solution. The samples generated at various concentrations were mixed with 3.9 ml of DPPH radical. The radical scavenging rate was then computed by recording the absorbance at 517 nm.

The samples generated at various concentrations were mixed with $7.5x10^{-3}$M methanolic neocuprin solutions, 0.01 M $CuCl_2$ solution, and 1M CH_3COONH_4 (pH: 6.5) buffer. After half an hour of incubation, copper ion reduction capacity was

determined by absorbance measurement at 450 nm. Antioxidant analysis according to the CUPRAC method was performed according to the method of Apak et al. (Apak et al., 2007).

By combining 2.45 mM potassium persulfate solution with 2 mM ABTS solution, an ABTS radical was created. With the help of 0.1 M phosphate buffer (pH 7.4), the absorbance of the radical solution was stabilized to a range of 0.750 to 0.800 nm. To the samples prepared at various concentrations, 2 ml of ABTS radical was added. At 734 nm, absorbances were measured following a 30-minute incubation period (Bursal et al., 2013; Re et al., 1999).

2.4. Antimicrobial activity test

Antimicrobial activity analyses by Disk Diffusion technique (National Committee for Clinical Laboratory Standards., 1997) were performed on *Klebsiella pneumoniae* ATCC 13883, *Escherichia coli* ATCC 11229, *Pseudomonas aeruginosa* ATCC 9027, *Staphylococcus aureus* ATCC 25923 and *Candida albicans* ATCC 10231. Thus, the effect of nanoparticles on gram-negative bacteria, gram-positive bacteria, and fungi was investigated.

2.5. Statistical Analysis

Trials were conducted in triplicate. Statistical analysis was examined by one-way ANOVA. As a result, Both RAgN and BAgN showed significant antioxidant activity and antimicrobial activity. In both analyses, the significance values were $p<0.05$.

3. Results and Discussion

Pictures of the *Rosa damascena* flowers and *B. crataegina* fruits are given in Figure 1.

R. damascena flowers *B. crataegina* fruits

Fig. 1. *Rosa damascena* flower and *Berberis crataegina* fruit

UV-VIS spectrum of silver nanoparticles synthesized from *R. damascena* flower and *B. crataegina* fruit between 300-600 nm is given in Figure 2.

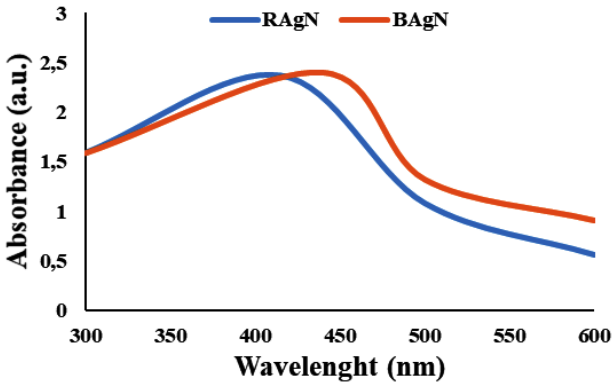


Fig. 2. UV-VIS spectrum of RAgN and BAgN

According to the UV-VIS results, characteristic surface plasmon resonance (SPR) peaks of silver nanoparticles, corresponding to silver nanoparticle biosynthesis, were observed at 415 and 440 nm (Arshad et al., 2022).

SEM images of silver nanoparticles synthesized from *R. damascena* are given in Figure 3. Although RAgN nanoparticles are extremely small, *R. damascena* gave very efficient results in the synthesis of silver nanoparticles. *R. damascena* gave a reaction product similar to the sludge and slime appearance with $AgNO_3$ solution.

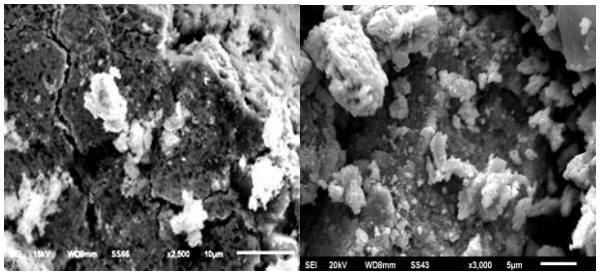
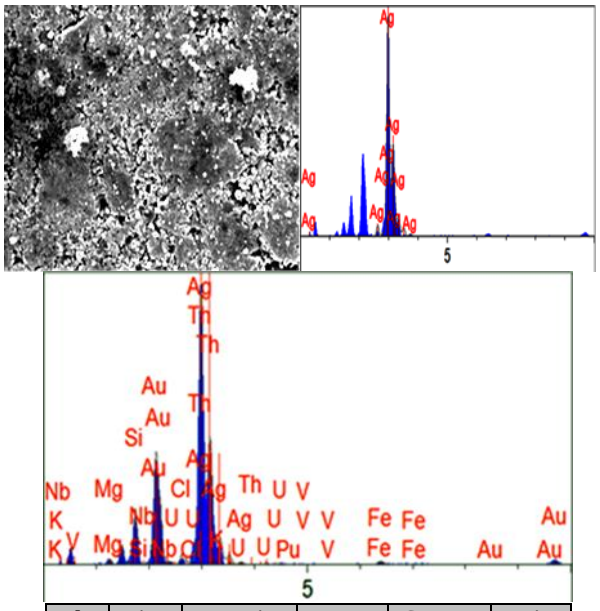


Fig. 3. SEM images of RAgN

SEM-EDX results and graph of RAgN synthesized from *R. damascena* are given in Figure 4.



Elt.	Line	Intensity (c/s)	Error 2-sig	Conc	Units
Mg	Ka	27.58	3.169	0.329	wt.%
Al	Ka	93.12	4.115	0.993	wt.%
Si	Ka	277.56	5.461	2.665	wt.%
V	Ka	3.96	2.935	0.076	wt.%
Fe	Ka	27.88	2.984	0.780	wt.%
Nb	La	84.43	4.287	2.478	wt.%
Ag	La	1,355.48	10.633	55.722	wt.%
Au	La	29.83	2.523	24.456	wt.%
Th	Ma	279.22	6.438	12.501	wt.%
			Total	100.000	wt.%

Fig. 4. SEM-EDX analysis results of RAgN

FTIR analysis results of RAgN are given in Figure 5.

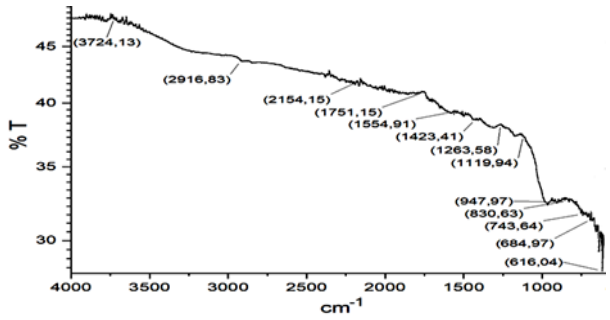


Fig. 5. FTIR analysis results of RAgN

3724.13, 2916.83, 2348,88, 2154.15, 1751.15, 1554.91, 1423.41,1263.58, 1119.94, 947.97, 830.63, 743.64, 684.97, 616.04 cm^{-1} intermolecular bonds gave vibrational peaks. The vibration peak of 3724.13 cm^{-1} in the FTIR analysis result of RAgN indicates the tension of the O-H bond in alcohols and phenols (Hosseini et al., 2020; Şahin et al., 2022). Alkanes' saturated C-H bond is what causes the 2916.83 cm^{-1} signal to appear further, the 2348.88, 2154.15 cm^{-1} vibration peaks are due to the C≡N bonds in the peptide bonds (Şahin et al., 2022). A vibration peak of 1751.15 cm^{-1} indicates the presence of a C=O bond, and a vibration peak of 1554.91 cm^{-1} indicates a C=C bond (Hosseini et al., 2020). 1263.58, 684.97, and 616.04 cm^{-1} peaks indicate the C-O bonding (Periasamy et al., 2022). The FTIR results show the presence of different molecular groups that stabilize the silver nanoparticle (Adebayo-Tayo et al., 2022).

The XRD plot of the RAgN nanoparticle is given in Figure 6.

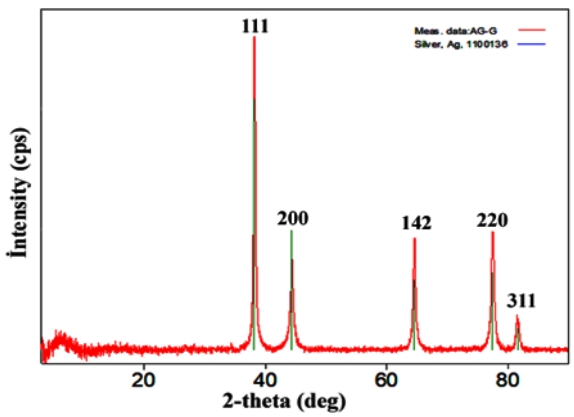


Fig. 6. The XRD graph of RAgN

2θ angles for RAgN were found as 38.141, 44.341, 64.498, 77.436, 81.54. According to the Debye–Scherrer equation: (D=kλ/βcosθ), the crystal size of RAgN was calculated as approximately 20.59 nm, while k: 0.9, λ:0.154 (Giri et al., 2022). A face-centered cubic structure was obtained from the XRD model of RAgN (Giri et al., 2022). The RAgN characteristic peaks (111), (200), (220), and (311) conform to Four Bragg's standard data (JCPDS No. 89-3722) (Baker et al., 2005; Giri et al., 2022; Shameli et al., 2010).

SEM images of (BAgN) silver nanoparticles synthesized from *B. crataegina* fruit are given in Figure 7.

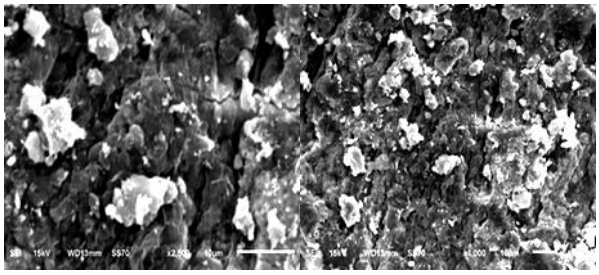
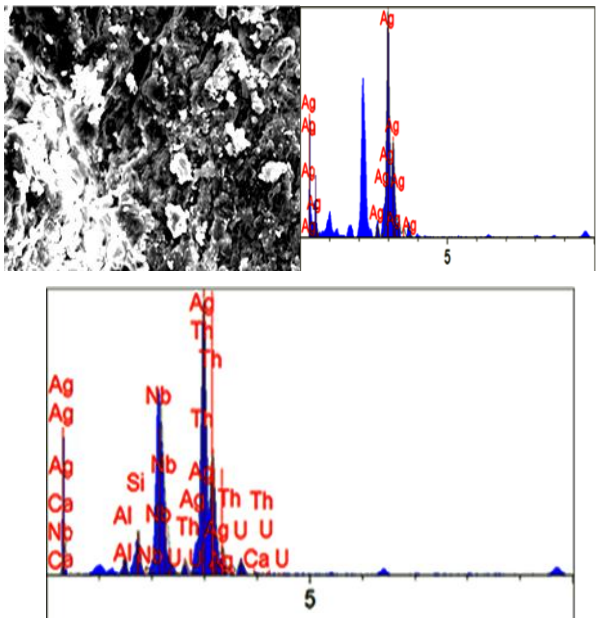


Fig. 7. SEM results of BAgN

SEM-EDX results of silver nanoparticles synthesized from *B. crataegina* fruit are given in Figure 8.



	Line	Intensity (c/s)	Error 2-sig	Conc	Units
Al	Ka	55.92	4.642	1.757	wt.%
Si	Ka	148.45	5.209	3.815	wt.%
Ca	Ka	49.27	4.035	1.916	wt.%
Nb	La	594.39	7.550	25.350	wt.%
Ag	La	1,030.29	9.421	61.453	wt.%
Th	Ma	62.37	4.927	5.709	wt.%
			Total	100.00	wt.%

Fig. 8. SEM-EDX results of BAgN

FTIR analysis results of silver nanoparticles synthesized from *B. crataegina* fruit are given in Figure 9.

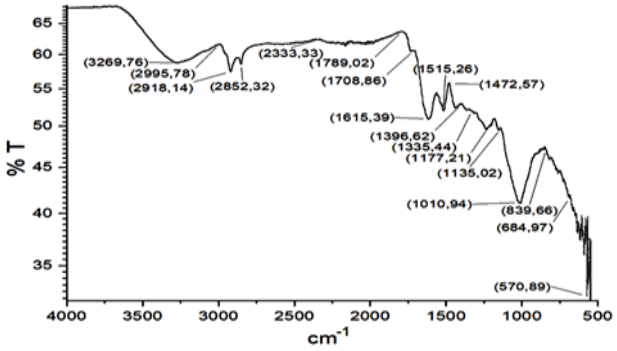


Fig. 9. FTIR results of BAgN

The molecular bonds of BAgN gave vibration peaks in the range of 3269-570 cm^{-1}. There are 3269.76, 2995.78, 2852.32, 2918.14, 2333.33, 1789.02, 1708.86, 1615.39, 1515.26, 1472.57, 1396.62, 1335.44, 1177.21, 1335.44, 1177.21, 1135.02, 1010.94, 839.66, 684.97, and 570.89 cm^{-1} many vibration peaks. 3269.76, cm^{-1} vibration peak shows the tension of the O-H bond (Hosseini et al., 2020). The peaks of 2995.78, 2852.32, and 2918.14 cm^{-1} show the C-H bond, while the vibrational peak of 2333.33 cm^{-1} shows the C≡N bond (Şahin et al., 2022). In addition, vibration peaks of 1789.02, and 1708.86 cm^{-1} may indicate the C=O bond, and vibration peaks of 1515.26, and 1472.57 cm^{-1} may indicate the C=C bond (Hosseini et al., 2020). 1396.62, 1335.44, 1177.21, 1335.44, 1177.21, 1135.02, 1010.94 cm^{-1} peaks indicate the C-O bond (Periasamy et al., 2022). Numerous functional groups that stabilize BAgN are shown by FTIR findings (Adebayo-Tayo et al., 2022). XRD analysis results of BAgN are

given in Figure 10.

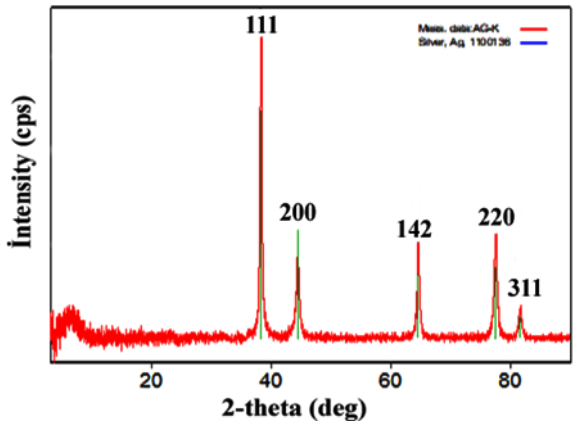


Fig. 10. The XRD graph of BAgN

2θ angles of BAgN; 38.181, 44.357, 64.503, 77.417, 81.683. The crystal size of BAgN was determined to be roughly D=18.04 nm using the Debye-Scherrer equation. In accordance with standard values, the XRD data show that the BAgN (111), (200), (220), and (311) peaks are face-centered cubic (Giri et al., 2022).

Antioxidant analysis results of RAgN and BAgN are given in Table 1.

Table 1. Antioxidant analysis results of RAgN and BAgN

Methods	RAgN (µg/ml)	BAgN (µg/ml)	Trolox (µg/ml)
DPPH(IC_{50})	502,9	618,45	22,29
CUPRAC($A_{0,5}$)	68,96	70,42	18,75
ABTS(IC_{50})	270,21	562,59	15,20

According to the antioxidant analysis results of RAgN, BAgN, and Trolox as standard antioxidants, the concentrations that inhibit 50% of free radicals in both DPPH and ABTS methods (IC_{50}) and the concentrations corresponding to 0.5 absorbance in the CUPRAC method ($A_{0.5}$) are given in Table 1. IC_{50} values were calculated from the graph created using different concentrations and % radical inhibition values of the samples. In the CUPRAC technique, the increase in absorbance is directly proportional to the amount of antioxidant capacity. In this method, a graph was created using absorbance values corresponding to different concentration values. From this graph, the concentration corresponding to 0.5 absorbance ($A_{0.5}$) was calculated. According to these results, RAgN showed better antioxidant properties than BAgN.

The antimicrobial analysis results of RAgN and BAgN are given in Figure 11.

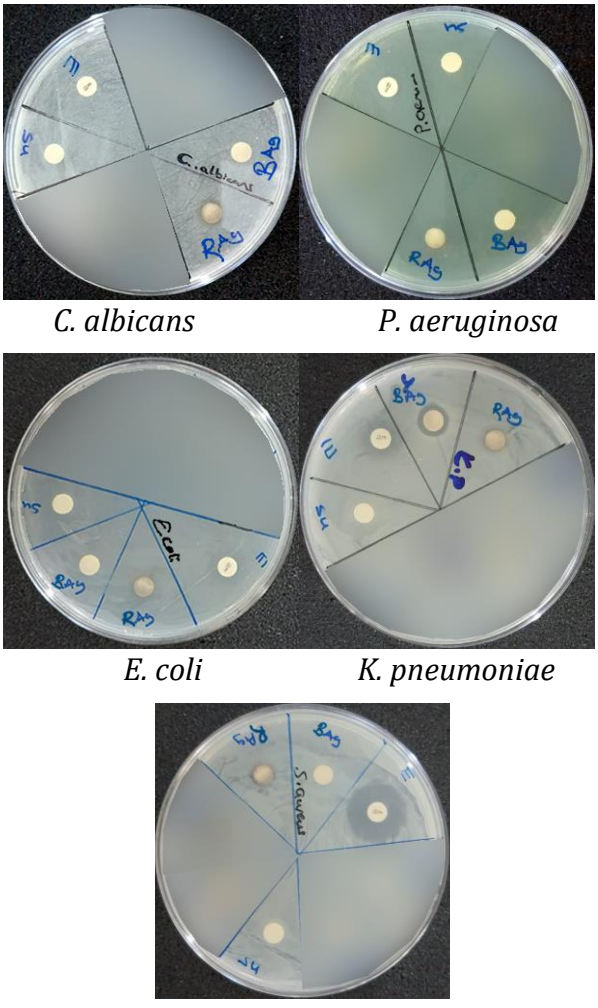


Fig. 11. The antimicrobial analysis results of RAgN and BAgN

Table 2. The antimicrobial analysis results of RAgN and BAgN (zone diameters (mm))

Microorganisms	Erythromycin (15µg)	BAgN (40mg/ml)	RAgN (20mg/ml)
P. aeruginosa	8.0	-	8.5
K. pneumoniae	10.0	7.5	8.0
E. coli	14.0	-	8.0
S. aureus	19.5	-	10.0
C. albicans	14.0	-	8.5

The antioxidant and antimicrobial properties of silver nanoparticles synthesized with two anthocyanin-rich samples gave different results. Silver nanoparticles synthesized from *R. damascena* flowers and *B. crataegina* fruit both exhibited antioxidant properties. RAgN nanoparticles exhibited lower antioxidant properties than Trolox and higher than BAgN. According to these results, the components or phenolic compounds of *R. damascena* that participate in the silver nanoparticle structure may have more antioxidant properties than the silver nanoparticle-forming components of *B. crataegina* fruit. Because bioactive components and phenolic compounds are important in terms of antioxidant properties (Balasundram et al., 2006). *R. damascena* flower extract has been reported to have strong radical scavenging and antioxidant capacity according to DPPH radical reduction and phosphomolybdenum methods (Özkan et al., 2004). In terms of antimicrobial properties, RAgN showed more antimicrobial effects than BAgN (Figure 11 and Table 2). Due to the high antioxidant and antimicrobial properties of RAgN, it has been revealed that *R. damascena* flower extract has been reported to inhibit *Aeromonas hydrophila, Bacillus cereus, Enterobacter aerogenes, Enterococcus feacalis, Escherichia coli, Klebsiella pneumoniae, Mycobacterium smegmatis, Proteus vulgaris, Pseudomonas aeruginosa, P. fluorescens, Salmonella enteritidis, S. typhimurium, Staphylococcus aureus* and *Yersinia enterocolitic* (Özkan et al., 2004). *R. damascena* flowers are an extremely suitable plant for producing nanoparticles with antioxidant and antimicrobial features. In the literature, the ability of silver nanoparticles to fight bacteria synthesized from *Rosa damascena* flowers was investigated and its inhibition effect on *S. aureus, K. pneumonia,* and *E. coli* bacteria was determined (Peron et al., 2021). Along with the outcomes that corroborate these outcomes, the inhibitory effect of silver nanoparticles synthesized from *R. damascena* flowers on *P. aeruginosa* and *C. albicans* was also revealed in this study. While BAgN showed antioxidant properties, it showed an inhibition effect only on *K. pneumoniae* bacteria. Chitosan-based film synthesized from *B. crataegina* fruit previously showed antioxidant and antimicrobial properties (Kaya et al., 2018). In experiments, BAgN showed antioxidant properties, but its antimicrobial effect was low. The antioxidant and antimicrobial properties of *B. crataegina* fruit have been previously examined and it has been reported that it has a strong antioxidant property in addition to its antimicrobial properties (Ercan, 2024). This might be the result of *B. crataegina* fruit's antioxidant components contributing more to the creation of nanoparticles.

Using different samples as reducing agents in silver nanoparticle synthesis can significantly affect the antioxidant and antimicrobial activity. Silver nanoparticles are agents that can be used in the treatment of various wounds and burns (Bold et al., 2022; Gherasim et al., 2020). Because of their antioxidant qualities, silver nanoparticles made from *R. damascena* flowers and *B. crataegina* fruit may be considered potential ingredients in these creams. RAgN can be used safely in antimicrobial products that come into touch with the skin because of its exceptional antibacterial properties.

4. Conclusion

Herbal samples used for silver nanoparticle synthesis by green synthesis affect the activity of the nanoparticle. Silver nanoparticles synthesized from both *R. damascena* and *B. crataegina* fruits are materials that can be safely used in health and cosmetics due to their antioxidant and antimicrobial properties. Especially pink *R. damascena* can stand out because it is a strong antimicrobial in silver nanoparticle synthesis. Silver nanoparticles of this flower and fruit, which are widely found in nature and have a pleasant taste, can be an

important material for products that can be used in different areas such as food, health, and cosmetics.

Disclosure statement

The authors state that they have no conflicts of interest.

Author Contribution

LE, Nanoparticle synthesis, antioxidant analyses and evaluations

ÜTE, Antimicrobial analysis and evaluations

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Lavandula Stoechas extract; Synthesis of Silver Nanoparticles (Nature-Friendly Green Synthesis Method) Characterization Antimicrobial Activity and *In Silico* Molecular Docking Study

Yunus BAŞAR[1*], Aybek YİĞİT[2], Ayşe KARACALI TUNÇ[3], Büşra Merve SARITAŞ[4]

[1]Research Laboratory Practice and Research Center, Igdir University, 7600, Igdir, Turkiye
[2]Department of Pharmacy Services, Tuzluca Vocational School, İğdır University, 7600, Igdir, Turkiye
[3]Department of Medical Microbiology, Faculty of Dentistry, Iğdır University, 7600, Igdir, Turkiye
[4]Department of Dental Services, Vocational School of Health Services, Iğdır University, 7600, Igdir, Turkiye
*Corresponding author : ybasar7631@hotmail.com

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Abstract

Nanoparticles are structures that are applicable to a wide range of fields. The most significant characteristic of these structures is their structural and dimensional diversity, which varies the synthesis process based on the area that will be incorporated into several sections. Although there are other synthetic techniques, the green synthetic technique is the most often used. By employing only natural processes instead of chemicals, this method synthesizes products leaving no by-products. The technique is also highly preferred as it is affordable. In this respect, the green synthesis approach was utilized in this study to investigate the synthesis characteristics, phytochemical content, and evaluate the antibacterial activity of silver nanoparticles from *Lavandula stoechas*, which is commonly used as a sweetener in food and beverages. Characterization tools like FT-IR, UV-Vis, and fluorescence spectroscopy were employed. The phytochemical content was examined by HPLC. According to the HPLC analysis result; resveratrol and caffeic acid were detected as the main components. Furthermore, theoretical investigation was conducted into the glucosamine-6-phosphate synthase inhibitory characteristics of the resveratrol molecule, which was identified as the main constituent based on the HPLC outcome.

Key Words: *Lavandula stoechas,* resveratrol, glucosamine-6-phosphate synthase, HPLC, FT-IR, UV, nanoparticles.

1. Introduction

For some decades now, silver (Ag) Agand Ag^+ have been applied in areas such as food and beverage preservation and wound treatment due to their antibacterial capabilities (Simončič & Klemenčič, 2016; Franci et al., 2015). For the synthesis of metal nanoparticles, such as AgNPs, there are three recognized or favored ways; these include physical, chemical, and green biological processes (Abdelghany et al., 2018; Malekzadeh et al., 2018). Nanomaterials (superior metallic particles), such as microbially resistant antibiotics, have attracted much attention due to their better

performance compared to ions (Theivasanthi & Alagar, 2011; Mohanta et al., 2017). These structures are highly preferred due to their unique physical, chemical and dimensional properties (McNamara & Tofail, 2017). Silver has been used in creams, solutions, medicinal and dental materials lately. Among its many iadvantageous qualities, silver is a good option for suppressing infectious germs (Theivasanthi & Alagar, 2011; Mohanta et al., 2017). As an alternative to chemical and physical synthesis methods, the biological synthesis of nanoparticles which has gained popularity recently is thought to be the most straightforward and long-lasting (Ahmed et al., 2017; Rajeshkumar & Bharath, 2017; Mohanta & Behera, 2014).

Lavandula stoecha, a member of the *Lamiaceae* family, is consumed by many Asian and European families as herbal medicine as well as preferred as a healthy diet (Hakim et al., 1991; Zuzarte et al., 2008; Canli et al., 2019). In industries like cosmetics and pharmaceuticals, *L. stoechas* is commonly used. Antibacterial properties (Teixeira et al., 2012), antioxidant activity (Ferreira et al., 2006), and numerous advantageous polyphenol components (Pereira et al., 2015; Celep et al., 2018) are all known to be present in *L. stoechas* extract. In biological systems, enzyme inhibitors function as a control mechanism. Furthermore, they are crucial in clarifying enzyme action mechanisms and metabolic pathways (Feldhammer et al., 2013). A web-based technique called molecular docking forecasts a ligand-protein complex's preferred orientation when it is bound or non-bonded together. Additionally, it is an in silico method for predicting the molecule's binding activity and affinity to protein targets (Nisha et al., 2016; Yenigün et al., 2023).

Two steps went into the design of our study. First, an environmentally friendly green synthesis approach was employed to create silver nanoparticles (AgNPs) using black pepper extract, a substance rich in polyphenols. Furthermore, various techniques were employed to examine the synthesized product's antibacterial properties and characterisation. In the second step of the chemical content analysis of the crude extract, content analysis was performed by HPLC and the main component was determined. Then, the interaction between the main component and the glucosamine-6-phosphate synthase (bacterial) enzyme was calculated theoretically by molecular docking (MolDock). The study's findings provide insight into possible medicinal applications of *L. stoecha*. There aren't many publications in the literature on the synthesis of nanoparticles with *L. stoechas* extracts. As a result, the present work will open up possibilities for future research.

2. Material and Methods

2.1. Reagents

Pure water, ethyl alcohol, $AgNO_3$, Filter paper (Pore Diameter: 0.22 µm) were obtained from Merck.

2.2. Preparation of *L. stoechas* Plant Extract

L. stoechas was taken, properly cleaned in clean water, and left outside in the sun for two days. Subsequently, the extracted samples were finely diced and ground utilizing a professional blender. After that, the ground samples were put in a 250 mL conical flask with 100 mL of pure water, covered with aluminum foil, and let to spin continuously at 600 rpm in a magnetic stirrer for 15-20 minutes at a temperature of between 100 and 105 ^{o}C. In previously prepared 250 mL conical flasks, the solution obtained at the conclusion of the heating procedure was filtered using filter paper with a pore size of 0.22 mm. After the filtration procedure was complete, the filtrate was kept for subsequent usage at +4 ^{o}C (Devi et al., 2019) (Figure 1).

2.3. Synthesis of AgNPs Nano Particles

A 10 mM 100 mL $AgNO_3$ solution was prepared with pure water in a 250 mL conical flask. 80 mL of *L. stoechas* plant extract, previously kept ready, was added to the $AgNO_3$ solution, covered completely with aluminum foil, and mixed in a magnetic stirrer at 600 rpm at room temperature for 12 h. At the end of the heating process, centrifugation was started (3000 rpm, 10 minutes). During this process, the washing process was carried out by adding pure water twice and then ethyl alcohol or methyl alcohol once. Finally, the solid sample obtained was completely dried every other day in an oven at 60 °C to be used in characterizations. The color of the mixture turning gray reveals that Ag^+ ions are reduced to Ag metal (Öztürk et al., 2022; Erenler et al., 2023).

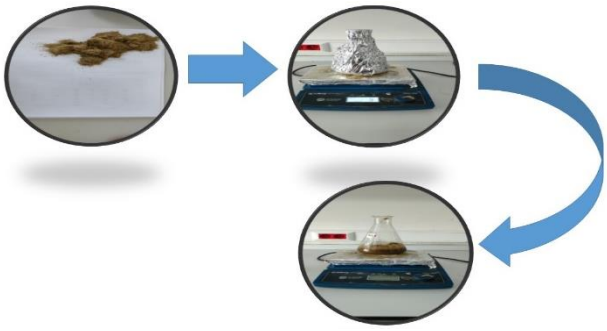

Figure 1. İmage showing the extraction of *L. stoechas* plant

2.4. Phenolic content analysis by HPLC

High-performance liquid chromatography (HPLC) was used to determine the phenolic content of the crude extract. Chromatographic conditions were optimized to achieve optimal separation for the compound and overcome imprinting effects. DAD Signal was performed at a wavelength of 300/200 nm, Ref; 500/100 nm, and chromatographic separation was performed with a reversed-phase Hi-plex (300x7.7) 8-micron analytical column. The column temperature was set at 30 ºC. Elution gradient, eluent A; 83% water (0.1 formic acid) and eluent B; 17% acetonitrile (0.1 formic acid), the solvent flow rate was set to 0.8 mL/min, and the injection volume was set to 10 µL. Also, 19 phenolic compounds were used as standards. Sample preparation: 10 mg tasted samples were dissolved with methanol and 1 mL was taken with an automatic pipette. It was then filtered through a 0.45-micrometer filter. It was diluted with water at a ratio of 1:1 and injected into the device. As a result of the calculation, it was calculated by entering the dilution factor.

2.5. Moleculer Docking Studies

The 3D structure and minimum energy of the Resveratrol molecule were made in the ChemDraw program (Başar et al., 2023; Yenigün et al., 2023). The enzymes chosen for this docking investigation were glucosamine-6-phosphate synthase [PDB ID: 1MOQ] synthase. Resveratrol interactions with glucosamine-6-phosphate were determined using the Molegro Virtual Docker (MVD) program (Y. Başar et al., 2024). 2D and 3D images of the interactions were taken with the BIOVIA Discovery Studio Visualizer program. Also, The AutoDock Vina program was used to calculate the binding affinities (Başar et al., 2024).

2.6. Antimicrobial Assay by Agar Well Diffusion Method

Sterilized Lavandula stoechas nanoparticles were weighed and dissolved in DMSO by sonication. Stock solution was prepared at a concentration of 1024 µg/mL. Mueller Hinton Agar medium was prepared and sterilized, 20 mL was poured into each petri dish, and the agar was allowed to dry. The passages of the bacteria to be used in our study, taken from the stock at -80 °C, were cultured twice and their antimicrobial susceptibility was evaluated by the disc diffusion method from subculture. Bacteria were diluted with physiological saline and prepared to 0.5 Mc Farland turbidity and were planted equally throughout the medium. AgNP impregnated with 20 µl empty

disk was placed on the medium and the antimicrobial effect was evaluated by measuring the zone diameters at the end of the incubation period. The experiment was repeated 3 times and the averages were evaluated. The solvent DMSO was used as a negative control.

2.7. Characterizations; UV-Vis. and Fluorescence Spectrometer

The Cary 60 UV-Vis Spectrophotometer was used as a model instrument for spectroscopic investigations. In addition, fluorescence experiments were conducted using the Cary Eclipse Fluorescence Spectrometer, an Agilent model equipment. Future analysis of the plant will benefit from the obtained photographs.

2.8. Characterizations; FT-IR

Utilizing the Agilent Cary 630 FT-IR model FT-IR device, this characterization phase was carried out.

3. Results and Discussion

water extract of *L. stoechas* plant was obtained. The phytochemical content of the extract was determined by HPLC. Silver nanoparticle study was applied to the extract, and its characteristic properties were determined with UV and FT-IR. In addition, in silico molecular docking was performed to observe the interactions of the glucosamine-6-phosphate enzyme with the resevratrol molecule (Figure 3), which was determined as the main component in the HPLC analysis.

3.1. HPLC Analysis

In HPLC phenolic content analysis, 15 phenolic compounds were detected (Figure 2). According to the analysis results, resveratrol (98.597 ng/ul), caffeic acid (61.945 ng/ul) and chlorogenic acid (21.910 ng/ul) are the compounds detected in the highest amount (Table 1). The amount and types of phenolics in the plants; Vary depending on the climate where the plant grows, altitude, harvest season, as well as the extraction method.

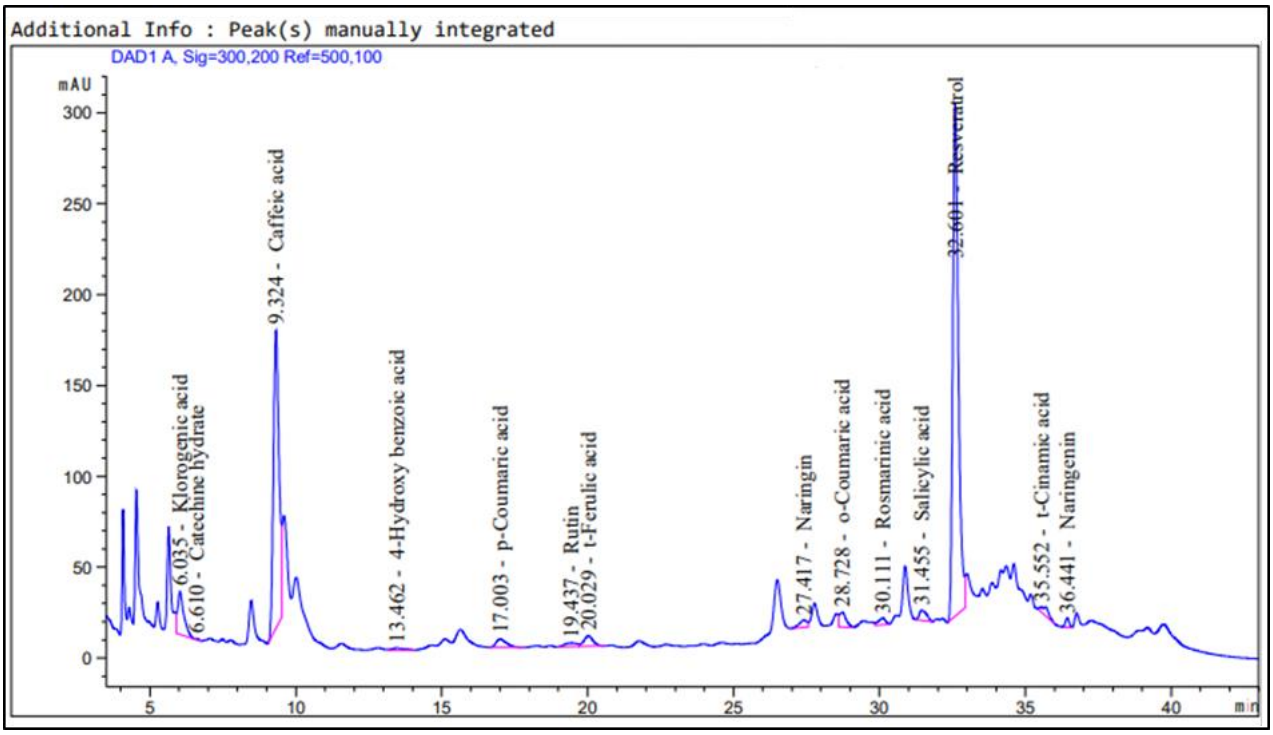

Figure 2. HPLC chromatogram of the crude extract

Table 1. HPLC phenolic content analysis

No	Retention time	Compounds	Amount (ng/ul)
1	6.035	chlorogenic acid	21.910
2	6.610	catechine hydrate	0.172
3	9.324	caffeic acid	61.945
4	13.462	4-hydroxy benzoic acid	0.991
5	16.382	vanillin	-
6	17.003	p-coumaric acid	3.110
7	19.437	rutin	3.705
8	20.029	t-ferulic acid	4.316
9	23.378	hydroxycinnamic acid	-
10	27.417	naringin	7.131
11	28.728	o-coumaric acid	2.898
12	30.111	rosmarinic acid	1.870
13	31.455	salicylic acid	4.160
14	32.601	resveratrol	98.597
15	34.787	quercetin	-
16	35.552	t-cinamic acid	2.295
17	36.441	naringenin	1.820
18	39.384	chrysin	-
19	40.770	flavones	-

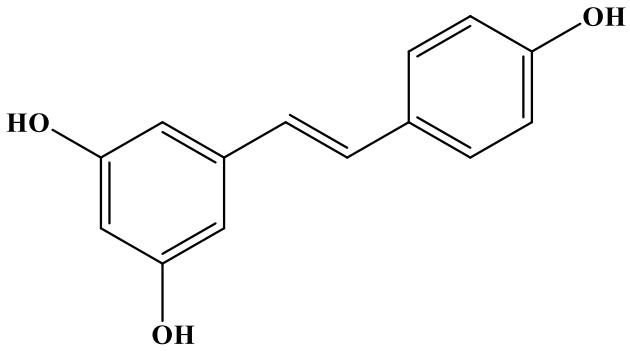

Figure 3. Structure of the resveratrol molecule

3.2. FT-IR:

The –OH and –NH groups for both samples were matched with the peaks seen 3242 and 3321 cm^{-1}, the carboxyl group's C=O group was matched with the peaks seen 1625 and 1617 cm^{-1}, and Ag-OH groups were matched with the peaks seen at about 1013 cm^{-1}. (Geoprincy et al., 2013; Salunke et al., 2014; Nasir et al., 2016; Rani et al., 2017; Bhagyaraj and Krupa, 2020; Dua et al., 2023; Mahmoudi et al., 2020; Şuică- Bunghez et al., 2024) (Figure 4).

3.3. UV-Vis

Two peaks are seen to be formed for AgNPs and the 287 nm extract. Furthermore, it is noted that the extraction yields an additional 327 nm peak (Kumar et al., 2016; Mahmoudi et al., 2020). Analysis in the 350–800 nm wavelength region of emission was achieved using fluorescence spectroscopy. It is noted that the pictures acquired using fluorescence spectroscopy exhibit some shift. It is believed that a contact brought about by AgNP production is the cause of this condition (Rasheed et al., 2023; Chelly et al., 2024) (Figure 5).

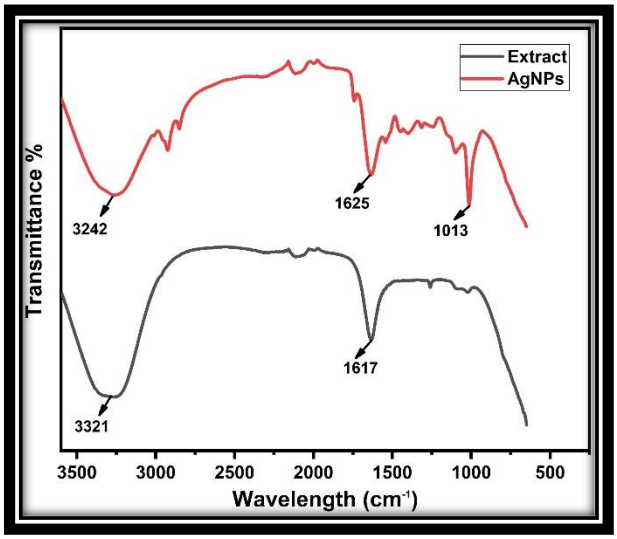

Figure 4. FT-IR image of *L. stoechas* plant extract and AgNPs

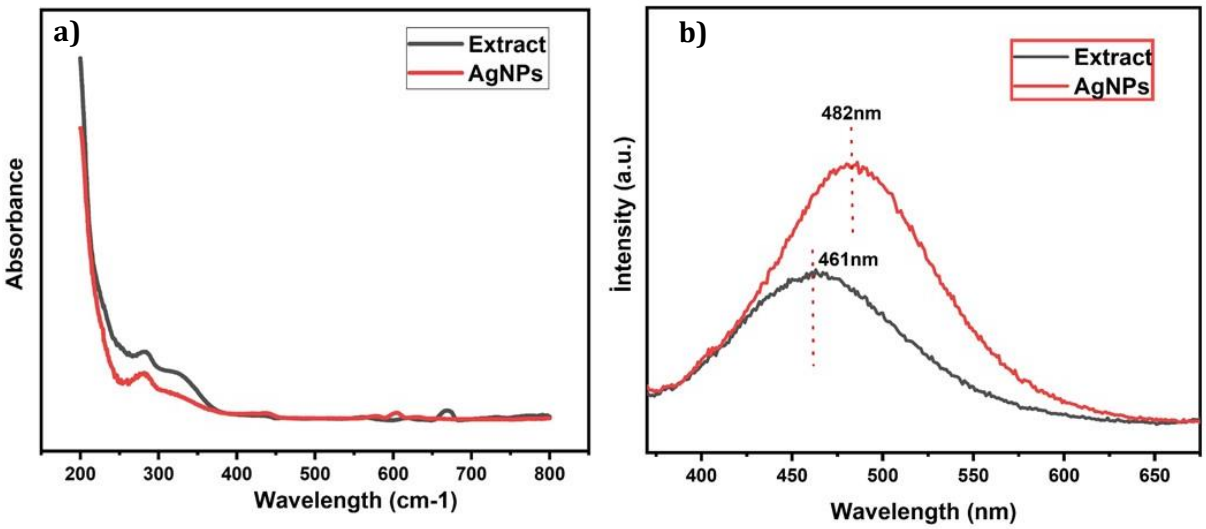

Figure 5. UV-Vis (a) and Fluorescence (b) image of *L. stoechas* plant extract and AgNPs

3.4. Molecular Docking Result

The design and development of pharmaceuticals is costly and time-consuming. With the development of new technologies, new tools are emerging to avoid this difficult process. Molecular docking is a computerised method for predicting protein-ligand interactions and calculating binding positions and energies for the target molecule. Based on the results obtained, it helps to identify the molecule (ligand) as a drug candidate and bring it to a stage that informs in vivo studies (Fan et al.,2019; Singh et al.,2022).

Glucosamine-6-phosphate synthase is known as a promising target for antimicrobial agents and antidiabetics. The enzyme glucosamine-6-phosphate synthase plays an important role in the biosynthesis and synthesis of peptidoglycan in the bacterial cell wall. This enzyme is not used as an antibacterial agent, but due to its role in peptidoglycan synthesis it is of interest for the development of antibiotics. By inhibiting it with a specific ligand, glucosamine-6-phosphate synthase disrupts bacterial cell wall formation, which leads to the death of the bacterial cells (Fikrika et al., 2016; Stefaniak et al., 2022). According to the HPLC analysis result, the resveratrol molecule (Figure 3) was detected in the highest amount in the crude extract; Its interaction with the glucosamine-6-phosphate synthase enzyme was determined theoretically. Additionally, the MolDock score and binding energy of the interactions were calculated.

Resveratrol molecule interacted with glucosamine-6-phosphate synthase by six conventional-hydrogen bonds with amino acid SER316, ARG472, ASN522, VAL519, ALA520, TYR312, one pi-anion with amino acid ASP474, and one pi-alkyl with amino acid ARG472 (Figure 6-Table 2). Resveratrol molecules with glucosamine-6-phosphate synthase interactions were determined as a MolDock score of -90.81, with binding energies of -5.80 kcal/mol. According to the results, it can be said that the resveratrol molecule is effective as an inhibitor of glucosamine-6-phosphate.

3.5. Agar Well Diffusion Method

The present study aimed to evaluate the antimicrobial effects of the *L. stoechas* AgNPs and Gentamicin and antibiotic standard strains of *S. aureus* ATCC 25923, *E. coli* 25922, *P. aeruginosa* ATCC 27853, *E. feacalis* ATCC 29212 *using* the well diffusion method, the diameter of the growth inhibition zone was measured for AgNPs at a concentration of 1024 µg/mL against four bacteria. Gentamicin was used as a positive control, and a precise measurement was made for it.

Table 2. Interaction categories, types, and distances of molecular insertion of the resveratrol molecule with glucosamine-6-phosphate synthase

No	Name	Distance	Category	Type	Transmitter	From Chemistry	Receiver	To Chemistry
1	A:SER316:HG - :[001:O3	1.93855	Hydrogen Bond	Conventional Hydrogen Bond	A: SER316:HG	H-Donor	:[001:O3	H-Acceptor
2	A:ARG472:HN - :[001:O2	2.3882	Hydrogen Bond	Conventional Hydrogen Bond	A: ARG472:HN	H-Donor	:[001:O2	H-Acceptor
3	A:ASN522:HN - :[001:O1	2.27169	Hydrogen Bond	Conventional Hydrogen Bond	A: ASN522:HN	H-Donor	:[001:O1	H-Acceptor
4	:[001:H11 - A:VAL519:O	2.20973	Hydrogen Bond	Conventional Hydrogen Bond	:[001:H11	H-Donor	A:VAL519:O	H-Acceptor
5	:[001:H11 - A:ALA520:O	2.28076	Hydrogen Bond	Conventional Hydrogen Bond	:[001:H11	H-Donor	A:ALA520:O	H-Acceptor
6	:[001:H12 - A:TYR312:O	2.74218	Hydrogen Bond	Conventional Hydrogen Bond	:[001:H12	H-Donor	A:TYR312:O	H-Acceptor
7	A:ASP474:OD2 - :[001	3.47614	Electrostatic	Pi-Anion	A:ASP474:OD2	Negative	:[001	Pi-Orbitals
8	:[001 - A:ARG472	3.87475	Hydrophobic	Pi-Alkyl	:[001	Pi-Orbitals	A:ARG472	Alkyl

The values of the growth inhibition zone in millimeters are displayed in Figures, which provide the results. It showed the highest antimicrobial effect against *S. aureus* 12.00±1.00 mm. On the other hand, it showed the lowest inhibitory effect against *E. feacalis*, with a growth inhibition zone diameter of approximately 9.50±1.00 mm. These findings shed important light on the *L. stoechas* AgNPs antibacterial activity and suggest that they might be used in place of antibiotics to treat bacterial infections (Fig. 7).

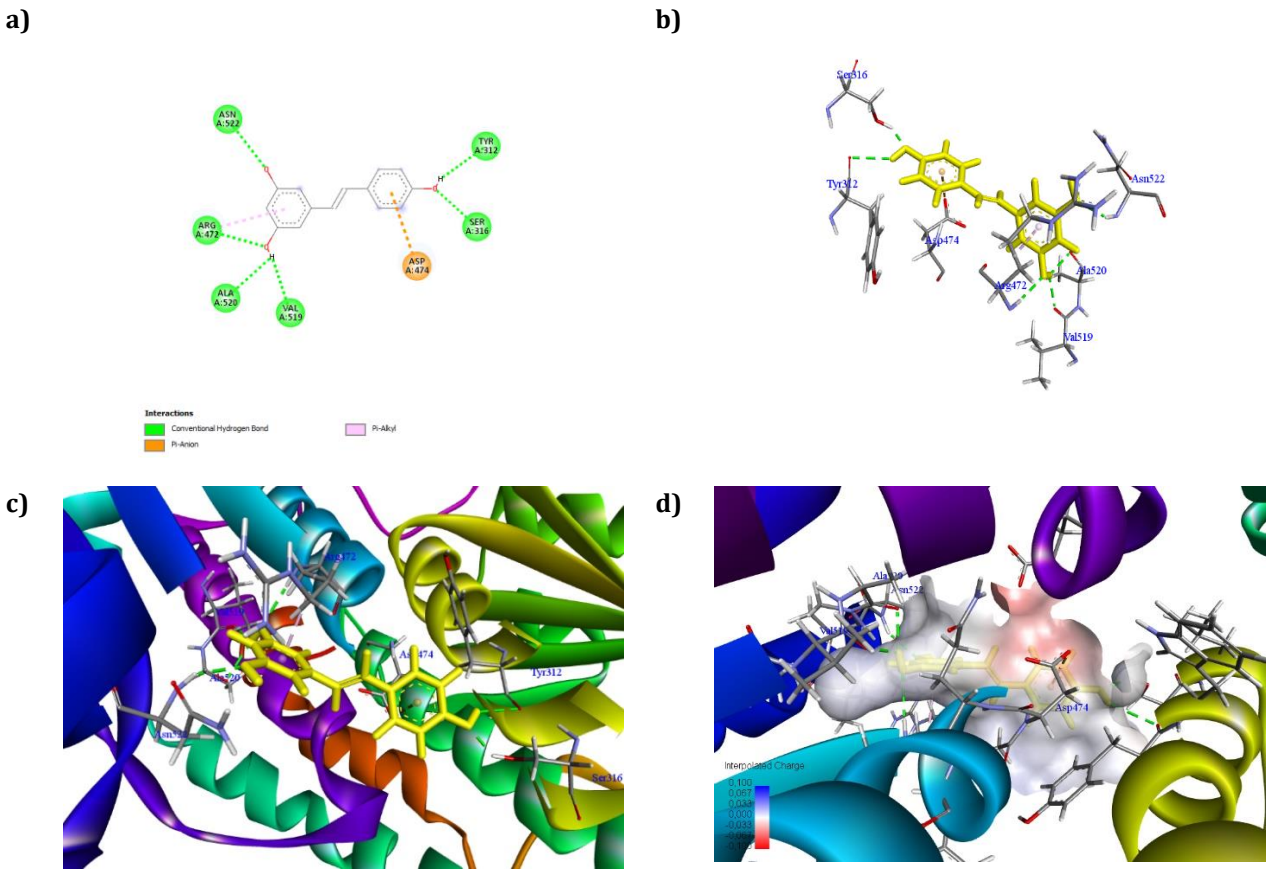

Figure 6. Resveratrol-glucosamine-6-phosphate synthase interaction a) 2D images b) general view c) 3D images d) interpolated load view

Using natural approaches, we produced AgNPs from the high polyphenol content plant, *L. stoechas*, more economically and without leaving any chemical by-products in the environment. UV-Vis spectrophotometry can be used to identify the reduction of Ag^{+} ions to metallic Ag^{0} in this situation. Additionally, it was discovered that S. Aureus was more negatively impacted by it in studies of nanoparticle antibacterial activity. Additionally, the high reduction potential of *L. stoechas* extract was linked to an increase in AgNP production via an increase in silver nitrate concentration. The extract of *L. stoechas* includes flavonoids, phenolic acids, and phytosterols. It belongs to the Lamiaceae family of mints, which also includes mint, and has numerous active metabolites that can be used as capping and reducing agents (Nitzsche et al., 2004; Gonçalves & Romano, 2013).

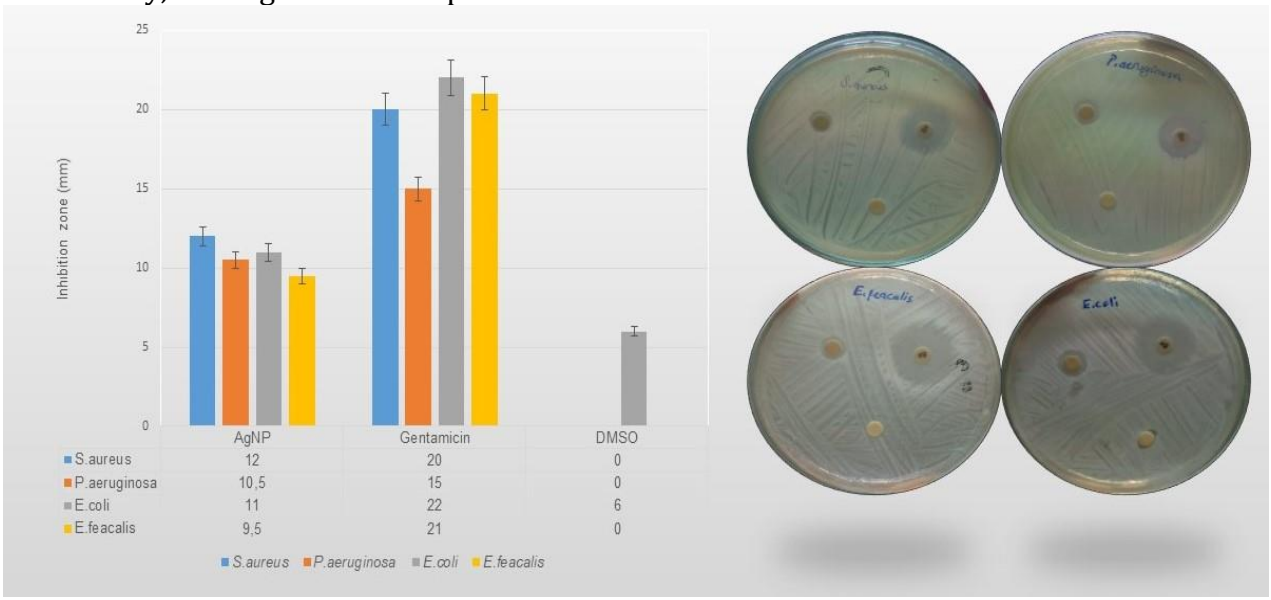

Figure 7. Image of the antimicrobial activity study performed by the Agar Well Diffusion method (inhibition zone, mm)

4. Conclusion

Since the 1990's, scientists have been calling for the synthesis of nanoparticles using the environmentally benign green synthesis approach. *L. stoechas* extract was chosen as the reducing agent in this work because it was more cost-effective, quicker, and environmentally friendlier than other methods for synthesizing silver nanoparticles. The shape of the produced AgNPs was investigated in addition to their structural characteristics. The fact that these AgNPs work well against *S. aureus, P. aeruginosa*, *E. coli*, and *E. faecalis* further supports the nanoparticle's potent antibacterial activity. As a result, in this study, resveratrol compound was determined in the highest amount as the main compound in the HPLC device. In addition, in antimicrobial activity studies, it was determined by the disk diffusion method that it showed the highest antimicrobial effect against *S. aureus* but the lowest effect against *E. feacalis*. In the interaction of glucosamine-6-phosphate synthase and resveratrol, the MolDock score was determined to be -90.81 and the binding energies -5.80 kcal/mol. It is assumed that this study will provide ideas for further research.

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Author Contribution

All authors declare equal contribution to the design and experimental work, interpretation of the resultsand editing the manuscript.

Conflicts of Interest

The authors declare no conflicts of interest during the accomplishment of this research. None of the authors has any financial and/or personal relationships with other people or organizations that could inappropriately influence (bias) their work.

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Phytochemical Investigation, Antioxidant, and Enzyme Inhibitory Activities of Blackberry (*Rubus fruticosus*) Fruits

Ali Hasan SAQEE[1], Nuraniye ERUYGUR[2*]

[1] Department of Pharmacognosy, Graduate School of Health Sciences, Selcuk University, 42250, Konya, Türkiye
E-mail: alisaqi200684@gmail.com, ORDID ID: 0009-0007-2574-5593
[2] Department of Pharmacognosy, Faculty of Pharmacy, Selcuk University, 42250, Konya, Türkiye, E-mail: nuraniye.eruygur@selcuk.edu.tr, ORDID ID: 0000-0002-4674-7009
*Corresponding author : nuraniye.eruygur@selcuk.edu.tr

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Abstract

This scientific paper explores the phytochemical composition of blackberry (*Rubus fruticosus* L.) fruit and investigates its antioxidant and enzyme inhibitory activities. Blackberry is known for its rich nutritional profile and potential health benefits, making it a subject of interest in the field of functional foods and natural medicine. The study aims to provide valuable insights into the bioactive compounds present in blackberry fruit, their antioxidant properties, and their potential as enzyme inhibitors. According to the results, it was determined that the fruit extracts had strong antioxidant and moderate enzyme inhibition activity. HPLC analysis showed that the fruit extract generally contained *o*-coumaric acid (7.74-30.87), procatechin (16.44 and 36.91 in BE and BA, respectively), ellagic acid (0.51-3.03) and quercetin (0.22-8.50) as major components.

Key Words: Blackberry, phytochemicals, antioxidant activity, enzyme inhibition, functional foods, HPLC

1. Introduction

Antioxidants are compounds that help neutralize harmful molecules called free radicals. Free radicals are produced naturally in the body as part of normal metabolic processes, but they can also be generated in higher amounts due to factors like stress, pollution, unhealthy diet, and certain medical conditions (Lobo et al., 2010). Antioxidants help protect cells and tissues from oxidative damage, thereby reducing the risk of complications associated with diabetes. For example, antioxidants such as vitamin C, vitamin E, and beta-carotene can help preserve eye health and reduce the risk of diabetic retinopathy(Dal & Sigrist, 2016). The relationship between antioxidants and Alzheimer's disease revolves around oxidative stress, inflammation, and the damage caused by reactive oxygen species (ROS) in the brain (Manoharan et al., 2016).

Blackberry (*Rubus fruticosus* L.), a member of the Rosaceae family, is a widely consumed fruit known for its distinctive flavor and dark color. Previous research has highlighted its nutritional content, including vitamins, minerals, and dietary fibers. Additionally, blackberries are reported to contain various phytochemicals with potential health-promoting effects (Martins et al., 2023).

Inhibition of enzymes plays an important role in the treatment of diseases such as Alzheimer's disease, diabetes and Parkinson's(Arcone et al., 2023). Some of these diseases are caused by the irregular functioning of certain enzymes in the body. For this reason, inhibition of enzymes is very important in the treatment of these diseases. Alzheimer's disease is associated with the buildup of certain proteins in the brain. A buildup of a protein called beta-amyloid can lead to the death of nerve cells and ultimately cognitive impairment. At this point, enzyme inhibition can be used to prevent beta-amyloid production or accumulation (Ibach & Haen, 2004).

Diabetes occurs when the pancreas does not produce enough insulin or the body cannot use insulin effectively. Insulin is an enzyme that helps glucose enter cells. Inhibiting the enzymes α-glucosidase and α-amylase, which are responsible for breaking down carbohydrates during digestion, can effectively lower the rise in blood glucose after meals. This makes it a crucial approach for managing blood glucose levels in individuals with type-2 diabetes and those at risk. Recently, there has been a resurgence of interest in plant-based medicines and functional foods that can modify physiological responses, aiming to prevent and treat diabetes and obesity (Tundis et al., 2010).

Parkinson's disease is characterized by damage or loss of dopamine-producing cells in the brain. Dopamine is a neurotransmitter that plays an important role in movement control and emotional reactions. In the treatment of Parkinson's disease, studies are being conducted on the inhibition of enzymes to increase dopamine production or reduce dopamine destruction(Nagatsu et al., 2022). There are studies in the literature on the analysis of the antioxidant and phytochemical content of blackberry fruit. However, previous studies were carried out on a single type of extract, and no additional studies on enzyme inhibition activity were found. Therefore, this study aimed to investigate the phytochemical content, antioxidant and enzyme inhibition of blackberry fruit, which is frequently consumed in our daily lives. In this way, the beneficial effects of blackberry fruit on health will be evaluated and the basis will be formed through scientific study for the development of nutritional supplements derived from blackberry.

2. Material and Methods

2.1. Sample Collection and Preparation

Fresh blackberry fruits were harvested from a local orchard (From Konya province, Turkiye) and immediately transported to the laboratory. The fruits were cleaned, and freeze-dried to preserve their phytochemical composition.

2.2. Preparation of extracts

The freeze dried samples were cut into small pieces and soaked seperately in 50% acetone, 80% ethanol and 80% Methanol containing 0.01% HCl at ambient tempereture for 24h. The extract were filtered and concentrated by a rotary evaporator to obtaine dry acetone(BA), ethanol(BE) and methanol (BM) extracts, respectively.

2.3. Phytochemical Analysis

The phytochemical profile of blackberry was determined using various extraction techniques, including solvent extraction and chromatographic methods. Common phytochemicals such as flavonoids (Quettier-Deleu et al., 2000), phenolic acids (Clarke et al., 2013), and anthocyanins (Fredes González et al., 2014) were quantified by septrophotometric method. The phenolic profiling was also determined with HPLC (Fig. 1).

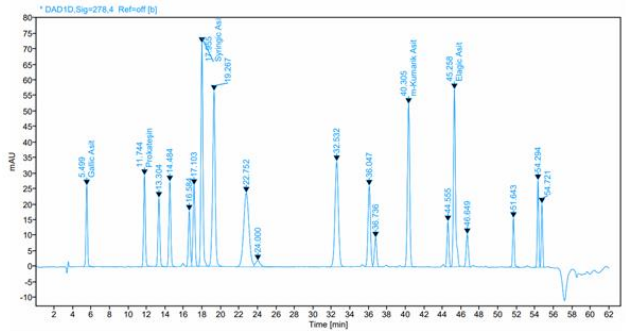

Figure 1. HPLC chromatogram of standard phenolic compounds

2.4. Antioxidant Activity Assays

The antioxidant potential of blackberry extracts was assessed using standard assays, including the 2,2-diphenyl-1-picrylhydrazyl (DPPH)(Wang et al., 2008) and ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (Chun et al., 2005) radical scavenging assay.

2.5. Enzyme Inhibitory Assays

Enzyme inhibitory activities, against key enzymes involved in various metabolic processes, such as α-amylase (Yang & Kong, 2016) and α-glucosidase (Bhatia et al., 2019) for carbohydrate metabolism, tyrosinase for skin whitening effect, and acetyl-, butyrylcholinesterase for neurological health (Šinko et al., 2007) were performed according to previously reported experimental methods.

The cholinesterase inhibitory activity (AChE and BChE) of the extracts was conducted using Elmann's method. The α-amylase activity was determined using starch as a substrate in a colorimetric reaction using 3,5-dinitrosalicylic acid. The α- glucosidase activity was determined using para-nitrophenyl-α- d-glucopyranoside as a substrate. Tyrosinase inhibitory was performed using L-DOPA as the substrate (Jeong et al., 2009). The tests were conducted via distribution of 140 µL of a solution of mushroom tyrosinase (250 U/mL in phosphate buffer) in each well, together with 25 µL of the extracts evaluated. Kojic acid was used as a positive control and DMSO alone was used as the negative control.

3. Results and Discussion

The present study was conducted to determine total phenol, flavonoid, anthocyanin content as well as the in vitro antioxidant and enzyme inhibitory activity of blackberry fruits.

3.1. Phytochemical Composition

Table 1 presents the findings on the total phenolic and flavonoid contents, as well as total anthocyanin content of various extracts of blackberry fruits.

Table 1. Total anthocyanin, flavonoid and phenolic content of blackberry acetone, ethanol and methanol extracts

Specimens	Extract yield (%, g/g)	Total anthocyanins (mg of Cyanidin-3-Glucoside E/g)	Total flavonoids (mg of QE/g)	Total phenolics (mg of GAE/g)
BM	11.05	4.828 ± 0.001	0.03± 0.008	25.37±1.13
BA	13.85	3.374 ± 0.081	1.95±1.08	36.62±0.59
BE	10.44	5.997 ± 0.003	2.18 ± 0.07	23.76±0.85

The total anthocyanin content of methanol, acetone and ethanol extract were 4.828 ± 0.0001, 3.374 ± 0.081 and 5.997 ± 0.003 mg/g cyanidin 3-glucoside equivalent, respectively. The acetone extract had the highest TPC (36.62±0.59 mg/g GAE), followed by BM extract (25.37±1.13 mg/g GAE) and BE extract (23.76±0.85 mg/g GAE). The total flavonoid content of methanol, acetone and ethanol extract were

0.03 ± 0.008, 1.95±1.08 and 2.18 ± 0.07 mg/g quercetin equivalent, respectively. In a previous study, TPC, TFC and TAC of blackberry were found as 5.58 mg GAE/g, 11.83 mg RE/g and 3.99 mg catechin/g dry weight, respectively (Huang et al., 2012). In another study, TPC and TFC were found to be 87.25 mg GAE/g and 48.97 mg CE/g, respectively (Tan & Chang, 2017). HPLC analysis of phenolic compounds was performed by determining As can be seen from the HPLC result (Fig. 2-4), there is a difference in the profile of phenolic compounds found in the extracts.

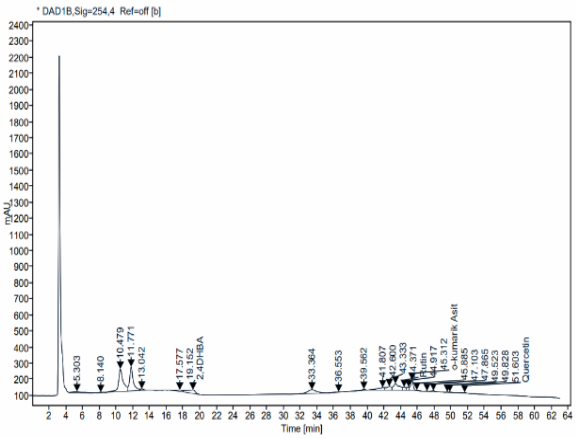

Figure 2. HPLC chromatogram of blackberry acetone extract

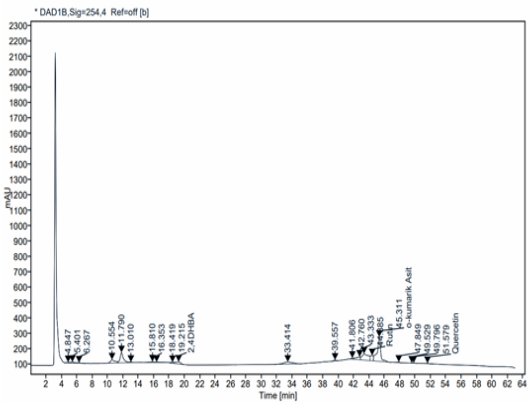

Figure 3. HPLC chromatogram of blackberry ethanol extract

While o-coumaric acid was found in all extracts (Table 2), the highest level was detected in methanol and ethanol extracts. Procatechin was found to be highest in acetone extract.

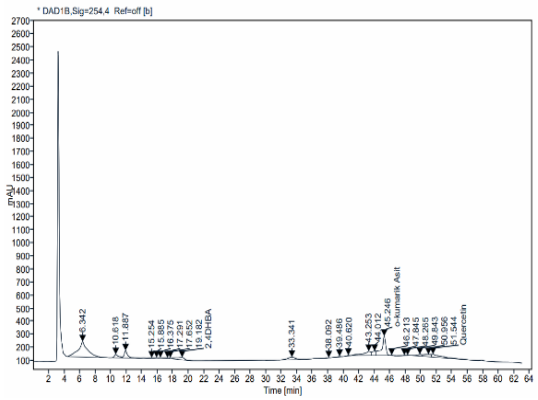

Figure 4. HPLC chromatogram of blackberry methanol extract

Table 2. Phenolic content of blackberry fruit extracts by HPLC j (µg/mg)

Name	RT (min)	BA	BE	BM
Gallic acid	5.499	-	-	-
Procatechin	11.744	36.909	16.443	-
Chlorogenic acid	13.304	-	-	-
4-hydroxy benzoic	14.484	3.039	-	-
2,5- dihydroxy	15.914	-	-	-
Vanilic acid	16.584	-	-	-
Cafeic acid	17.103	-	-	-
Syringic acid	17.955	-	-	-
2,4- dihydroxy	19.088	-	1.217	2.562
Vanilin	19.270	0.768	0.435	0.711
4H1,3 - benzoic acid	22.752	-	-	-
2,3- dihydroxy	24.011	-	-	-
p-Coumaric acid	32.532	-	-	-
Ferulic acid	36.047	-	-	-
Sinapic acid	36.736	-	-	
m-coumaric acid	40.305	-	-	0.200
Rutin	44.555	3.995	7.077	-
Elagic acid	45.258	0.508	1.571	3.029
o-coumaric acid	45.557	7.744	27.990	30.873
Salisilic acid	46.649	--	-	-
Quercetin	51.643	0.375	0.223	8.504
Caempherol	54.294	-	-	-
Apigenin	54.721	-	-	-

3.2. Antioxidant Activity

According to the antioxidant activity results of blackberry fruit extracts, a proportional increase in radical scavenging activity was observed depending on the increase in extract concentration. When compared at the low concentration (75 µg/mL), acetone extract showed higher activity in terms of both DPPH (55.45%) and ABTS (72.45%) radical scavenging activity (Fig. 2, 3).

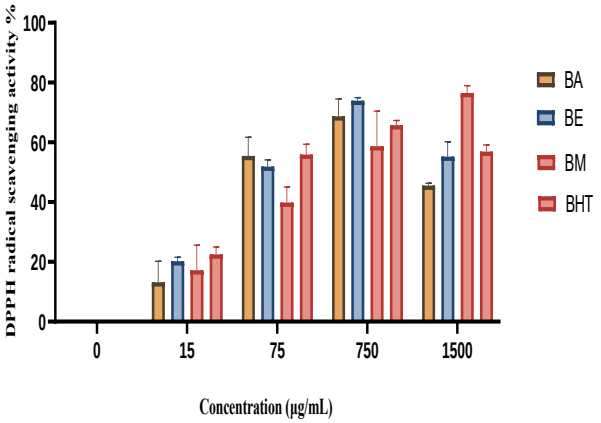

Figure 5. DPPH radical scavengign activity of blackberry acetone, ethanol and methanol extracts

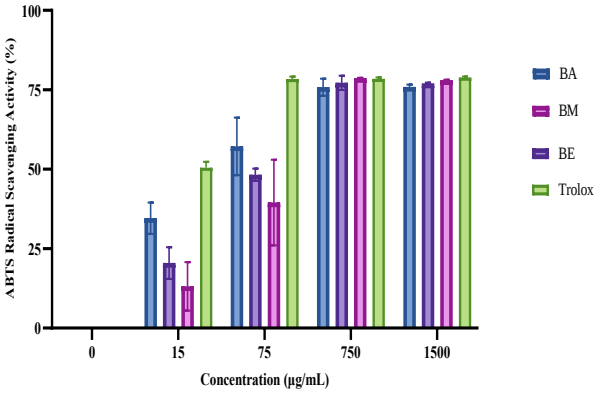

Figure 6. ABTS radical scavengign activity of blackberry acetone, ethanol and methanol extracts

The DPPH radical scavenging activity of BA, BE and BM extracts shown to be IC_{50} value of 319.6± 0.34, 179.9±0.40 and 235.1±0.52 µg/mL, respectively. The ABTS radical scavenging activity of BA, BE and BM extracts shown to be IC_{50} value of 69.01± 0.50, 115.3±0.60 and 157.0±0.71 µg/mL, respectively. Blackberry extracts demonstrated significant antioxidant activities in all tested assays, suggesting the potential for free radical scavenging and reducing oxidative stress. The DPPH and ABTS radical scavenging potential of different blackberry cultivars methanol extract were showed 108.43-146.89 mg TE/g and 126.00-177.11 mg TE/g, respectively (Sariburun et al., 2010).

3.3. Enzyme Inhibitory Activities

The inhibitory effects of blackberry extracts on α-amylase, α-glucosidase, and acetylcholinesterase activities will be discussed, highlighting their potential role in managing diabetes and supporting cognitive function. In general, acetone extract showed higher AChE (10.11%), BChE (15.58%), α-amylase (25.84%) inhibitory activity while methanol extract demonstrated greater α-glucosidase (588.46) and tyrosinase (58.62%) inhibitory activity followed by ethanol extract (Table 3). In a study, blackberry crude extract showed inhibitory activity against α-amylase and α-glucosidase with the IC_{50} value of 1.56 and 57.03 mg/ml, respectively (Tan & Chang, 2017). Polyphenols from berries have the potential to modulate starch digestion and berry components can be substitute for acarbose or used as pharmaceutical enzyme inhibitor (Boath et al., 2012).

Table 3. Enzyme inhibitory activity of blackberry acetone, ethanol and methanol extracts (%inhibition at 1 mg/ml concentration)

Specimens	AChE	BChE	α-amylase	α-glucosidase	Tyrosinase
BM	7.19±3.26	7.19±3.26	13.29±4.43	88.46±0.18	58.62±3.00
BA	10.11±3.33	15.58±1.16	25.84±1.55	70.52±0.57	54.14±1.70
BE	4.01±1.65	10.73±2.15	12.85±5.03	83.95±2.05	56.71±5.62
Galanthamine	33.09±2.78	20.20±3.37	-	-	-
Acarbose	-	-	39.72±4.44	70.45±4.51	-
Kojic acid	-	-	-	-	61.96±1.45

The results of this analysis indicated that it is important to point out that blackberry fruit is an excellent raw material for obtaining biologically valuable products. As far as we are concerned, this is the first study to investigate phytochemical profiling and enzyme inhibition activity of black berry fruits with different extracts.

4. Conclusion

This study provides a comprehensive analysis of the phytochemical composition of blackberry fruit and its antioxidant and enzyme inhibitory activities. The findings suggest that blackberry may serve as a valuable source of bioactive compounds with potential health benefits. Further research is warranted to explore the therapeutic applications of blackberry in preventing or managing various health conditions.

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Author Contribution

Conceptualization, A.S. and N.E.; methodology, software, validation, and formal analysis, N.E.; data curation, A.S.; writing—original draft preparation, A.S.; writing-review and editing, visualization, and supervision, N.E.; funding acquisition, N.E. All authors have read and agreed to the published version of the manuscript..

Conflicts of Interest

The authors declare no conflicts of interest.

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In vitro Antioxidant and *In Silico* Wound Healing Activity of Quercus Infectoria Dry Extract

Mohammed Ismail BENLAZAR[1,2*], **Soumia MERZOUG**[2,3]

[1]Hemobiology and blood bank Department, Dr. T. Damerdji Hospital of Tlemcen, Algeria
[2]Pharmacy Department, Faculty of Medicine, Abou Bekr Belkaid University, Tlemcen, Algeria
[3]Laboratory of Therapeutic Chemistry, Faculty of Medicine, Abou Bekr Belkaid University, Tlemcen, Algeria
*Corresponding author e-mail: benlazarmohammedismail@gmail.com

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Abstract

In the present study we were interested in the healing activity of Quercus infectoria gall extract. We started by preparing the extract by two methods: maceration and digestion. Then, polyphenol and tannin contents were determined. The antioxidant activity of the extract was evaluated *in vitro* using DPPH radical scavenging assay. The last part of this study concerns the study of the healing activity in silico by molecular docking assisted by the Shrödinger program and this via the inhibition of GSK3-β, an enzyme involved among others in the healing process. The extract obtained shows a high tannin content, which explains most of its antioxidant activity. The in-silico study, revealed that ellagic acid, isocryptomerin, propyl gallate, ethyl gallate, and methyl gallate are likely inhibitors.

Key Words: Quercus infectoria, tannins, healing activity, antioxidant activity, molecular docking, GSK3-β

1. Introduction

The gall oak or Aleppo oak (Quercus infectoria), is a shrub of the genus Quercus belonging to the family of Fagaceae (Shrestha et al., 2014). It is a common shrub in Iran, Syria and Turkey and is also found in Greece (Fabre et al., 1992). Phytochemical analysis of the aqueous extract of galls indicates the presence of polyphenolic compounds (gallic acid, methyl gallate, ethyl gallate, propyl gallate, syringic acid and ellagic acid), flavonoids (amentoflavone and isocryptomerin), triterpenoids (roburic acid, methylbetulate, methyloleanolate and nyctanthic acid) and sterols (β-sitosterol). Tannins are the main constituents of the dry extract. In the last decades, Quercus infectoria galls have been increasingly studied due to their wide spectrum of physiological and pharmacological properties. Several biological activities have been reported, such as wound healing (Elham et al., 2021), anti-inflammatory (Kaur et al., 2004), and anti-tumor activities (Kuo et al., 2014). It has been reported that the inhibition of glycogen synthase kinase-3β (GSK3-β) promotes wound healing (Harish et al., 2008). This study aims to investigate both the *in vitro* antioxidant activity and the *in silico* wound healing activity of the extract by docking its constituent molecules to GSK3-β.

2. Material and Methods

2.1. Plant material

The galls of Quercus infectoria were obtained from a local herbal shop in the city of Tlemcen (Algeria). They were ground with an electric coffee grinder. The powder obtained is kept in a dark place until it is used. The plant material was identified on the basis of its macroscopic characteristics.

2.2. Extraction of plant material

The extraction of compounds was carried out by two methods:

2.2.1. Extraction by maceration: 45 g of gallnut powder was introduced into an Erlenmeyer flask. 400 mL of a hydroacetone solution (water-acetone 60/40 : v/v) was added. Magnetic stirring was maintained for 24 hours at room temperature.

2.2.2. Extraction by digestion: 45 g of gallnut powder was introduced into 400 mL of distilled water. The whole was heated for 2 hours at 50°C and then left to macerate for 24 hours while maintaining magnetic stirring.

The filtrates were concentrated until a dry extract was obtained, this was then reduced to powder.

2.3. Quantification of tannins

This determination was carried out according to the indirect method described by Lastra et al. (2000). This method consists of two parts: a determination of total polyphenols (TPP), followed by residual polyphenols (RPP), after precipitation of the tannins by 20% gelatin in a NaCl-saturated solution.

The total polyphenol content is given by the following formula:

Tanin content = TPP content- RPP content

The analysed solution was prepared by dissolving 0.1 g of dry extract in 100 mL of distilled water. The total phenolic compounds were quantified by spectrophotometry using the Folin–Ciocalteu reagent. 10 mL of distilled water was added to 0.5 mL of the prepared solution. 0.5 mL of Folin–Ciocalteu's reagent and 1 mL of sodium carbonate (10%) were added to the previous mixture. Absorbance was measured at 750 nm after 1 hour against a standard curve of gallic acid. The residual polyphenols were then quantified after the precipitation of tannins. 10 mL of the prepared solution, 15 mL of 20% salted gelatin and 1 g of kaolin were mixed. The previous mixture was kept under magnetic stirring for 30 min in order to allow a better precipitation of tannins. The mixture was separated by centrifugation (10 min 4000 rpm). The surnageant containing the residual polyphenols was then analysed following the same protocol as previously described.

2.4. Antioxidant activity determination using DPPH radical scavenging assay

The determination of the antioxidant activity of the extract was carried out using the method described by C. Sánchez-Moreno et al. (1998). 1,1-diphenyl-2-picrylhydrazyl (DPPH) was defined as a stable free radical which forms a violet-coloured solution. In the presence of anti-radical compounds, the DPPH radical was reduced and changes the colour from violet to yellow. The absorbances measured at 515 nm were used to calculate the percentage of inhibition of the DPPH radical. 50 µl of each solution of the extracts at different concentrations (from 0.0625 to 1 mg/ml) were added to 1.95ml of the methanolic solution of DPPH (0.04 g/l).

In parallel, a negative control is prepared by mixing 50µl of methanol with 1.95ml of the methanolic solution of DPPH. The absorbances are measured at 515 nm against a blank prepared for each concentration after a 30-minute incubation in the dark at room

temperature. The positive control is represented by a solution of ascorbic acid whose absorbance was measured under the same conditions as the samples, and for each concentration, the test is repeated three times. The results were expressed as a percentage of inhibition (I%).

$$I\% = (〚Abs〛_{control} - 〚Abs〛_{sample}) / 〚Abs〛_{control} \times 100$$

2.5. Molecular docking

Automated docking was used to determine the orientation of inhibitors bound to the active site of GSK3-β. The Schrödinger Maestro programme was employed. GSK3-β (EC: 2.7.1.37) represented by PDB code 1Q5K co-crystallised with 1-[(4-methoxyphenyl)methyl]-3-(5-nitro-1,3-thiazol-2-yl) urea was selected as the biological target for our research theme (Bhat et al., 2003). Grid generation was achieved in the presence of the co-crystallized reference inhibitor CID: 448014. Before the actual docking step, we performed a redocking using the co-crystallised reference ligand: 1-[(4-methoxyphenyl)methyl]-3-(5-nitro-1,3-thiazol-2-yl)urea (TMU). Cross-docking was also performed for a ligand already known to have inhibitory activity on GSK-3β: staurosporine (STU), obtained from the PubChem database. Redocking and cross-docking protocols were employed in order to evaluate the accuracy of docking procedures. For the screening step, we limited ourselves to molecules from the gallnut, so a small chemical library of thirteen molecules was prepared. These were downloaded from the PubChem website (www.pubchem.ncbi.nlm.nih.gov) in SDF format and prepared. The ligands were minimised using the OPLS3 force field implemented in Maestro's Ligprep application. Their ionisation and tautomerization states were predicted by Epik 2.2 at pH 7±2.

3. Results and Discussion

3.1. Extraction of plant material

These findings demonstrate that extraction using maceration in a hydroacetone solution yields a greater amount of material than digesting. This is attributed to the presence of acetone, a polar solvent that is suitable for the extraction of tannins.

Table 1. Extraction yields

	Maceration	Digestion
Yields (%)	47.69	44.16

3.2. Quantification of tannins: The tannin content was determined by indirect spectrophotometry in the visible range using gallic acid as standard. Total and residual polyphenol contents are calculated from the equation $y = 1.152x + 0.046$ ($R^2 = 0.990$) of the gallic acid calibration curve Abs=f(C).

Table 2. Quantification of tannins

TPP content (*)	594.62 ± 0.030
RPP content (*)	222.22 ± 0.002

(*): mg Gallic acid equivalent/g dry matter

Total tannin content = 62.63 % of total polyphenols.

These results show that the gallnut is an important source of tannins, which represent 62.63 % of the total polyphenols in the dry extract. This value is comparable to that reported by Zhang et al. (2023), i.e., 70% for Quercus chenii and Quercus aliena shell extract.

3.3. Antioxidant activity determination using DPPH radical scavenging assay

The antioxidant activity of the gallnut extract was determined by calculating the concentration of the extract inhibiting 50%

of the DPPH radicals (IC50). In this test, ascorbic acid was used as a standard. The results obtained are shown in Figure 1.

The IC50s of gallnut extract and ascorbic acid were calculated from the equations y=140.1x+5.900 (R2=0.984) and y=290.2x+2.540 (R2=0.978) respectively.

$$IC\ 50_{Extract} = 0.16 \pm 0.01\ g/L$$

$$IC\ 50_{Ascorbic\ acid} = 0.31 \pm 0.2\ g/L$$

From these results, gallnut extract has twice the antioxidant activity of the ascorbic acid used as a reference. This value was in accordance with that reported by Arina et al. (2019), i.e., 0.13 g/L for gallnut extract prepared by decoction at 50°C, and Kamarudin et al. (2021) i.e., 0,14 g/L for the aqueous gallnut extract. The antioxidant activity of gallnut extract was mainly due to the presence of phenolic compounds (594.62 ± 0.030 mg EAG/g dry extract), secondary metabolites with pronounced antioxidant properties.

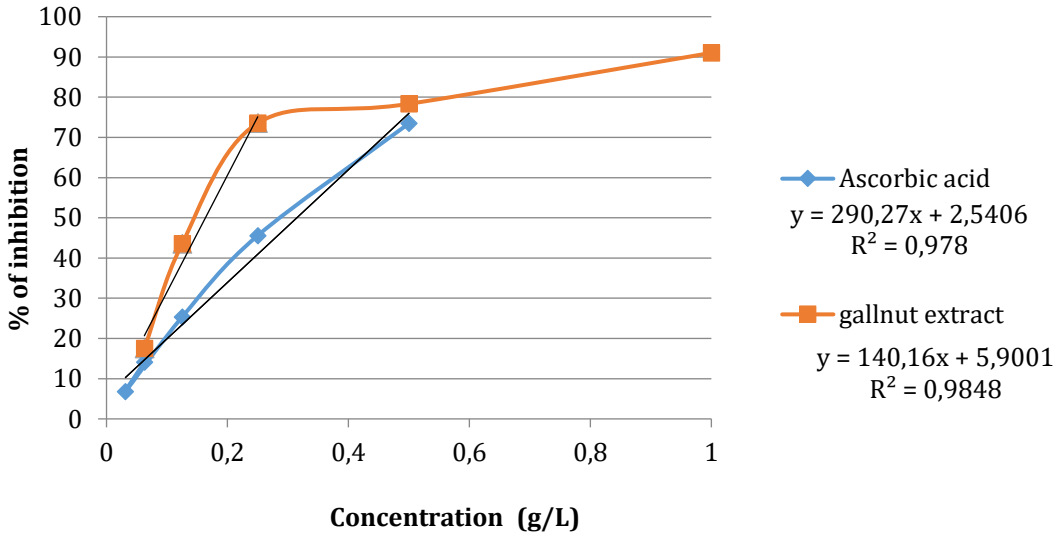
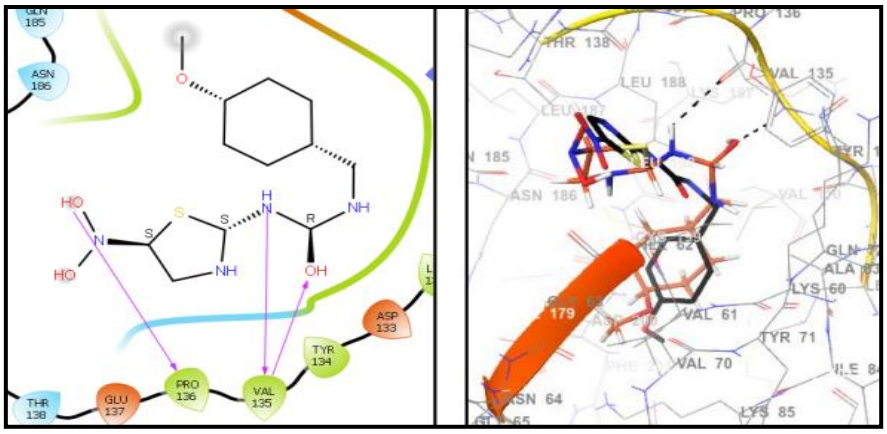


Figure 1. Percentage of DPPH inhibition versus concentration.

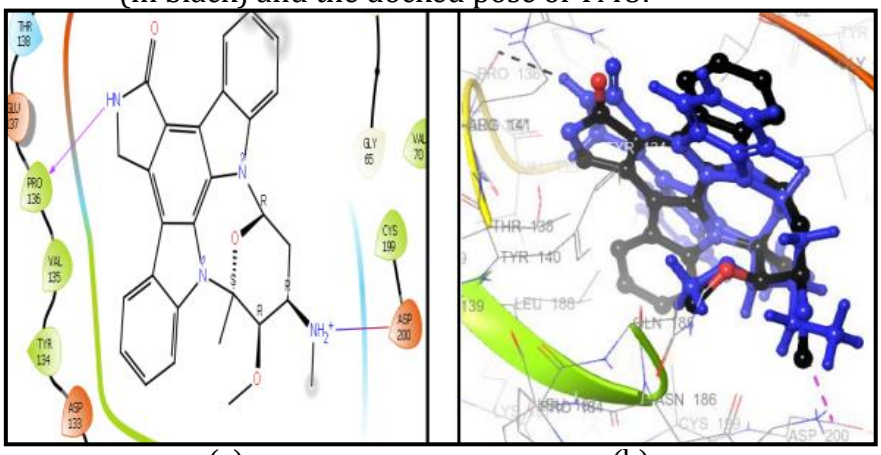
3.4. Molecular docking

The reference ligand (TMU) shares with the active site of the enzyme two "donor-acceptor" bonds with the amino acid Val 135 and a third "donor" bond with the amino acid Pro 136. The interaction energy is estimated at -7.55 Kcal/mol. It was observed that the root-mean-square deviation (RMSD) for the redocking protocol was 1.7164 Å. Similarly, for the cross-docking, the RMSD of staurosporine (STU) was found to be 0.5594 Å. It has an electrostatic bond with Asp 200 and a donor bond with Pro 136. The interaction energy is estimated at -5.25 Kcal/mol. We note that the two RMSDs of the poses are lower than 2 Å, which validates the approach of the docking protocol.



(a) (b)

Figure 2. a) 2D visualization of the co crystallized and docked poses of TMU **b)** 3D visualization of the superposition of the co crystallized pose (in black) and the docked pose of TMU.



(a) (b)

Figure 3. a) 2D visualization of the docked poses of STU
b) 3D visualization of the superposition of the co crystallized (black) and docked poses of STU

Table 3. Scoring results

Ligand	Glid score (Kcal/mol)	State penalty
Ellagic acid	-10.13	0.07
Isocryptomerin	-8.89	0.04
Propylgallat	-8.43	0.05
Ethylgallat	-8.31	0.05
Méthylgallat	-7.8	0.05
Syringic acid	-6.64	0
Amentoflavone	-6.46	0.12
Methylbutulate	-3.64	0
Nycthantic acide	-2.96	0
Roburic acid	-2.75	0
β-sitosterol	-2.61	0
Gallic acid	-1.56	0.01
Methyleolealonate	-1.31	0

Table 4. Detected interactions of the top five scoring ligands

Ligand	Detected interactions from 2D diagram
Ellagic acid	O Val135 NH (1HD) NH Val135 O (1HA) NH Lys85 O (1HA)
Isocryptomerin	O Val135 NH (1HD) NH Val135 O (1HA) NH Lys60 O (HA) Phenyl Arg141 (HI)
Propylgallat	O Val135 NH (1HD) NH Val135 O (1HA)
Ethylgallat	O Val135 NH (1HD)
Methylgallat	O Val135 NH (1HD) NH Val135 O (1HA)

The scoring results obtained and detected interactions of complexes exhibiting significantly lower energy than those obtained by the reference ligand are shown in Tables 3 and 4.

According to these results, the complexes formed with ellagic acid, isocryptomerin, propylgallate, ethylgallate, and methylgallate show energies significantly lower than those obtained by the reference ligand (-7.55 Kcal/mol). Ellagic acid gave the best score (-10.13 Kcal/mol), suggesting that the polyphenol family has strong inhibitory power. Propylgallate, ethylgallate, and methylgallate show scores of -8.43, -8.31, -7.80 Kcal/mol, respectively, these increasing values depend on the length of the esterified alkyl chain. In fact, a three-carbon chain has a higher score than a one-carbon chain.

All ligands with a higher score than the reference share hydrogen bonds with the Val 135 residue. According to El Kerdawy et al. (2019), this binding must be retained to achieve effective inhibition of GSK-3β kinase. Interactions with other residues at the binding site will only increase the strength of the binding interaction (El Kerdawy et al., 2019).

In addition to the two bonds with Val 135, ellagic acid has a hydrogen bond with Lys 85. As for isocryptomerin, it presents, in addition to the two bonds with Val 135, a hydrogen bond with Lys 60 and a hydrophobic interaction between the phenyl group and Arg 141. The latter two interactions further strengthen the binding of the ligand to the active site of the enzyme. According to M. Arfeen et al., the study of the most active and selective compound showed that selectivity is determined by residues Lys 85, Arg 141, Thr 138, and Cys 199 thus allowing the design of selective GSK-3β inhibitors (Arfeen et al., 2016).

Ellagic acid and isocryptomerin share hydrogen bonds with residues Lys 85 and Arg 141 respectively, which allows them to be classified as selective inhibitors.

4. Conclusion

In vitro antioxidant activity and in-silico wound healing activity of *Quercus infectoria* dry extract were determined and presented in this paper. The extract obtained shows a high tannin content, representing 62.63% of total polyphenols. This content explains most of the antioxidant activity of our extract. The final step in our work concerns the study of gallnut healing activity *in silico* via inhibition of GSK-3β by molecular docking. Score energy and molecular interactions are the criteria for inhibitor selection. This study revealed that ellagic acid, isocryptomerin, propyl gallate, ethyl gallate, and methyl

gallate were likely inhibitors, justified by the low score energy these ligands possess towards the reference inhibitor.

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Author Contribution

All authors declare equal contribution to the design and experimental work, interpretation of the results and editing the manuscript.

Conflicts of Interest

The authors declared no conflict of interest.

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GC-MS/MS and 1H-NMR Analysis of Endemic *Campanula baskilensis Behçet (Campanulaceae) Leaf Fractions*

Sarmad MARAH[1], Tevfik OZEN[1]*, Ibrahim DEMIRTAS[2,3], Yaşar IPEK[4], Fatih GUL[2], Lutfi BEHCET[5]

[1]Ondokuz Mayıs University, Faculty of Science, Departman of Chemistry, Samsun-Türkiye
[2]Research Laboratories Application and Research Center (ALUM), Igdir University, Iğdır-Türkiye
[3]Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Ondokuz Mayıs University, Samsun, Türkiye
[4]Department of Chemistry, Faculty of Science, Çankırı Karatekin University, Uluyazı Campus, Çankırı, Türkiye
[5]Department of Biology, Faculty of Science and Art, Bingol University, Bingol-Türkiye
*Corresponding author: tevfikoz@omu.edu.tr

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Abstract

In this research, 1H-NMR and CG-MS/MS spectrophotometric techniques were used to elucidate the chemical profile of leaf fractions of Campanula baskilensis Behçet (Campanulaceae). A column chromatography system was used for the fractionation of the crude extract. Fifteen separate fractions (Fr1-15) were obtained throughout the fractionation procedure. Thirty-nine molecules were identified using both spectrometry techniques. Thirty-one compounds of these molecules were identified using GC-MS/MS and ^{1}H-NMR techniques, and eight compounds were listed as unknown. The highest quantity as a percentage recorded was by lupeyl acetate as following 77.80% in Fr6-2, 63.21% in Fr3-3, 43.81% in Fr4-3. 1-octadecene was determined as 36.23% in Fr3-2 and 32.14% in Fr4-1. Additionally, levels of 34.88% for hexadecanoic acid and 34.05% for borneol were recorded at Fr8. It is also noteworthy that Fr3-4 is formed at a percentage of 97.28 of unknown molecule. Molecular structures such as hexadecanoic acid (methyl palmitate), benzoic acid, eicosanoic acid, limonene, borneol, and hentriacontanol were supported by 1H-NMR analysis. Understanding the chemical constituents of C. baskilensis plant will provide opportunities to suggest broader research in the future. The identified molecules can be the subject of isolation and further original models for various in-vitro and in-silico bioassays or effective drug development and application.

Key Words: *C. baskilensis*, GC-MS/MS, ^{1}H-NMR, Fractionation

1. Introduction

The endemic *Campanula* species are found in limited regions with rocky terrain and edaphic and microclimatic condition (Yildirim et al., 2019). A wide range of chemical compounds has been identified in *Campanula* species, including flavonoids, phenolics, anthocyanins, polyacetylenes, phenylpropanoids, essential oils, acylated triterpenoids, glycosides, resins, and coumarins. Additionally, a variety of their subunit compounds are present, including fraxin, linalool, α-terpineol, lavandulyl acetate, (E, E)-allo-ocimene, β-pinene, α-cadinene, β-farnesene, β-caryophyllene, and myo-inosito (Anestis et al., 2023; Mohammed et al., 2023; Brandt et al., 2017; Kim et al., 2017). Chemical compounds synthesized and

stored in various parts of the plants have essential functions and possess high biological properties that add to the plant's vitality. Biologically active chemical substances found naturally in plants are called phytochemicals. Several previous studies have emphasized that the chemical components of plants performed extraordinary economic and medical importance (Newman and Cragg, 2016). Phenolics are secondary metabolite products of plants and the most common phytochemical category found in plants, and they also play an important role in the reproduction, growth, and metabolism of plants. They are also responsible for defense mechanisms against pathological viruses and fungal infections, parasites, and predators and contribute to the color of plants. Scattered over Iran-Turan, the Eastern Mediterranean, and Mediterranean phytogeographical areas, *Campanula*, one of the most prominent genera in the Campanulaceae family, it is represented by about 115 species in Türkiye. Several investigations aimed to identify the volatile components of the aerial parts of Campanula species and some of their morphology and phenological aspects. The importance of *Campanula* taxa, which are edible and decorative plants, has been uncovered via expanded research in addition to its medical advantages (Sarıkaya and Kavaklı, 2020). According to the results of epidemiological studies, increasing the consumption of plant-based foods, fruits, and vegetables is extremely important in preventing chronic diseases such as cancer, diabetes, cardiovascular diseases, Alzheimer's disease, and age-related functional decline. Fruits and vegetables contain phytochemicals, such as active phenolic compounds, which act as natural antioxidants (Wen et al., 2015; Liu, 2013; Eberhardt et al., 2000; Sun et al., 2002). The past few decades have seen an increase in passion for investigating secondary metabolites that promote health, such as phenol and carotenoid compounds, from many perspectives. The essential components of *Campanula* species and several secondary metabolism products were quantitatively measured using chemotaxonomic analysis techniques. As several earlier investigations have confirmed, aerial parts of the plant, particularly the leaves, are also thought to be an abundant source of such chemicals (Politeo et al., 2013).

This work aims to characterize polyphenolic and volatile compounds quantitatively for leaf fractions of the newly discovered endemic *C. baskilensis* Behcet. We used chemotaxonomic methods with the application of NMR techniques for the fractions that showed high purity and GC-MS/MS compared to more than 30 volatile and widely researched compounds. Access to the chemical content of this plant may enable us to form a comprehensive idea of the potentially bioactive compounds present in the other species within the same environment, especially since these plants are endemic to Türkiye in an environment that is difficult for other species to live in.

2. Material and Methods

2.1. Plant material and chemicals

A regionally localized and widespread species *C. baskilensis* Behçet is reported to exist over the Baskil (Elazığ) area in Türkiye 's Eastern Anatolia region. It was first collected by Prof. Dr. Lütfi Behçet, Bingol University's Faculty of Arts, Department of Biology (Behçet and İlçim, 2018).

2.2. Fractionation

Fractionation processes using solvents with different polarities, from lowest to highest polarity, were applied to obtain separate fractions that contain a single or few compounds that carry the same physical properties as the extract. Also, in order to reach pure compounds, it is necessary to go through this step, which is considered somewhat preliminary to obtain individual compounds after performing the second

fractionation process, second fractions containing a group of compounds that may be close in polarity or size. Firstly, the triplet extraction process was applied to the *C. baskilensis* leaves (251 grams) using a solution mixture of methanol-chloroform 1:1. Once the 32 g of crude extract was obtained, it was applied to the impregnated silica column (100 grams of silica gel (silica 60) was prepared for chromatographic processes by eluting it with hexane) with hexane. Additionally, based on the increasing polarity of the elution, methanol 6.6, ethyl acetate 4.4, and chloroform 2.7 solvents were utilized in that order. Nine significant fractions were obtained from this rudimentary fractionation (Basar et al., 2023). Then, based on the results of thin-layer chromatography, the second stage of fractionation was applied for more accurate separation using the Sephadex (LH-20) column to reach isolated compounds, using mixed solvent systems (methanol, chloroform, ethyl acetate, and hexane) as shown in Figure 1.

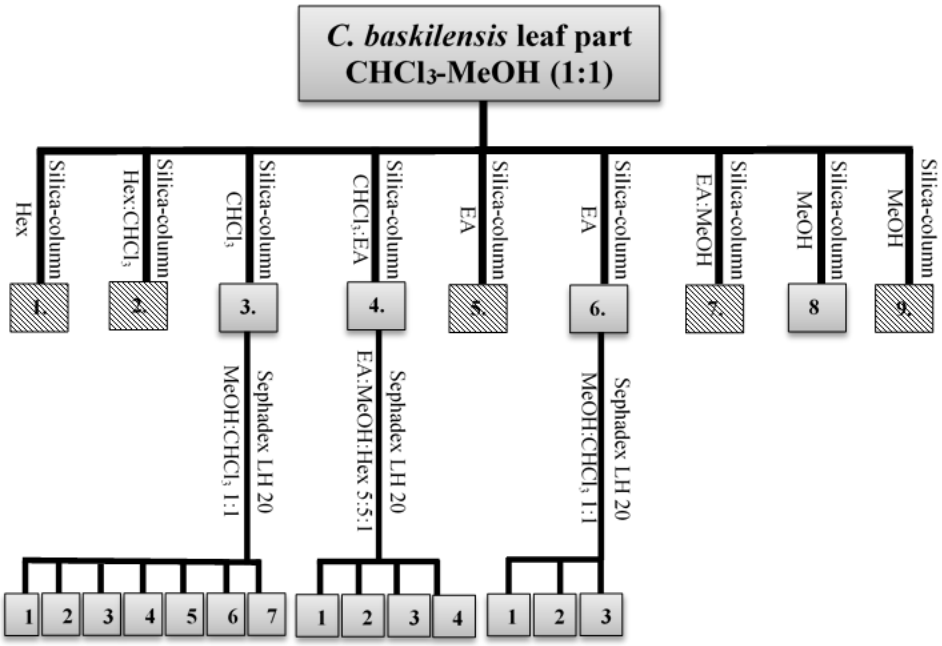

Figure 1. Chemical characterization fractionation scheme; methanol-chloroform (MeOH:CHCl3), hexane (Hex) and ethyl acetate (EA)

Comparing the thin layer chromatography findings for each sub-fraction allowed the combination of the similar ones. Fractions with similar content were combined by applying thin-layer chromatography (TLC), and nine sub-fractions were obtained. A 60-40 ethyl acetate-hexane solvent system was used for TLC. Anisaldehyde was used as the TLC reagent. Subfractions of each fraction were combined with similar ones by TLC. These re-obtained fractions were subjected to chromatography with Sephadex in a solvent system using only methanol (Ipek et al., 2017). These applications allowed us to get fifteen highly pure fractions that could be subjected to characterization methods Figure 1.

2.3. GC-MS/MS analysis

All applied samples underwent esterification to determine the chemical constituents of the *C. baskilensis* fractions' leaf part. Samples at the ratio of 1:10 were mixed with hexane for the esterification procedure. 5 mL of 1M KOH (dissolved in MeOH) was then added, and the mixture was forcefully agitated for 30 seconds using a vortex device. The outcome

of the reaction indicated that 1 mL of the upper phases, which contained fatty acid methyl esters that had formed in the mixtures (the hexane phase), should be taken and filtered into vials using a syringe to pass through a 0.45-micron filter. The mixture's constituents were then subjected to a phytochemical analysis using GC-MS. Analyses were performed using a mass detector model Agilent 5975C with the triple-axis detector and Agilent Technologies Brand 7890A model GC-MS equipment. The following analytical parameters and GC temperature programming were used in GC analyses of fractions: the initial temperature was set to 100°C for 10 minutes, then a constant 20°C/minute to 180°C for 15 minutes, followed by a constant 20°C/minute to 300°C for 30 minutes. The ion temperature of the MS detector is 280°C. Agilent J&W HP-5ms Ultra Inert on GC column (5%-phenyl-methylpolysiloxane) specifically tested for inertness in the analysis of active compounds (30m X 320 µm X 0.25 µm) has been carried out (Yenigun et al., 2024; Ozen et al., 2017).

2.4. ^{1}H-NMR analysis

The Agilent-Premium Compact 14.1 Tesla 600 MHz Frequency NMR equipment was utilized to identify the structures of the fractions' molecules. The amount of protons in a molecule and how each proton interacts with its neighbor protons may be seen in the ^{1}H-NMR spectrum (Ipek et al., 2017; Ozen et al., 2017; Yenigun et al., 2023; Yenigun et al., 2024).

3. Results and Discussion

Identifying the chemical composition of the fractions' contents and/or isolating molecules, as well as determining the chemical structures using appropriate analytical processes, paves the way for future studies regarding the biological effectiveness of our species. Here in our study, all *C. baskilensis* leaf` fractions and sub-fractions were analyzed using GC-MS/MS and ^{1}H-NMR analysis. The main reason for performing a second fractionation was to obtain separate molecules whose composition is easy to determine using the analysis as mentioned earlier methods. Based on the results of TLC, we selected the Fr8 from the initial fractionation and the remaining fractions from the second fractionation by merging similar fractions after the second stage.

3.1. GC-MS/MS analysis

As seen in Table 1 and Figure 2, GC-MS/MS data for the volatile components for all of the fractions indicates the existence of approximately forty compounds, most of which could be identified. The compounds that were most prevalent among the fractions were as follows: borneol, 1-hexadecene, and hexadecanoic acid where these compounds determined in eleven fractions, followed by tetradecanoic acid, 1-octadecene and 1-octadecanol where this compounds determined in ten fractions. Another remarkable finding was the high level of lupeyl acetate detected in multiple fractions: Fr6-2 at 77.80%, Fr3-3 at 63.21%, and Fr4-3 at 43.81%, followed by 1-octadecene at 36.23% for Fr3-2 fraction and 32.14% for Fr4-1 fraction. In addition, Fr8 recorded high levels of hexadecanoic acid at 34.88% and borneol at 34.05%. It is also striking that Fr3-4 has a large proportion of unknown molecules as 97.28% in addition to borneol as 2.72% . As for the rest of the compounds, they were less prevalent, lower than 30% in all remain fractions. By comparing the results obtained with the results of previous studies for different Campanula species in the literature, we note that these species contain a wide chemical diversity: In a previous study, 53 prominent compounds were identified as the volatile components of C. portenschlagiana detected by GC-MS/MS; such as Linalool, Nonanal, α-terpineol, Pentadecane, and Caryophyllene oxide (Politeo et al., 2013).

Table 1. The volatile composition (%) of the fractions obtained by GC-MS/MS

Compounds	RT (min.)	3-1	3-2	3-3	3-4	3-5	3-6	3-7	4-1	4-2	4-3	4-4	6-1	6-2	6-3	8
Limonene	12.28	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	4.26	nd	29.81	nd
Benzoic acid	14.67	nd	nd	nd	nd	nd	3.64	nd	nd	nd	nd	nd	nd	nd	nd	nd
Borneol	17.21	nd	nd	nd	2.72	2.18	nd	5.60	16.06	3.42	1.35	1.07	8.82	1.02	15.00	34.05
2-tert-Butyl-4-isopropyl-5-methylphenol	27.71	nd	nd	nd	nd	nd	2.63	8.33	3.02	3.15	1.48	nd	nd	nd	nd	nd
Isoaromadendrene epoxide	29.59	nd	nd	nd	nd	nd	nd	nd	nd	1.26	nd	nd	nd	nd	nd	nd
Tetradecanoic acid	30.89	1.87	0.98	nd	nd	nd	1.91	3.66	5.17	13.69	7.03	4.37	3.38	nd	8.49	nd
Unknown	31.57	nd	nd	nd	nd	2.04	nd	nd	4.07	nd	1.92	nd	19.33	nd	nd	nd
1-Hexadecene	31.74	1.18	22.40	2.23	nd	9.71	1.85	16.68	6.39	5.89	7.28	3.70	8.47	nd	nd	nd
Pentadecanoic acid	32.08	0.20	nd	nd	nd	nd	nd	nd	4.44	nd	nd	4.48	nd	nd	nd	nd
2-Pentadecanone, 6,10,14-trimethyl-	33.19	1.22	0.36	nd	nd	4.95	nd	nd	nd	1.76	2.67	nd	15.99	nd	nd	nd
Hexadecanoic acid	33.16	11.97	3.59	2.97	nd	nd	14.10	13.53	13.50	18.46	10.32	16.85	nd	nd	8.69	34.88
1-Octadecene	33.90	nd	36.23	7.02	nd	6.88	13.57	23.16	32.14	6.61	6.91	12.33	nd	nd	9.67	nd
9-Octadecenoic acid	34.91	nd	nd	nd	nd	nd	nd	2.51	nd	nd	8.02	nd	nd	nd	nd	6.89
Octadecanoic acid	35.16	8.16	1.30	nd	nd	nd	nd	2.28	nd	6.30	nd	3.69	nd	nd	nd	8.21
1-Octadecanol	37.05	1.08	nd	nd	nd	1.75	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Eicosanoic acid	37.70	24.30	nd	nd	nd	2.14	7.49	nd	nd	1.75	1.54	4.50	9.88	nd	nd	8.13
Unknown	37.92	nd	nd	nd	nd	nd	nd	1.84	nd	nd	nd	nd	nd	nd	nd	nd
1-Octadecanol	38.69	1.03	14.83	1.99	nd	1.68	6.43	4.88	9.44	1.48	2.16	nd	nd	nd	nd	nd
Heneicosanoic acid	39.27	0.89	nd	nd	nd	11.64	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Stigmast-5-en-3-ol	39.80	nd	nd	nd	nd	nd	21.88	nd	nd	nd	nd	nd	nd	nd	nd	nd
Unknown	40.20	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	1.12	8.77	nd	nd	nd
1-Tricosanol	40.65	29.04	nd	nd	nd	nd	2.55	5.77	nd	nd	nd	nd	nd	nd	nd	nd
Docosanoic acid	41.44	nd	nd	nd	nd	nd	2.28	nd	nd	nd	nd	2.30	nd	10.16	nd	nd
Viminalol	41.76	nd	nd	10.77	nd	12.68	nd	1.87	nd	nd	3.81	nd	nd	nd	nd	nd
Unknown	42.40	nd	nd	nd	nd	3.01	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
1-Pentacosanol	43.18	1.28	7.44	nd	nd	nd	3.00	1.78	5.78	nd	nd	nd	nd	nd	nd	nd
Unknown	43.75	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	1.16	7.50	nd	nd	nd
Unknown	44.81	nd	nd	1.65	97.28	1.95	nd	1.78	nd	nd	nd	nd	nd	nd	nd	nd
1-Tetracosanol	46.62	5.44	2.15	nd	nd	2.23	nd	1.79	nd	nd	nd	4.60	nd	nd	nd	nd
Tetracosanoic acid	48.06	7.44	nd	nd	nd	nd	nd	nd	nd	nd	1.70	nd	nd	nd	nd	nd
Viminalol isomer	48.90	nd	nd	10.17	nd	nd	nd	nd	nd	nd	nd	nd	5.92	11.02	nd	nd
9,12-Octadecadienoic acid,2,3-bis[(trimethylsilyl)oxy]propyl, ester	49.67	nd	nd	nd	nd	nd	6.95	nd	nd	nd	nd	nd	nd	nd	nd	7.84
Lupeyl acetate	50.10	nd	nd	63.21	nd	17.37	nd	nd	nd	nd	43.81	nd	nd	77.80	nd	nd
Nonacosanol	51.17	nd	3.71	nd	nd	nd	nd	nd	nd	nd	nd	30.11	nd	nd	nd	nd
Octacosanoic acid	52.83	nd	nd	nd	nd	nd	nd	nd	nd	17.98	nd	9.74	nd	nd	nd	nd
Unknown	54.86	nd	nd	nd	nd	19.80	nd	nd	nd	18.25	nd	nd	nd	nd	nd	nd
Unknown	56.53	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	7.67	nd	nd	nd
1-Hentriacontanol	57.21	3.78	6.99	nd	nd	nd	8.79	1.96	nd	nd	nd	nd	nd	nd	28.33	nd
Hexacosanoic acid	59.64	1.11	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd

*nd: not detected

In another study, the essential oil components obtained from *C. glomerata* L. subsp. were analyzed using GC-MS, and 48 compounds representing 89.0% of the total volatile components were characterized, the main components were hexadecanoic acid (24.51%), docosane (15.9%), isocitronellene (12.6%), heneicosane (4.6%), hexahydrofarnesyl acetone (3.2%), 9-tricosene (1.6%), octadecanol (1.4%), caryophyllene oxide (1.3%), α-funebrene (1.2%), β-thujaplicinol (1.1%), pentadecanoic acid (1.1%), tricosane (1.1%), (2E,4E)-decadienal (1.0%), (E)-β-damascenone (1.0%) and (E)-caryophyllene (1.0%) (Sinek et al., 2012). In another study, the volatile components composition of *C. olympica* Boiss were analyzed using GC-MS; 19 components representing 94.0% of the total volatile components were characterized and the main components were 2E,6Z-farnesol (14.8%), 3,3-dimethyl-2[5-methoxy-3-methyl-2-pentylidenen]-1-cyclohexanone (12.1%), dihydro aromadendrane (11.6%), tetracosane (9.0%), pentacosane (7.9%), epoxy alloaromadendrene (5.9%) and cyclohexadecanolide (5.8%) (Tosun et al., 2011). The most noticeable peaks in the spectra of the different fractions were investigated in order to determine whose components were the most common, as shown in Figure 2 and Figure 3.

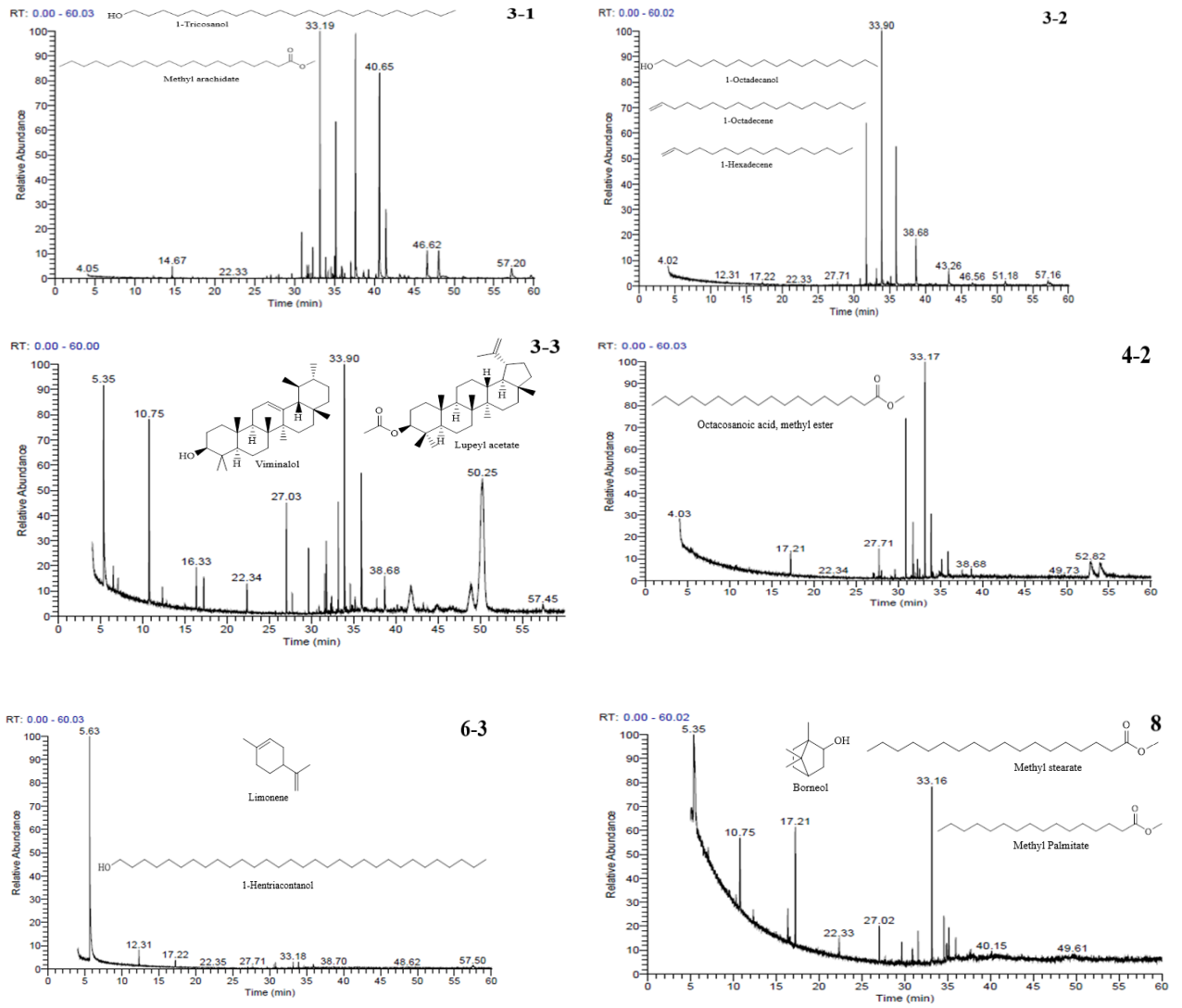

Figure 1. GC-MS/MS Spectrums for the most abundant molecule

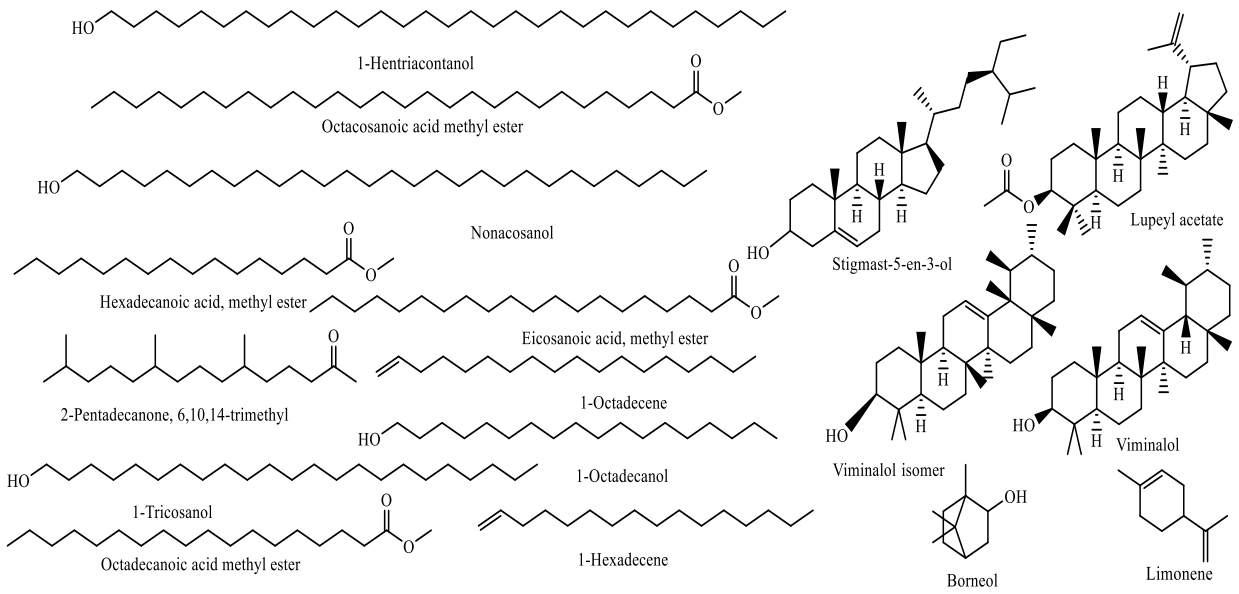

Figure 2. Molecular structures of the most abundant compounds in GC-MS/MS analysis

3.2. ^{1}H-NMR analysis

NMR analysis was performed on *C. baskilensis* leaf fractions depending on the most prominent peaks in conjunction with GC-MS/MS to determine the structures of the different volatile components, Table 1 and Figure 4. CH2 groups in the volatile compounds; as hexadecanoic acid, eicosanoic acid, and 1-tricosanol were resonate as multiplets between 1.2-1.5 ppm, CH3 groups were in the area below 1 ppm, and for the peaks above 2 ppm were s for those CH2 groups adjacent to the carbonyl group, CH2 groups in Fr3-1 were in resonance. For benzoic acid, we inferred through the characteristic hydrogen signals attached to carbon are split into triblets by the effect of neighboring CH2 protons, the other CH3 and CH2 groups appear to resonate between 0.5 to 1.8 ppm, respectively. Fr3-2 has the same groups and it was detected also via the characteristic hydrogen signals attached to carbon 3 split into triblets with the effect of neighboring CH2 protons, the other CH3 and CH2 groups appear to resonate between 0.5 to 1.8 ppm. Fr3-3 spectrum. Fr4-2 spectrum gave 0.9 ppm, the last carbon of the -CH3 fatty acid resonated as a triplet, and between 2.3-2.5 ppmCH2 group appeared to be adjacent to the carbonyl group and resonated like a triplet, Also, at 1.3 ppm hydrogens belonging to saturated hydrocarbons were resonated in the form of muliplets, and finally a mixture of hexadecanoic acid and octadecanoic acid has been observed in the same spectrum. Further, limonene, borneol, and hentriacontanol appear as follows in Fr6-3: olefinic protons resonated around 5.5 ppm, methyl groups resonated around 1 ppm, and hydrogens bonded to carbon adjacent to the hydroxyl group resonated around 4 ppm, while the CH2 groups in the molecules resonated in multiples around 1.5 ppm, the OH groups gave a signal around 3.5 ppm. Finally, Fr8 fraction spectrum showed sugar groups with high amounts due to the hydrogen groups that resonated between 3-4 ppm and the hydroxyl hydrogens that appeared between 4 to 5 ppm. However, it is worth mentioning that sugar groups are not seen in GC-MS/MS because they are not volatile. The reason for detecting some common volatiles found in multiple fractions, although with different solvents, is that the separation techniques used in this research can be considered classical techniques. It also has a kind of complexity, as there is a possibility of more than one compound mixing or coming down of more than one

compound that is different in degree of polarity from the solvent used due to the influence of many factors, including its molecular size and/or weight.

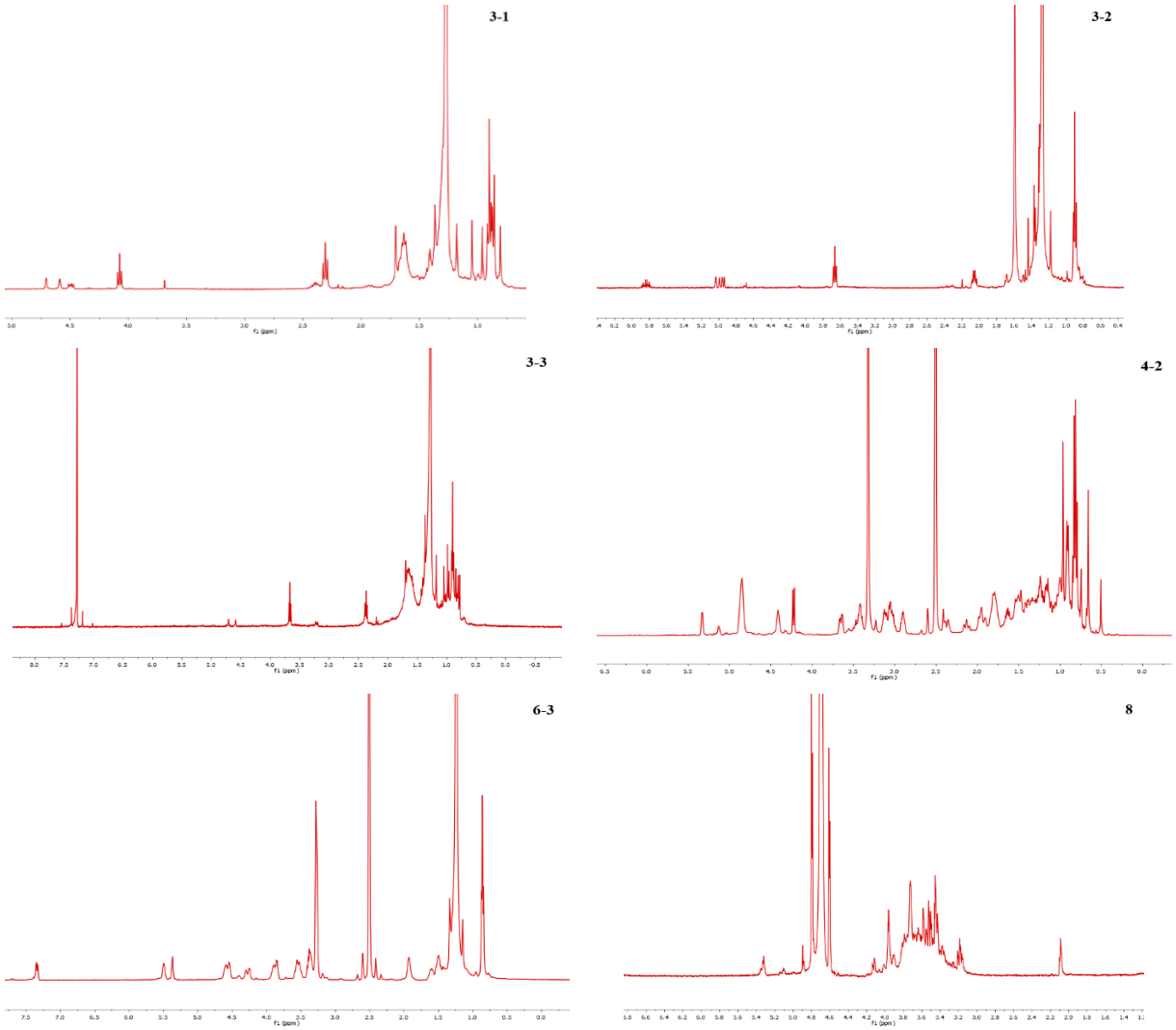

Figure 3. ^{1}H-NMR Spectrums for the most abundant molecules

The following observations were reached by comparing the 1H-NMR data obtained in this study with the data for the same chemicals published in the literature: for borneol in another study they track down δH values as; 1.81 (ddd, J = 16.2, 12.6, 10.5, 5.6 Hz, 2H, –CH–CH2-) 1.64 (t, J = 4.6 Hz, 1H, -CH-) 1.53–1.27 (m, 2H, –CH2-CH2-C-) 1.26–0.92 (m, 2H, –CH2-CH2-C-) 0.91–0.83 (m, 9H, 3 × -CH3) (Dong et al., 2021); 0.85 (3H, s, -CH3), 0.86 (3H, s, -CH3), 0.87 (3H, s, -CH3), 0.95 (1H, dd, H-6b), 1.25 (2H, m, H-5b, H-6a), 1.62 (1H, t, -OH), 1.70 (2H, m, H-3b, H5a), 1.90 (1H, m, H-3a), 2.28 (1H, m, H-4), 4.04 (1H, m, H-2) (Wang et al., 2014). Hexadecanoic acid (methyl palmitate) has been tracked using ^{1}H-NMR (CDCl3, 400 MHz) as the δ found to be: 3.59 (3H, s, OCH3), 2.23 (2H, t, J=8.0 Hz, CH2), 1.55 (2H, t, J=8.0 Hz, CH2), 1.19 (s, -(CH2)n-), 0.82 (3H, t, J= 8.0 Hz, CH3) (Mir et al., 2021). For the Stigmast-5-en-3-ol (β-sitosterol) in a previous study the findings of 1H-NMR (CDCl3, 400MHz) shown signals at δ 3.2 (1H, m, H-3), 5.26 (1H, m, H-6), 5.19 (1H, m, H-23), 4.68 (1H,m, H-22), 3.638 (1H, m, H-3), 2.38 (1H, m, H-20), 1.8-2.0 (5H, m) ppm, in addition to the other peaks were observed at δ 0.76-0.89 (m, 9H), 0.91-1.05 (m, 5H), 1.35-1.42 (m, 4H), 0.69-0.73 (m, 3H), 1.8-2.00 (m, 5H), 1.07-1.13 (m, 3H), 1.35-1.6

(m, 9H) ppm (Kamboj and Saluja, 2011). Moreover, for octacosanoic acid (methyl octacosanoate), 1H-NMR outcomes were recorded as δ = 3.65 (s, OCH3), 2.10 (t, J = 7.7 Hz, H-2), 1.41–1.11 [brs, -(CH2)24-], 0.72 (t, J = 7.3 Hz, CH3)(Wansi et al., 2009).

4. Conclusion

This study represents an exhaustive analysis of the volatile contents via GC-MS/MS and 1H-NMR for diverse fractions of the *C. baskilensis*' leaves. The fractions selected for these analyses were conducted based on their purity using the thin-layer chromatography results. More than thirty volatile compounds were identified due to the component analysis, and a few unknown chemicals with similar properties have been detected, too. Relying on spectroscopic techniques, such as NMR and GC-MS, is greatly beneficial for creating a volatile component and phytochemical content library of what natural sources possess. The quantitative and qualitative analysis of *C. baskilensis*'s volatile components provides information regarding the effectiveness of their extracts or chemical compositions and determines whether or not it is worthy of forward isolation and/or bioassay application. Our research has revealed that *C. baskilensis* possesses a diverse range of volatile components. Further investigation into its biological activity may be achieved using enhanced isolation techniques and experimental and theoretical evaluation.

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Author Contribution

All authors declare equal contribution to the design and experimental work, interpretation of the resultsand editing the manuscript.

Conflicts of Interest

The authors declare no conflicts of interest during the accomplishment of this research. None of the authors has any financial and/or personal relationships with other people or organizations that could inappropriately influence (bias) their work.

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A Narrowed Look into Plant-Derived Testosterone 5α-Reductase Inhibitors for Androgenetic Alopecia

Nurten ABACI KAPLAN*, Ilkay ERDOGAN ORHAN

Department of Pharmacognosy, Faculty of Pharmacy, Gazi University, 06330 Ankara, Türkiye
E-mail: nurtenabaci@gazi.edu.tr ORCID ID: 0000-0002-4144-7074
E-mail: iorhan@gazi.edu.tr ORCID ID: 0000-0002-7379-5436
*Corresponding author: nurtenabaci@gazi.edu.tr

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Abstract

Androgenetic alopecia (AGA) is the most prevalent cause of hair loss in men and women, resulting from a combination of genetic susceptibility and androgen hormones. Increased activity of testosterone 5α-reductase in hair follicles leads to increased synthesis of dihydrotestosterone (DHT). DHT's destructive effect on hair follicles is one of the leading reasons for hair loss. Conventional pharmacotherapeutic treatments, *e.g.* finasteride and minoxidil, are frequently accompanied by adverse effects and may be ineffective for certain people. Recent years have seen a rise in research on plant-derived testosterone 5α-reductase enzyme inhibitors. These herbal substances produced by various plant extracts and natural ingredients show promise in terms of fewer side effects and improved tolerability. This mini review investigates herbal testosterone 5α-reductase inhibitors for treatment AGA.

Key Words: Androgenetic alopecia, testosterone, 5α-reductase, 5α-reductase inhibitors, herbal 5α-reductase inhibitors

1. Introduction

Hair loss (alopecia) is a frequent and non-contagious condition in modern culture, affecting people of all sexes and ages. It can be caused by a variety of variables, including genetics, hormonal changes, stress, pharmaceutical usage, or side effects from any therapy. In other words, alopecia is a disorder caused by the weakening of hair follicles on the scalp or other parts of the body where hair grows for a variety of reasons. Hair loss has been a common worry for many people in many nations in recent years, mainly mostly to the pressures associated with contemporary living (Aukerman & Jafferany, 2023; Liu, Xu, Meng, Liu, & Liu, 2024). According to a statistical analysis performed at a dermatology clinic, the proportion of patients requesting treatment for hair loss headed from 1.24% in 2010 to 9.44% in 2020. The number of female patients requesting therapy for hair loss increased significantly during the previous 10 years. Furthermore, the study concluded that the prevalence of androgenetic alopecia (AGA) increased the most from 17% in 2010 to 32% in 2020. The study's clinic visits comprised 30.6% for AGA, 19.3% for alopecia areata, 15.4% for telogen effluvium, 14.9%

for seborrheic dermatitis, and 7.1% for lichen planopilaris (Lyakhovitsky *et al.*, 2023). A study of 226 patients aged 10 to 16 years old revealed that complaints of hair loss in pediatric patients doubled over 10 years. The rise was most typically observed among boys (Xiao *et al.*, 2006). According to a study (Al Najjar *et al.*, 2023), half of men and one-third of women will get alopecia over their entire lives. Hair loss is a common health issue in daily life. Typically, shedding between 25 to 125 hairs *per* day is considered typical. However, this number may vary by gender. Because there is no degeneration or lack of hair follicles, this condition is not deemed pathologic. However, shedding more than an average of 100 hairs each day might suggest pathological hair loss (Chumlea *et al.*, 2004; Katharina, Wolf-Bernhard, & Christoph, 2004; Semalty, Semalty, Joshi, & Rawat, 2011). Alopecia not only affects people's physical appearance, but also causes psychological, mental, and social problems. In other words, it may have a substantial influence on people's quality of life, mental health, and even self-esteem, as well as their social lives. Patients with alopecia have much greater rates of sadness and anxiety than others (Al Najjar *et al.*, 2023). A study conducted in Poland revealed that 60% of Polish men feel ashamed of hair loss, 81.3% experience stress in their daily lives, and 66.7% report a significant impact on their self-esteem (Adamowicz, Załęcki, Dukiel, & Nowicka, 2022). Therefore, novel and effective drugs to treat AGA and other types of hair loss are needed.

2. Method

A literature search was conducted on Scopus, Web of Science, Google Scholar Library, and PubMed to evaluate the relationship between AGA and the enzyme known as testosterone 5α-reductase. Various filters were used to search for articles in English. The following search terms were utilized: androgenetic alopecia, testosterone 5α- reductase, AGA treatment, AGA treatment methods, finasteride, herbal 5α- reductase inhibitors, and plants against alopecia.

3. Causes of Alopecia

Gender, age, and race may all affect the prevalence of alopecia. While numerous disorders can induce alopecia, several treatments can also lead to alopecia as a side effect (Al Najjar *et al.*, 2023). Hormonal changes (*i.e.* pregnancy, menopause), eating habits, obesity, autoimmune illnesses, hormonal problems, and stress could lead to alopecia. The decrease in estrogen levels after menopause also causes baldness. When alopecia develops as a result of another disease, the underlying cause should be identified. This method also helps cure disease-related alopecia. Alopecia may also result from a few autoimmune illnesses. Thyroid issues, such as hypothyroidism and hyperthyroidism, can lead to alopecia by inducing hair loss through a condition known as alopecia areata, in which the body views its own hair follicles as alien dangers. Stress can cause temporary hair loss problems, such as telogen effluvium. During times of high stress, changes in hormone levels occur in the body, which can lead to hair loss. Stress management and psychological assistance are essential for successfully treating this form of hair loss. Hair loss can also be caused by nutritional deficits, particularly those involving iron, zinc, B vitamins, or protein. Therefore, maintaining a balanced diet is vital. Alopecia can also be seen in polycystic ovary syndrome (PCOS), a common endocrine condition in women. In this condition, there is an excessive production of androgen hormones in women, which can lead to androgenetic-type hair loss.

Oxidative stress is characterized by an imbalance between normal oxidation and antioxidant defense mechanisms in cells, resulting in an increase in reactive oxygen species (ROS). ROS can cause damage to biomolecules such as DNA, proteins, and lipids within the cell. Oxidative stress is

known to play a role in many diseases, including aging, cancer, cardiovascular diseases, and neurodegenerative diseases. Hair follicles are protected against oxidative damage through antioxidant enzymes. However, in some cases, such as aging, hormonal changes or environmental factors, antioxidant defense mechanisms may be inadequate, leading to augmented oxidative stress. In this scenario, damage to hair follicles may increase, accelerating hair loss. The effect of DHT on hair follicles may also be a mechanism for oxidative stress. DHT can upsurge ROS production or weaken the antioxidant defense system in hair follicle cells. This can increase oxidative stress in hair follicles, accelerating hair loss. Additionally, the role of oxidative stress in the pathogenesis of AGA may be associated with cellular aging in hair follicles. Cellular aging involves the loss of cellular functions and even the acquisition of resistance to cell death. This can affect the normal cycle of hair follicles, leading to changes in the hair growth cycle and triggering hair loss. As a result, oxidative stress is thought to play a role in the pathogenesis of AGA and may accelerate hair loss. Antioxidants might potentially be beneficial in preventing and treating hair loss. Antioxidants can reduce oxidative damage to hair follicles, helping to keep hair healthier and prevent hair loss. Additionally, antioxidants may promote hair growth and support the renewal of hair follicles (Cwynar, Olszewska-Słonina, & Czajkowski, 2020; Prie, Iosif, Tivig, Stoian, & Giurcaneanu, 2016; Trüeb, 2009). Between October 2014 and May 2015, an in-depth analysis was undertaken at the Elias Dermatology Hospital and Dermatology Clinic in Bucharest (Romania), meticulously gathering plasma samples from 27 individuals grappling with AGA. This investigation precisely quantified levels of Trolox equivalent antioxidant capacity (TEAC), malondialdehyde (MDA), and total thiols within the plasma of each participant. Furthermore, it delved into assessing the activities of key antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) alongside TEAC activity in the erythrocyte samples. Remarkably, the conclusion of this exploration revealed a pronounced reduction in SOD activity among the AGA patients, alongside a notable decline in TEAC activity and an escalation in MDA levels within their plasma samples, signaling an upsurge in oxidative stress.

This pivotal research unequivocally verified oxidative stress's involvement within the dermal papillae of individuals afflicted by AGA (Prie *et al.*, 2016). Complementarily, a parallel investigation in Türkiye probed the linkage between oxidative stress and AGA, focusing on 33 male patients, aged 18 to 30, diagnosed with the condition. This study measured total oxidant levels (TOS), total antioxidant levels (TAS), and the oxidative stress index (OSI), uncovering significantly elevated TOS and OSI values in AGA sufferers, despite unchanged TAS levels, thereby identifying increased oxidative stress in these young individuals.

These investigations collectively underscore the critical role of oxidative stress in the etiopathogenesis of AGA, advocating for further molecular research to unravel the complexities of this condition more thoroughly (Kaya Erdogan *et al.*, 2017). Moreover, they propose the contemplation of incorporating topical or systemic antioxidants into AGA treatment protocols, highlighting a potentially pivotal approach to managing this condition.

4. Androgenetic alopecia (AGA)

AGA unfolds through a nuanced process whereby hair follicles progressively diminish in size, leading to the transformation of robust terminal hairs into delicate and vellus-like strands. This transformation triggers a reduction in the anagen phase's duration—the critical period that dictates hair length within the

cycle of hair growth—while extending the telogen phase. Such shifts not only render newly emerged hairs noticeably shorter but also contribute to the gradual miniaturization of hair follicles, paving the way for the emergence of a balding visage. As the follicles miniaturize, the hairs they produce become increasingly finer, shorter, and less vibrant in color. In certain instances, this process may also give rise to microinflammations within the follicles. Though AGA is predominantly categorized under non-scarring forms of alopecia, there exists a scholarly discourse suggesting its classification as both scarring and non-scarring, underscoring the complexity of its nature.

A distinct feature of AGA is the heightened sensitivity of hair follicles atop the head to androgens as time progresses, which leads to their gradual atrophy. This heightened sensitivity results in a marked shortening of the anagen phase and a prolongation of the telogen phase within the hair growth cycle. Consequently, the ongoing miniaturization of hair follicles manifests ultimately as baldness, illustrating a profound transformation in the landscape of scalp hair (Yorulmaz, 2016).

AGA stands as the predominant form of hair loss affecting both men and women. Its onset often traces back to adolescence, with its prevalence escalating progressively with age. Remarkably, statistics indicate that by the time individuals reach the age of 70, approximately 80% of men and 50% of women will have encountered the effects of AGA. This widespread condition underscores a significant aspect of the aging process, affecting a vast majority of the population to varying degrees (Al Najjar *et al.*, 2023). The pioneering research conducted by Hamilton on men with hypogonadism, characterized by delayed or absent sexual maturation, has shed light on the intricate relationship between hair loss and hormonal fluctuations, with a specific focus on variations in testosterone (Fig. 1) levels. This work has unveiled a compelling insight that the incidence of AGA is virtually nonexistent in men who underwent castration before reaching puberty or those suffering from hypogonadism. However, the external administration of testosterone to these individuals was found to potentially initiate AGA.

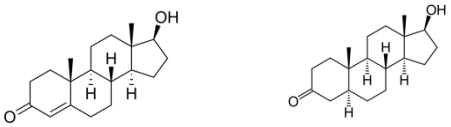

Fig. 1. Testosterone (left) and dihydrotestosterone (right)

These observations have led to the hypothesis that the genesis of AGA may be intricately linked to a complex interplay among androgens, genetic predispositions, and age-related factors, suggesting a multifaceted foundation for this common form of hair loss (Hamilton, 1951). The prevalence of AGA exhibits notable variability across different racial groups, with the highest occurrence observed in Caucasians, followed by Asians, and exhibiting the lowest incidence among Eskimos. This distinctive distribution underscores a profound genetic linkage with AGA, highlighting the influence of genetic factors on its manifestation. The patterns of hair loss associated with AGA also differ markedly between genders. In men, the condition primarily affects the temporal, frontal, and vertex (crown) regions of the scalp, leading to a receding hairline and a characteristic "horseshoe" pattern of baldness. Conversely, in women, AGA manifests through a diffuse thinning and shedding of hair, predominantly in the frontal and vertex regions, without a pronounced recession of the hairline. Hair loss in women tends to concentrate at the vertex and crown areas, and when the frontal region is more significantly impacted, the pattern of loss may resemble the shape of a "Christmas tree." This gender-specific variation in the manifestation of AGA further illustrates the complexity of the

condition and its underlying genetic and hormonal influences (Al Najjar *et al.*, 2023; Aukerman & Jafferany, 2023; Chan & Cook, 2018; Fus-Mazurkiewicz, Nowiński, Sak, Mazurkiewicz, & Król, 2024; Nestor, Ablon, Gade, Han, & Fischer, 2021).

To classify AGA, the Hamilton-Norwood scale was developed for the first time in 1951 and continues to be utilized today. This scale categorizes hair loss in men into 7 clinical stages (Norwood, 1975). Additionally, the Ludwig classification, established in 1977, grades AGA in women across three stages. This methodical approach in categorizing AGA stages has significantly contributed to the understanding and treatment of hair loss, providing a structured framework for both diagnosis and evaluation of progression in affected individuals (Ludwig, 1977). As the name suggests, the fundamental cause of AGA lies in the abundance of androgen receptors on the scalp and the hair follicles' sensitivity to androgen hormones. Particularly, DHT, a product of the reaction catalyzed by the testosterone-5α-reductase enzyme, generates a stronger androgenic signal compared to testosterone. Consequently, DHT leads to the miniaturization of hair follicles, resulting in hair thinning and loss. Consistent with this pathophysiological mechanism, individuals with AGA have elevated levels of 5-α-reductase type II, a crucial enzyme in the conversion of testosterone to DHT. In 1916, Osborn proposed that AGA could be an autosomal dominant disease, suggesting its transmission from one generation to the next (Osborn, 1916). However, Küster & Happle (1984) revisited the genetics of AGA in 1984, challenging Osborn's hypothesis by arguing that it was not sufficiently substantiated with examples, thus casting doubt on its validity. They suggested a polygenic inheritance pattern as more plausible. Studies to date support the notion that AGA possesses polygenic traits, yet its pathophysiology and genetics remain not fully understood. It has been proposed that the androgen receptor gene plays a significant role in AGA, indicating that the disorder is multifactorial, involving several genes and influenced by environmental factors as well (Ellis, Sinclair, & Harrap, 2002). There is substantial evidence and research supporting the role of androgens in the development of AGA. Even though testosterone levels in individuals with and without AGA may be similar, those with AGA tend to have higher levels of free testosterone, DHT, and active testosterone. Moreover, individuals lacking the testosterone-5α-reductase type II enzyme show a lower prevalence of AGA (Devjani, Ezemma, Kelley, Stratton, & Senna, 2023; Piraccini & Alessandrini, 2014). Yet, the pathophysiology and molecular mechanisms of AGA are still not entirely elucidated. The diagnosis of AGA is established through the patient's history and specific tests. The area of hair loss and its severity are determinative. An early diagnostic feature of AGA is an increase in fine, vellus hairs and a reduction in hair shaft diameter by approximately 20% compared to normal (De Lacharrière *et al.*, 2001; Kaliyadan, 2022; Lolli *et al.*, 2017). Various scales are used to assess the severity of hair loss. The "Norwood-Savin Scale" is one of the most commonly used for classifying hair loss in men, while the "Ludwig Scale" or "Sinclair Scale" is used for women. Diagnosis begins with a physical examination, where the hairline, hair density, pattern of hair loss, and other signs on the scalp are evaluated. This helps identify the typical signs of androgenic alopecia, such as a receding hairline and thinning hair at the crown. Examination with a dermatoscope can reveal characteristic findings of AGA, including thinning of hair follicles, miniaturization of the hair, and changes in scalp pigmentation. Laboratory tests to assess androgen levels, particularly free and total testosterone and dehydroepiandrosterone sulfate (DHEAS), can also assist in the diagnosis (Alves, 2017;

Devjani *et al.*, 2023; Piraccini & Alessandrini, 2014).

4.1. Treatment of AGA

Despite its widespread occurrence, while the diagnosis of AGA is often straightforward, there is no standard treatment guideline for managing the condition. Initiating treatment at the earliest possible stage is crucial. The selection of a treatment approach in AGA takes into account factors such as feasibility, effectiveness, and cost. The primary goal in treating AGA is to halt the miniaturization of hair follicles and to enhance hair density. Typically, AGA treatments aim to slow hair loss, increase hair density or promote hair regrowth.

Currently, oral finasteride and topical minoxidil are the only two American Food and Drug Administration (FDA)-approved medications for AGA treatment. In 1997, the FDA approved the use of finasteride at a daily dose of 1 mg for men with mild to moderate AGA. Nevertheless, due to its teratogenic effects, it is advised that women either do not use finasteride or use it in conjunction with suitable contraception methods. Numerous clinical studies and meta-analyses have validated the effectiveness of oral finasteride in AGA treatment.

Minoxidil is available as a 2% solution (primarily for women) and a 5% solution or foam (primarily for men) with the recommended dosage being 1 ml applied once or twice daily. The foam formulation, which does not contain propylene glycol, tends to irritate the scalp less. Initially used as an antihypertensive medication, minoxidil was observed to cause hypertrichosis as a side effect, which led to its application in AGA treatment. In the scalp, the sulfotransferase enzyme converts minoxidil to its active metabolite, minoxidil sulfate. Although the exact mechanism of action of minoxidil remains partially understood, several hypotheses exist.

Besides these FDA-approved treatments, numerous other therapeutic methods without FDA approval are currently employed in AGA management. Treatment is not limited to pharmacotherapy but also includes various physical and complementary therapeutic approaches (Alzaid, 2023; Devjani *et al.*, 2023; Ellis *et al.*, 2002; Feldman *et al.*, 2023; Fus-Mazurkiewicz *et al.*, 2024; Liu *et al.*, 2024; Lolli *et al.*, 2017). In recent years, the FDA has approved the combined treatment method of topical minoxidil and oral finasteride for AGA management (Trilisnawati *et al.*, 2021). The effects of finasteride and minoxidil treatment begin to manifest after 6 months of use. Continuous lifelong treatment is necessary, as cessation of the medication results in the resumption of hair loss.

Herbal extracts and pure substances are likewise employed in the treatment of AGA. For instance, pure substances like beta-sitosterol and biochanin A (Fig. 2), as well as extracts from *Serenoa repens* (W.Bartram) Small, *Panax ginseng* C.A.Mey., *Curcuma aeruginosa* Roxb., *Cucurbita pepo* L., and *Trifolium pratense* L., have exhibited positive effects by inhibiting DHT formation.

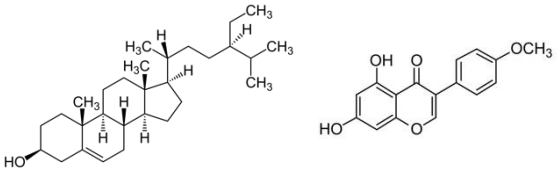

Fig. 2. Beta-sitosterol (left) and biochanin A (right)

Rosemary (*Rosmarinus officinalis* L.) extract reduces hair loss by enhancing perifollicular vascularization, while *Camellia sinensis* L. extract displayed anti-apoptotic activity, thereby reducing hair loss (Feldman *et al.*, 2023; Karaca & Akpolat, 2019; Nestor *et al.*, 2021; Ntshingila, Oputu, Arowolo, & Khumalo, 2023; Trilisnawati *et al.*, 2021). *Nigella sativa* L. seeds and the fixed oil of the seeds possess antioxidant and anti-

inflammatory activities. Thymoquinone, constituting 30-48% of its content, displayed a notable anti-inflammatory activity by suppressing pro-inflammatory cytokines like tumor necrosis factor-α (TNF-α), interleukins (IL)-3, 4, and 5. In a randomized, double-blind, placebo-controlled study conducted on 10 patients with telogen effluvium type alopecia, oil derived from *N. sativa* was applied topically daily for 3 months. The study concluded that within 3 months, there was a significant intensification in hair density and hair shaft thickness, and many patients experienced a reduction in inflammation at the hair roots (Rossi *et al.*, 2016).

Procapil™ is a complex that contains biotinoyl tripeptide-1, apigenin, and oleanolic acid ((Fig. 3). Its effects on nourishing hair follicles, preventing hair loss, and stimulating new hair growth have been proven in clinical studies. Unlike FDA-approved minoxidil and finasteride, Procapil™ does not have any reported side effects. Apigenin, a flavonoid found in Procapil™, promotes vasodilation in the capillaries of hair follicles, thereby enhancing blood flow and nutrition to the hair roots (Guerrero & Ch, 2011).

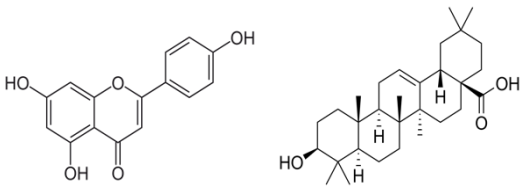

Fig. 3. Apigenin (left) and oleanolic acid (right)

However, detailed and definitive studies on the sole effect of apigenin on hair growth are still lacking. Oleanolic acid in the formulation inhibits types I and II of 5α-reductase, preventing the formation of DHT. Biotinoyl tripeptide-1 has antioxidant activity and prevents the aging of hair roots, thereby inducing the growth of new hairs (Karaca & Akpolat, 2019).

Developed by a Swiss company, Redensyl™ is a compound used to promote hair growth. It consists of various components such as dihydroquercetin glucoside, epigallocatechin gallate glucoside, glycine, and zinc. Dihydroquercetin glucoside and epigallocatechin gallate EGCG) glucoside in its content stimulated hair follicle cells and dermal papilla, inducing hair growth. Glycine is an amino acid found specifically in the proteins of the hair structure, playing a crucial role in hair metabolism (Jenkins & Powell, 1994). Zinc helps strengthen the hair structure and facilitates the binding of cysteine to keratin in the hair (Hsu & Anthony, 1971). Redensyl™ is used to prevent hair loss and promote the growth of new hair follicles.

Capixyl™, developed by a French company, is an innovative blend of red clover extract (*Trifolium pratense* L.) and biomimetic peptides. It has recently become quite popular for helping prevent hair loss (Loing, Lachance, Ollier, & Hocquaux, 2013). In a study conducted in Türkiye, Capixyl™, Procapil, and Redensyl™ were combined and evaluated against minoxidil (5%) in 120 male patients diagnosed with AGA. The combination of Capixyl™, Procapil™, and Redensyl™ exhibited considerably high success compared to that of minoxidil. While 64.7% of patients treated with the Capixyl™, Procapil™, and Redensyl™ mixture reported a significant or moderate improvement, only 25.5% of patients treated with minoxidil (5%) observed a significant or moderate improvement (Karaca & Akpolat, 2019).

In the treatment of AGA, a diverse array of therapeutic options is available, targeting various aspects of the condition's pathophysiology. These treatments range from pharmacological interventions to procedural approaches, aiming to slow hair loss, stimulate hair growth or improve hair density. Table 1 shows the mechanism of action and side effects of the methods and active substances used in the treatment of AGA.

4.2. Relationship between AGA and testosterone-5α-reductase enzyme

Testosterone, the primary male sex hormone responsible for the development of male sexual characteristics, is produced and released by the testes, ovaries, and adrenal glands in the human body. Although present in both sexes, testosterone levels are significantly higher in males. The hormone's production is regulated by the luteinizing hormone (LH) and follicle-stimulating hormone (FSH), which are secreted by the pituitary gland located in the lower part of the brain. Testosterone plays a crucial role in numerous bodily functions, including sexual maturation and reproduction, increasing muscle mass, maintaining bone health, and metabolizing fats.

DHT, a hormone synthesized in the cytoplasm through the catalysis of testosterone by the 5α-reductase enzyme, plays a vital role in various physiological processes in males, such as sexual development and hair formation. While androgens facilitate the development of hair follicles in areas like the beard, underarms, and groin, they paradoxically inhibit hair follicle development on the scalp, leading to hair loss. Excessive amounts of DHT can contribute to health issues such as hair loss and prostate enlargement. The link between hair loss and testosterone is primarily explained through DHT, which binds to androgenic receptors in hair follicles, leading to follicle miniaturization and subsequent hair loss. In AGA, a genetic predisposition increases the sensitivity of hair follicles' androgenic receptors to DHT, thereby causing hair loss.

The enzyme testosterone-5α-reductase, which catalyzes DHT formation, exists in two isoforms, both embedded within the membrane and facilitating the conversion of testosterone to dihydrotestosterone. These enzymes, encoded by different genes, are known as testosterone-5α-reductase type I and type II. While type I exhibits maximum activity at pH 6.5, the type II enzyme operates optimally at pH 4.5, with nicotinamide adenine dinucleotide phosphate (NADPH) acting as a cofactor in this enzymatic reaction. Testosterone-5α-reductase, a membrane-bound enzyme, enhances its activity by reducing the double bonds of steroid substrates in an NADPH-dependent manner.

Moreover, the testosterone-5α-reductase type I is predominantly localized in androgen-independent organs such as skin, liver, brain, and sebaceous glands, whereas the type II enzyme is found in androgen-dependent organs such as the prostate, epididymis, and hair follicles. AGA treatment typically targets the inhibition of the testosterone-5α-reductase type II enzyme, given its localization in tissues relevant to AGA (Burns, Breathnach, Cox, & Griffiths, 2008; Sperling, 2008).

In a pivotal study conducted in 1994 by Dallob *et al.*, the role of the testosterone-5α-reductase type II enzyme in the pathogenesis of AGA was established, marking a significant step towards the current use of finasteride in AGA treatment approved by the FDA (Dallob *et al.*, 1994). Androgens exert various effects on human skin, including the growth of sebaceous glands and the elongation and development of hair. While androgens are not the sole influencers—thyroid hormones and glucocorticoids also play roles—it is widely acknowledged that androgens are the primary regulators of these processes (Inui, Fukuzato, Nakajima, Yoshikawa, & Itami, 2003; Madaan, Verma, Singh, & Jaggi, 2018).

Today, the active ingredients used in AGA treatment, *e.g.* finasteride and dutasteride, function by inhibiting the testosterone-5α-reductase. However, these drugs can have numerous side effects, including erectile

dysfunction, abnormal ejaculation, reduced ejaculation volume, severe myopathy, impaired muscle development, testicular pain, abnormal sexual function, and gynecomastia. Testosterone 5-α-reductase inhibitors are not only utilized in the treatment of AGA but are also applied in managing benign prostatic hyperplasia and prostate cancer and as part of hormone replacement therapy for transgender women (Alzaid, 2023; G.-S. Choi *et al.*, 2022; Dallob *et al.*, 1994).

Table 1: Mechanisms of action and side effects of the active substances used against AGA

Active Substance	**Mechanism of Action**	**Side Effects**
Minoxidil (Topical)	Induces arteriole vasodilation and stimulates cell proliferation and acts as a potassium channel opener.	Local irritation, itching, dry skin, and erythema
Latanoprost (Topical)	A prostaglandin analog that extends the anagen (growth) phase of the hair.	Irritation
Dutasteride (Oral)	Inhibits type I and II testosterone 5α-reductase enzymes, preventing the formation of DHT.	Erectile dysfunction, abnormal ejaculation, gynecomastia, and myopathy
Flutamide (Oral)	An antiandrogen that reduces the effect of testosterone.	Bloating, headache, and breast tenderness
Spironolactone (Oral)	An antiandrogen that reduces the effect of testosterone.	Postural hypotension
Finasteride (Oral)	Inhibits type II 5α-reductase enzyme, preventing the formation of DHT.	Erectile dysfunction, abnormal ejaculation, gynecomastia, myopathy, and psychological disorders
Bicalutamide (Oral)	An antiandrogen that reduces the effect of testosterone.	Bloating, headache, and breast tenderness
Botulinum Toxin Type A (Injection)	Blocks the effect of DHT on hair follicles.	Temporary sagging in the muscles near the injection site, and headache
Platelet-Rich Plasma (PRP)	Platelets in the plasma induce hair growth and repair through growth factors and cytokines.	Headache and bleeding at the application site
Low-Level Laser Therapy (LLLT)	Stimulates cellular proliferation and vasodilation through the induction of nitric oxide, promoting hair growth.	Urticaria
Microneedling	Stimulates the release of growth factors that promote angiogenesis.	Pain and discomfort

4.2. Plant-derived testosterone-5α-reductase inhibitors

Plant-derived natural compounds with testosterone 5α-reductase inhibitory effect are used to slow down hair loss in conditions like AGA or to combat situations such as prostate enlargement by blocking the conversion of testosterone to DHT. Previous studies have demonstrated that extracts from a variety of plants such as *Urtica dioica* Linn., *Caesalpinia bonducella* Fleming., *Tribulus terrestris* Linn., *Pedalium murex* Linn., *Sphaeranthus indicus* Linn., *Cuscuta reflexa* Roxb., *Citrullus colocynthis* Schrad., *Benincasa hispida* Cogn.,

Phyllanthus niruri Linn., *Echinops echinatus* Linn., *Ocimum basilicum* L., *Oryza sativa* L., *Polygonum multiflorum* Thunb., *Piper nigrum* L., *Piper cubeba* Bojer., *Carthamus tinctorius* L., *Phyllanthus emblica* L., *Rhinacanthus nasutus* (L.) Kurz., *Cornus officinalis* Siebold & Zucc., *Cinnamomum verum* J.Presl., *Panax ginseng* C.A.Mey., *Rosmarinus officinalis* L., *Thuja occidentalis* L., *Serenoa repens* (W.Bartram) Small., *Scutellaria baicalensis* Georgi., *Glycyrrhiza glabra* L., *Pueraria thomsonii* Benth., *Equisetum debile* Roxb. ex Vaucher., *Pueraria lobata* (Willd.) Ohwi., *Quercus acutissima* Carruth., and others have been shown to inhibit the testosterone-5α-reductase *in vitro*. Additionally, the extracts prepared from fungi such as *Ganoderma lucidum* (Curtis) P. Karst, *Polygonum multiflora* Thunb., *Platycladus orientalis* (L.)Franco, and *Cynomorium songaricum* Rupr. have also been shown to inhibit testosterone-5α-reductase both *in vitro* and *in vivo*. These findings suggest the potential therapeutic use of these natural compounds in the treatment of conditions like androgenetic alopecia, where the inhibition of the testosterone 5α-reductase could be promising.

Indeed, while a broad array of plant species has been investigated for their potential as herbal testosterone 5α-reductase inhibitors, more detailed and focused studies have been conducted on a few samples. These in-depth investigations are crucial for understanding the mechanisms through which these plants exert their inhibitory effects on testosterone 5α-reductase, as well as for determining their efficacy, optimal dosages, and potential side effects. A closer look at some of the species that have been subject to more detailed studies is given below:

- *Serenoa repens* (W.Bartram) Small (Saw Palmetto)

One of the most extensively studied plants in relation to testosterone 5α-reductase inhibition is saw palmetto. Research has revealed that its extract can effectively inhibit both isoforms of the testosterone 5α-reductase. Clinical trials have explored its use in treating BPH and AGA, with some studies demonstrating its ability to improve urinary symptoms and hair growth with minimal side effects.

- *Camellia sinensis* (L.) Kuntze (Green Tea)

Green tea, particularly its constituent epigallocatechin gallate (EGCG), has been researched for its antioxidant properties and its role in inhibiting the testosterone 5α-reductase enzyme. Detailed studies have focused on its potential to prevent hair loss in AGA by reducing DHT levels. Research also explores green tea's broader health benefits, including its anti-inflammatory and anticarcinogenic properties.

- *Urtica dioica* L. (Stinging Nettle)

Stinging nettle root extract has been the subject of several studies due to its ability to block DHT by inhibiting the testosterone 5α-reductase enzyme. Research has focused on its synergistic use with other natural inhibitors and its application in treating BPH symptoms and hair loss.

- *Cucurbita pepo* L. (Pumpkin Seed)

Pumpkin seed oil has been examined for its phytosterol content and its ability to inhibit DHT production. A few studies have specifically looked at its role in treating AGA, with results indicating improvements in hair count and hair thickness.

- *Panax ginseng* C.A.Mey

Ginseng has been studied not only for its general health benefits but also for its specific action as a testosterone 5α-reductase inhibitor. Research includes exploring its effects on promoting hair growth in AGA patients, likely due to its inhibitory action on DHT as well as its ability to stimulate hair follicle cells.

These detailed studies contribute significantly to our understanding of how natural compounds can be used to combat conditions related to DHT overproduction. However, it is important to note that while these findings are promising, more research, including large-scale and controlled clinical trials, is needed to fully establish the efficacy, safety, and clinical applications of these herbal remedies (Chaiyana *et al.*, 2017; Chittur, Parr, & Marcovici, 2011; Cho, Bae, & Kim, 2010; H.-M. Choi *et al.*, 2016; Dhanotia, Chauhan, Saraf, & Dixit, 2011; Fischer, Hipler, & Elsner, 2007; Hirata, Tokunaga, Naruto, Iinuma, & Matsuda, 2007; Karunasagara *et al.*, 2020; Koseki *et al.*, 2015; Kumar, Rungseevijitprapa, Narkkhong, Suttajit, & Chaiyasut, 2012; Lee *et al.*, 2011; Murata, Noguchi, *et al.*, 2012; Murata *et al.*, 2013; Murata, Takeshita, Samukawa, Tani, & Matsuda, 2012; Nahata & Dixit, 2014; Patel, Nag, Sharma, Chauhan, & Dixit, 2014; Upadhyay, Ghosh, & Singh, 2012; Zhang *et al.*, 2016).

In an investigation aimed at determining the inhibitory effects of *Stauntonia hexaphylla* (Thunb.) Decne. leaf ethanol extract on the enzyme testosterone 5α-reductase, researchers employed both *in vitro* and *in vivo* methodologies. The experimental models for benign prostatic hyperplasia (BPH) were established using the testosterone-treated LNCaP cell line and Sprague Dawley rats to simulate the condition in both test tubes and live subjects. Results from the study indicated that *S. hexaphylla* extract effectively diminished the levels of DHT and the expression of testosterone 5α-reductase type 2, suggesting a strong inhibitory action by the extract. Hederacoside D was identified as the principal active compound within the extract, which is presumed to be responsible for these inhibitory effects (Hong et al., 2020). *In vivo* experiments conducted with the petroleum ether extract of *Garcinia kola* Heckel seeds have determined that the extract not only inhibits the enzyme testosterone 5*α* reductase but also possesses antioxidant activity. This finding adds to the therapeutic potential of the *Garcinia kola* Heckel seeds, suggesting their dual role in enzyme inhibition and antioxidation, which could be beneficial in treating AGA (Winner, Polycarp, Ifeoma, & Chinedum, 2016). An *in vivo* study was conducted to evaluate the inhibition of the testosterone 5*α*-reductase enzyme by the ethanol extract of the tubers of the plant *Colocasia esculenta* (L.) Schott. The study involved forty-five male albino rats. By the conclusion of the study, the extract was found to possess multiple bioactive properties, including anti-inflammatory, anti-alopecic (anti-hair loss), testosterone 5*α*-reductase inhibitory, anti-androgenic, lipoxygenase inhibitory, and hypocholesterolemic activities. The major compounds identified in the extract included hexadecanoic acid methyl ester, octadecanoic acid, 9-octadecenoic acid, hexanedioic acid, bis(2-ethylhexyl)ester, and 3,5-di-t-butyl phenol. Further research is needed to pinpoint which specific compounds are responsible for these effects (Tusubira et al., 2023). The inhibitory potential of *Eclipta alba* (L.) L. on testosterone 5*α*-reductase enzyme activity was the focus of an *in vitro* study. To assess this potential, extracts of *E. alba* were prepared using both methanol and petroleum ether. The study compared the inhibitory effects of these extracts to those of finasteride, a widely recognized testosterone 5*α*-reductase inhibitor. During the extraction process, *β*-sitosterol was isolated specifically from the petroleum ether extract of *E. alba.* Enzyme inhibition assays revealed the IC_{50} values for the petroleum ether extract and *β*-sitosterol to be 150.76 ± 4.56 µg/mL and 77.09 ± 3.07 µg/mL, respectively, signifying their inhibitory efficacy. For comparison, the IC_{50} value for finasteride was notably lower at 0.246 ± 0.02 µg/mL. The results underscore the substantial inhibitory capacity of the petroleum ether extract of *E. alba*, particularly due to the prominent presence of β-sitosterol, suggesting a robust

foundation for further exploration of its anti-androgenic properties (Tusubira et al., 2023).

4. Conclusion

The present mini-review reports the relationship between androgenetic alopecia and the testosterone 5α-reductase enzyme, as well as the effectiveness of plant-derived testosterone 5α-reductase enzyme inhibitors in treating AGA. AGA is a prevalent hair loss disorder caused by the interplay of genetic predisposition and androgen hormones. The testosterone 5α-reductase contributes significantly to the pathophysiology of AGA by converting testosterone to DHT, an active androgen derivative. Traditionally, testosterone 5α-reductase enzyme inhibitors (*e.g.* finasteride and dutasteride) used to treat AGA are intended to prevent hair loss by lowering DHT production. However, several of these drugs can cause major side effects, and their long-term use may be restricted. As a result, there is increased interest in plant-derived testosterone 5α-reductase enzyme inhibitors. According to the literature, various herbal substances have the capacity to block testosterone 5α-reductase and so can be utilized to treat AGA. For example, there is strong evidence that herbal treatments like saw palmetto, green tea extract, ginseng extract, chamomile extract, and turmeric inhibit the testosterone 5α-reductase and thereby prevent hair loss. While these herbal substances show promise as alternative treatments for hair follicle preservation and hair loss prevention, additional research is needed to determine their clinical effectiveness and safety. The completion of this research may result in the development of more effective and safe AGA treatment alternatives. Furthermore, in order to maximize the efficiency of these herbal substances, combination treatments and appropriate dosages must be determined. The appraisal of the research emphasizes plant-derived testosterone 5α-reductase enzyme inhibitors as talented alternatives in the treatment of AGA. This paper scrutinizes the potential of herbal testosterone 5α-reductase enzyme inhibitors to give some idea in this area. Finally, the use of plant-derived testosterone 5α-reductase enzyme inhibitors in the therapy of AGA is encouraging.

Abbreviations

AGA: Androgenetic alopecia, **CAT**: Catalase, **DHEAS**: Dehydroepiandrosterone sulfate, **DHT**: Dihydrotestosterone, **EGCG**: Epigallocatechin gallate, **FDA**: Food and Drug Administration, **FSH**: Follicle-stimulating hormone (FSH), **GPx:** Glutathione peroxidase, **IL**: Interleukin, **LH**: Luteinizing hormone, **MDA**: Malondialdehyde, **NADPH**: Nicotinamide adenine dinucleotide phosphate, **OSI**: Oxidative stress index, **PCOS**: Polycystic ovary syndrome, **ROS**: Reactive oxygen species, **SOD**: Superoxide dismutase, **TAS**: Total antioxidant levels, **TEAC**: Trolox equivalent antioxidant capacity, **TNF-α**: Tumor necrosis factor-α, **TOS**: Total oxidant levels

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Endemic *Achillea* Species in Türkiye: Phytochemical Contents and Pharmacological Activities

Ceylan DÖNMEZ [1*], **Beyza YILMAZ** [2]

[1] Deparment of Pharmacognosy, Faculty of Pharmacy, Selçuk University, Selçuklu 42130 Konya, Türkiye
[2]Deparment of Pharmacognosy, Faculty of Pharmacy, Selçuk University, Selçuklu 42130 Konya, Türkiye
*Corresponding author : akaceylan@gmail.com, ceylan.donmez@selcuk.edu.tr

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bstract

Asteraceae is the world's largest family of flowering plants, consisting of 23000 species distributed worldwide in 13 subfamilies, 44 tribes and more than 1600 genera. *Achillea* L. is one of the youngest evolutionary genera of the family Asteraceae with a worldwide widespread distribution of more than 100 species. This genus is represented by 110-140 species worldwide, centered in South East Europe and South West Asia, and extending through Eurasia to North America. In Türkiye, the genus is represented by 48 species with 54 taxa, 24 (about 50 %) of which are endemic to Türkiye. *Achillea* species have used for treatment of fatigue, inflammatoion, spasms, cold, bleeding, pneumonia, skin disorders, rheumatic pains. Furthermore, the species has diuretic and emmenagogic using in traditional medicine. *Achillea* species have various phytoconstituents such as sesquiterpenes, essential oils, flavonoids and phenolic acids. Flavonoids and phenolic acid derivatives are the most significant effective metabolites of *Achillea* species. Phenolics and flavonoids have been noticed to apply in wide range of pharmacological properties, including anti-cancer, anti-inflammatory, and antioxidant effects. In this review, endemic *Achillea* species were reviewed in terms of phytochemical content and pharmacological activities.

Key Words: *Achillea*, yarrow, endemic species, yarrow phytochemicals, biological activities of yarow

1. Introduction

Asteraceae is the world's largest family of flowering plants, consisting of 23000 species distributed worldwide in 13 subfamilies, 44 tribes and more than 1600 genera. The family is naturally distributed on all continents except Antarctica and is thought to have its phylogenetic origin in South America. the Asteraceae family is represented by 1209 species, 447 of which are endemic species, with an endemism rate of 37 % in the flora of Türkiye. Asteraceae family has largest number of endemics in Türkiye. *Achillea* L. is one of the youngest evolutionary genera of the family Asteraceae with a worldwide widespread distribution of more than 100 species. Türkiye is one of the main diversity centers of the genus *Achillea*. This genus is represented by 110-140 species worldwide, centered in South East Europe and South West Asia, and extending through Eurasia to North America (Tekin and Akdere, 2021). In

Türkiye, the genus is represented by 48 species with 54 taxa, 24 (about 50 %) of which are endemic to Türkiye. *Achillea* genus is divided into four sections: *Achillea* (with 13 species and, 4 subspecies), *Babounya* (with 30 species and 2 subspecies), *Otanthus* (only one species), and *Ptarmica* (with 4 species). The basic chromosome number in *Achillea* is nine and polyploidy often occurs. The karyological studies revealed that the genus has chromosome numbers of 2n = 18, 36, 54 and (Kiran et al., 2012; Tekin and Akdere, 2021).

The list of *Achillea* species recorded in the flora of Türkiye is given alphabetically and, endemic species in the plant list are marked with an asterisk (*) (Fig. 1.) (Davis 1975; Arabacı, 2012; Aytaç et al., 2016; Semiz et al., 2022). The name of the genus comes from the ancient use of the Trojan hero Achilles as a wound healer. Different *Achillea* species have traditionally used in Türkiye Iranian traditional medicine for healing abdominal pain, wound healing, as diaphoretic, diuretic carminative, emmenagogue and tonic agent (Eruygur et al., 2019). *A. millefolium*, a popular species among *Achillea* members, are widely used in European traditional medicine for healing of skin problems (wound, inflammations etc.), gastrointestinal disorders, hepatobiliary complaints. There are numerous reports describing the anti-inflammatory, antinociceptive, human erythrocyte and leukocyte protective, antispasmodic, antimicrobial and antioxidant effects of the compounds of many *Achillea* species. *Achillea* species have used as tonic, anti-inflammatory, anti-spasmodic, diaphoretic, diuretic and emmenagog agents and have traditionally been used to treat bleeding, pneumonia, rheumatic pain and wound healing. Unbalanced antioxidant systems lead to various problems such as inflammation, neurodegenerative and tumoral disorders. Clinical studies have indicated that some *Achillea* species have potential against episiotomy wound, multiple sclerosis, ulcerative colitis, irritable bowel syndrome, primary dysmenorrhea, oral mucositis etc. (Salehi et al., 2020). Ethanolic extracts from *A. setacea* showed potent anti-inflammatory and antinociceptive activity against *in vivo* carrageenan-induced hind paw oedema model in mouse without causing any gastric disease. Studies have shown that they can reduce the risk of inflammation-related diseases (Küpeli et al., 2007).

Achillea species have several constituents; flavonoids and phenolic acids, sesquiterpenes and essential oils. The most important therapeutical metabolites of *Achillea* species are considered as phenolic acids and flavonoids (Uzun and Arslan, 2020). Non-endemic *A. cretica* has been shown to be highly potent against gram-positive microorganisms *Staphylococcus aureus* and *Bacillus cereus*. *A. cretica* has used for healing wounds contaminated with bacterial infections in traditional medicine by its antibacterial properties. Carvone, caryophylladienol-II, *β*-maaliene, neo-intermedeol, spathulenol, selina-3,11-diene-6-ol, palmitic acid have been characterized as the main compounds of the essential oil of this species (Küçükbay et al., 2012).

A. adenii Aytaç & M.Ekici
A. aleppica DC. subsp. *aleppica*
A. aleppica DC subsp. zederbaueri (Hayek) Hub-Mor.*
A. alimeana Semiz & Uysal sp. nov. of sect. Santolinoidea DC.
A. armenorum Boiss. & Hausskn.*
A. arabica Kotschy.
A. baltai H.Duman & Aytaç
A. biebersteinii AFAN.
A. biserrata M.Bieb.
A. boissieri Hausskn. ex Boiss.*
A. brachyphylla Boiss. & Hausskn.*
A. cappadocica Hausskn. & Bornm.*
A. clypeolata Sibth. & Sm.
A. coarctata Poir.
A. cretica L.
A. crithmifolia Waldst. & Kit.
A. cucullata Bornm.*
A. falcata L.
A. filipendulina Lam.
A. formosa (Boiss.) Sch. Bip. subsp. *formosa*
A. formosa (Boiss.) Sch. Bip. subsp. *amanica* (Rech.f.) Ehrend &Y. Guo
A. fraasii Sch. Bip.*
A. goniocephala Boiss. & Balansa*
A. grandifolia Friv.
A. gypsicola Hub.-Mor.*
A. hamzaoglui Arabacı & Budak
A. ketenoglui H.Duman*
A. kotschyi Boiss. subsp. *kotschyi*
A. kotschyi Boiss. subsp. *canescens* Bässler*
A. latiloba Ledeb. ex. Nordm.
A. lycaonica Boiss. & Heldr.*
A. magnifica Heimerl ex Hub.-Mor.*
A. maritima (L.) Ehrend. & Y.P.Guo subsp. maritima
A. membranacea (Labill.) DC.
A. millefolium L. subsp. *millefolium* L.
A. millefolium L. subsp. *pannonica* (Scheele) Hayek
A. milliana H. Duman*
A. monocephala Boiss. & Balansa*
A. multifida (DC.) Griseb.*
A. nobilis L. subsp. *densissima* (O. Schwarz ex Bässler) Hub-Mor.*
A. nobilis L. subsp. *kurdica* Hub-Mor.*
A. nobilis L. subsp. *neilreichii* (A. Kern.) Velen
A. nobilis L. subsp. *sipylea* (O. Schwarz) Bässler*
A. oligocephala DC.
A. pannonica Scheele
A. phrygia Boiss. & Balansa*
A. pseudoaleppica Hausskn. ex Hub-Mor.*
A. salicifolia Besser subsp. *salicifolia*
A. santolinoides Lag. subsp. *wilhelmsii* (K. Koch.) Greuter
A. schischkinii Sosn.*
A. setacea Waldst. & Kit.*
A. sieheana Stapf*
A. sintenisii Hub.-Mor*
A. sipikorensis Hausskn. & Bornm.*
A. sivasica Çelik & Akpulat
A. spinulifolia Fenzl ex Boiss.*
A. tenuifolia Lam.
A. teretifolia Willd.*
A. vermicularis Trin.
A. wilhelmsii C. Koch
*endemic species

Figure 1. *Achillea* species recorded in the flora of Türkiye.

Some *Achillea* species are also consumed as diet such as additives, beverages, vegetables, and spices, and horticulture and cosmetic industry. Previous phytochemical works have revealed that the *Achillea* species are rich in terpenic compounds and phenolics, such as flavonoids, phenolic acids, and lignans as potential bioactive compounds. Flavonoid derivatives (like quercetin, luteolin apigenin) are mainly found as flavones and flavonols in the genus *Achillea*. In addition to flavonoid derivatives, hydroxycinnamic acids (caffeic acid, chlorogenic acid, dicaffeoylquinic acid) and hydroxybenzoic acids (syringic acid, vanillic acid) have also been characterized and quantified in some *Achillea* species (Agar et al., 2015). The essential oils by using hydro-distillation have been usually obtained from dried aerial parts of 31 *Achillea* species. n-hexane was used to recover the essential oil of some oil-poor species in during distillation. Oil yields modified between 0.01 %-1.2 % (Başer, 2016).

2. Phytochemicals and biological activities of Endemic *Achillea* species

Both phytochemical investigations and biological activity studies on endemic species "*A. armenorum* Boiss. & Hausskn.", "*A. brachyphylla* Boiss. & Hausskn.", "*A. kotschyi* Boiss. subsp. *canescens* Bässler", "*A. milliana* H. Duman", "*A. nobilis* L. subsp. *densissima* (O. Schwarz ex Bässler) Hub-Mor.", and "*A. nobilis* L. subsp. *kurdica* Hub-Mor." could not be found in the articles. The endemic species that were studied are described in the rest of the article.

2.1. *A. aleppica* DC subsp. *zederbaueri* (Hayek) Hub-Mor.

In a study examining the antimicrobial and antifungal activities of *A. aleppica* DC subsp. *zederbaueri* (Hayek) Hub-Mor. extracts (ethanolic, methanolic and aqueous extracts)

by disc diffusion, assay against several microorganisms, it was seen that all extracts reduced the zone diameter on Pseudomonas aeruginosa. The 2,2-diphenyl-1-picrilhydrazile (DPPH) free radical scavenging ability of aqueous and ethanolic extracts of *A. aleppica* subsp. *zederbaueri* were found to be in the range of 37.80-47.60 % and 86.30-90.60 %, respectively, at the concentration range between 50-500 µg/mL (Barış, 2016).

2.2. *A. boissieri* Hausskn. ex Boiss.

As a result of a study examining the antioxidant effect, the methanolic *A. boissieri* extract showed remarkable DPPH free radical scavenging activity (68.51 % at 37.5 mg/L) comparable with references (butylated hydroxyanisole (BHA), α-tocopherol and butylated hydroxytoluene (BHT)). Whereas the ferric reducing capacity of the extract was found to be moderate, the chelating capacity of the extract was found to be lower. Total phenol content of the plant extract was determined as 23.63 ± 0.17 mg gallic acid equivalents/gram extract; total flavonoid content of the extract was found as 29.70 ± 0.03 (mg quercetin equivalent/gram extract) (Tekin et al., 2021).

2.3. *A. cappadocica* Hausskn. & Bornm.

The main compounds in the fatty acid of *A. cappadocica* were determined as oleic (34.7 percent), palmitic (23.1 percent), and linoleic acids (20.6 percent). The methanolic and aqueous extracts exhibited higher 2, 2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS) cation radical scavenging effect compared to α-tocopherol and BHT at 100 mg/L. The acetone extract inhibited butyrylcholinesterase enzyme about 70.62 % at 200 mg/L concentration. The acetone and methanolic extracts demonstrated moderate antimicrobial activity (Ertaş et al., 2014).

2.4. *A. cucullata* Bornm.

The results of the studies noted that *A. cucullata* can be used in the pharmaceutical and food industry as an important source of natural antioxidants. The ethanolic extract from the aerial parts of *A. cucullata* demonstrated potent anticholinesterase inhibitor, antioxidant, antidiabetic and mild antimicrobial effects. As reported in previous studies, *A. cucullata* contains 1,8-cineole isoborneol and camphor (Eruygur et al., 2019).

2.5. *A. fraasii* Sch. Bip.

A. fraasii, native to Southeastern Europe and Türkiye, is a plant that has demonstrated to antimicrobial activity. According to the literature, *A. fraasii* has several phytochemicals, such as flavonoids, sesquiterpene lactones, tannins, and that antibacterial, antioxidant, anti-inflammatory and antifungal activities (Tunca-Pinarli et al., 2023).

2.6. *A. goniocephala* Boiss. & Balansa

The chitosan-tripolyphosphate nanoparticles from *A. goniocephala* chloroform extract showed higher anti-cancer (on MCF-7 and HT-29 cells lines) and antioxidant (DPPH, ferric reducing antioxidant power (FRAP), cupric reducing antioxidant capacity (CUPRAC) tests) activities compared to unencapsulated extracts (Taşkın et al., 2021).

2.7. *A. gypsicola* Hub.- Mor.

1,8-cineole, α-terpineol, borneol, camphor piperitone and sabinaketone were identified as major phytochemicals in the essential oil of *A. gypsicola* obtained in 0.65 % yield (Başer, 2016; Açikgöz and Kara, 2019).

2.8. *A. ketenoglui* H.Duman

The methanolic *A. ketenoglui* plant extract was detected to have high phenolic and flavonoid content. In these study, it's IC_{50} values of antioxidant and cytotoxic activities were found as 40.03±0.38, 263-350 µM respectively. According to phytochemical analysis results, apigenin, baicalin, casticin, chlorogenic acid, eupatorin, genistin, and luteolin were found in the methanolic extract. When scientists observed gen expressions (HCT 116 and HT-29 cells lines for colorectal cancer treatment, an increase in p53 expression and caspase-3 was found in both cell lines treated with the extract (Ayan et al., 2022). In the hydrodistilled essential oils of *A. ketenoglui* analyzed by GC/MS (Gas Chromatography-Mass Spectrometry), the main components in the oil were determined as borneol (14.1 %) and terpinen-4-ol (14.5 %) (Baser et al., 2001).

2.9. *A. kotschyi* Boiss. subsp. *kotschyi* subsp. *canescens* Bässler

The results of these studies provide important data on the phenolic compounds, antioxidant, cytotoxic and wound healing potential of methanolic extracts from non-endemic *A. kotschyi* Boiss. subspecies "*A. kotschyi* subsp. *kotschyi*". According to phytochemical investigations, it was considered as an important source of flavonoids like apigenin, hesperidin, hyperoside, kaempferol, rutin, and luteolin. In terms of total phenolics, this species extract contains significantly higher amounts. It exhibited a very pronounced wound healing potential and moderate cytotoxic activity at very small concentrations. To summarise the results of the present investigation, *A. kotschyi* subsp. *kotschyi* is a precious source of phenolic asit such as chlorogenic acid, and flavonoids had significant antioxidant, cytotoxic and wound healing activities. Therefore, it can be used in the development of additives, food, new drugs and cosmetics products (Agar et al., 2015). The oil of this species was rich in *p*-cymene~hexadecanoic acid, caryophyllene oxide and 1,8-cineole, respectively increasing, (Başer, 2016). However, no literature studies have been found regarding endemic "*A. kotschyi* Boiss. subspecies *A. kotschyi* Boiss. subsp. *kotschyi* subsp. *canescens* Bässler".

2.10. *A. lycaonica* Boiss. & Heldr.

In the hydrodistilled essential oils of *A. lycaonica* analyzed by GC/MS, the main component in the oil was determined as trans-sabinene hydrate (9.3 %) (Baser et al., 2001). In another study conducted in 2008 using the GC and GC-MS methods, L-camphor (43.19 %) was determined as a main component in the hydrodistilled essential oils of *A. lycaonica* (Azaz et al., 2008). In a study comparing three plant extracts, soxhlet extraction with ethyl acetate and maceration with chloroform were found to show the maximum total phenolic and total flavonoid contents with antioxidant capacity and antiurease activity. The phenolic compounds were determined to be caffeic, chlorogenic, dicaffeoylquinic, salicylic acids, and flavonoids such as apigenin, 8-hydroxy-salvigenin, quercetin, luteolin, rutin and naringenin. Among the solvent extraction processes, Soxhlet method was determined to have more recoveries compared to other approaches, and to show high effective antiurease activity in maceration with chloroform (Taşkın et al., 2017).

2.11. *A. magnifica* Heimerl ex Hub.-Mor.

The triterpenic compound magnificol was isolated from methanolic *A. magnifica* extract

(Ulubelen et al., 2007). A study, which was compared the antioxidant, anti-urease, anticholinesterase, and antiproliferative properties of various extracts of *A. magnifica*, demonstrated that chloroform extract has the highest antiproliferative and antioxidant activities. Elenolic acid, luteolin, eupatilin were obtained from this extract. When the cytotoxic capacities of the chitosan nanoparticles with chloroform extract were investigated, potent antiproliferative activity similar to that of the raw extract. The apigenin hexosides, ferulic acid derivative and diosmetin derivatives and vitexin were obtained from ethanolic plant extract (Taşkın et al., 2020). In another work, 22 different components such as 1,8-cineole, borneol, sabinyl acetate, camphor, germacrene and linalool, have been identified in the essential oil of *A. magnafica*. In it's fixed oil, components, linoleic, palmitic, ɣ-linolenic, oleic, behenic and caproic acids were determined as major components (Gedik et al., 2022).

2.12. *A. monocephala* Boiss. & Balansa

When the essential oils of *A. monocephala* flower and leaf were analysed, camphor and borneol in the leaf oil and 1,8-cineole borneol, camphor, and *α*-campholenal in the flower oil were determined as major compounds by comprehensive GC–time of flight mass spectrometry (TOF–MS) (Gogus et al., 2006). According to phytochemical analysis results by LC–MS/MS (Liquid Chromatography-Mass Spectrometry/Mass Spectrometry) method, it was found that ethanolic and methanolic-chloroform extracts of stems and aerial parts of *A. monocephala* have to flavonoids (apigenin, apigetrin, hesperidin, luteolin, isoquercitrin, rutin) and organic acids (chlorogenic, fumaric, malic, quinic, and vanillic acids) and. Additionally, total phenolic and flavonoid amounts, antioxidant, anti-tyrosinase, anticholinesterase, anti-urease and cytotoxic activities of *A. monocephala* exhibited significant results that could be a potential agent (Yılmaz et al., 2018).

2.13. *A. multifida* (DC.) Griseb.

In the water-distilled essential oils of *A. multifida* analyzed by GC and GC/MS, *α*-, *β*-thujone, camphor and sabinene were characterised as the main compounds. The antimicrobial activity of the essential oil was conducted by using a micro-dilution assay, minimum inhibitory concentration (MIC) was calculated as 62.5-250 µg/mL (Başer et al., 2002). Further in another study, MIC value of *A. multifida* flower extract was found to range from 50 to 75 µg/mL against three *Staphylococcus* bacteria (Karaalp et al., 2009). According to the study performed to evaluate the total phenolic amount, cytotoxic effects and antioxidant and antimicrobial capacities of heptane, chloroform and methanolic extracts from aerial parts of *A. multifida*, the phenolic compounds were isolated chlorogenic acid, dicaffeoyl quinic acid, quercetin hexoside, luteolin-7-O-glucoside, luteolin from methanolic plant extract. The chloroform extract showed strong cytotoxic activity (Taşkın et al., 2016).

2.14. *A. nobilis* L. subsp. *sipylea* (O. Schwarz) Bässler

There are a few study about *A. nobilis* L. subspecies. Studies on non-endemic *A. nobilis* subsp. *neilreichii* have antioxidant, antinociceptive and anti-inflammatory activities (Demirci et al., 2009). Fragranol, *β*-eudesmol and fragranyl acetate were isolated as the main compounds of *A. nobilis* subsp. *neilreichii* essential oil and Antioxidant and non-high antimicrobial activities were found

(Başer, 2016). As a result of a study examining the antioxidant effect, the methanolic *A. nobilis* subsp. *sipylea* extract showed low antioxidant capacity by DPPH radical scavenging activity, the ferric reducing and the chelating capacity. Total phenol amount of the extract was calculated as 17.33 ± 0.09 mg gallic acid equivalents/gram herbal extract; total flavonoid amount of the extract was determined as 18.20 ± 0.03 (mg quercetin equivalent/gram herbal extract) (Tekin et al., 2021). No studies on endemic "*A. nobilis* L. subsp. *densissima* (O. Schwarz ex Bässler) Hub-Mor." and "*A. nobilis* L. subsp. *kurdica* Hub-Mor. " species could be found.

2.15. *A. phrygia* Boiss. & Balansa

A. phrygia is a perennial herb, grows up to 0.45 meters in height and has golden yellow flowers. Studies have shown that *A. phrygia* has antioxidant by free radical scavenging activity. According to the experimentals results the plant is a potential natural antioxidants in medicinal preparations (Akcin et al., 2014).

2.16. *A. pseudoalepica* Hub.-Mor.

A. pseudoaleppica Hub.-Mor. is traditionally used in female disorders, menstrual problems and intestinal inflammations (with leaves and flowers), preventing hair-lossing and skin beauty (above-ground parts), frequent urination at night (flowers). It is thought that the capacity of preventing the inflammation, of the plant is related to its antioxidant capacity. *A. pseudoaleppica* contains high amounts of camphor. When the antioxidant, memory enhancing, antidepressant and anxiolytic activities of its essential oils have been examined *in vivo* animal model, significant activity results have been revealed such as healing neurological diseases including dementia and Alzheimer's disease, by confirming the potent ethnopharmacological use of many *Achillea* species. The extracts obtained from leaves of *A. pseudoaleppica*, especially the ethanolic extract, showed high amounts of flavonoids and phenolic constituents. Studies depict the medicinal values of *A. pseudoaleppica* as a promising source of phenolic compounds and antidiabetic, anti-cholinesterases and antioxidant properties (Yılmaz et al., 2023).

2.17. *A. sieheana* Stapf

Two different study showed that the essential oil obtained from aerial parts of *A. sieheana* was isolated by using hydro-distillation, main components of it were identified as Artemisia ketone, camphene, camphor and 1,8-cineole (Albayrak, 2013; Tabanca et al., 2004). Feruloylquinic acid, isorhamnetin, isovitexin, luteolin, luteolin glucoside, chrysoeriol were detected in ethylacetate extract, which was found to have a higher phenolic content compared to methanol, dichloromethane and n-hexane (Dikpınar et al., 2022).

Methanolic extract obtained from aerial parts of *A. sieheana* showed an effective DPPH radical scavenging activity with IC_{50} = 87.04 µg/mL, and a high reducing activity (71.08 %) on the oxidation of *β*-carotene (Albayrak, 2013). While a high anti-inflammatory effect was observed in dichloromethane and ethylacetate extracts, the highest antioxidant capacity was determined in ethylacetate extract (IC_{50} =96 µg/mL for DPPH, and IC_{50} =156 µg/mL for ABTS). Also, ethylacetate extract exhibited moderate *α*-glucosidase inhibitory activity (IC_{50} = 774 µg/mL) in the same study (Dikpınar et al., 2022). When the antimicrobial effects of the methanolic extract and the oil were also compared, the

results showed that both had high antimicrobial activity against 13 bacteria and two yeasts (Albayrak, 2013).

2.18. *A. sintenisii* Hub.-Mor.

In a study examining the antimicrobial effects of the *A. sintenisii* essential oil and methanol extract against 12 bacteria and two yeasts, *Candida albicans* and *C. krusei*, essential oil exhibited stronger activity than the other extracts tested. In this study, 32 different compounds such as borneol, camphor, eucalyptol and piperitone were determined in the essential oil using GC-MS analysis (Sökmen et al., 2003). The results of another phytochemical analysis study revealed the presence of quinic acid and chlorogenic acid in the extract obtained from aerial part of *A. sintenisii* by the LC/MS/MS. In the same study, it was revealed through experimental studies that the enzyme (elastase, hyaluronidase, and collagenase) inhibition capacity and antioxidant effect of the plant support wound healing activity (Eruygur et al., 2023).

In a study examining the effects of aqueous and ethanolic *A. sintenisii* extracts, such as cell culture analysis, collagen synthesis, fibroblast proliferation, hydrogen peroxide-induced problems, hyaluronidase enzyme inhibitory activities antibacterial, antioxidant, , results showed that the both plant extracts increased fibroblast proliferation without toxicity. While hyaluronidase inhibitory effects were observed in both extracts, it was stated that the aqueous extract supported collagen synthesis. It was found that the ethanolic and the aqueous extract demonstrated antibacterial activity against *Klebsiella pneumoniae* and *Bacillus subtilis* (Anlas et al., 2023). According to phytochemical analysis, phenolic compounds such as apigenin, isorhamnetin, luteolin, quercetin, rutin were isolated from plant by using HPLC and, high performance liquid chromatography-electrospray ionization quadrupole time-of-flight mass spectrometry- mass spectrometry (HPLC–ESI-Q-TOF-MS-MS) methods (Anlas et al., 2023; Şabanoğlu et al., 2017).

2.19. *A. sipikorensis* Hausskn. & Bornm.

It has been revealed that *A. sipikorensis* seeds containing a total of 3.83 % fixed oil contain palmitic acid and (lin)oleic acid as major fatty acids. These study showed that *A. sipikorensis* seeds could be used as a source of unsaturated fatty acids (Zonuz et al., 2017). Another study showed that borneol, camphor, caryophyllene oxide, cis-chrysanthenol, 1,8-cineol, were determined in the essential oil obtained from aerial parts of *A. sipikorensis*. The same study revealed that essential oil has significant antioxidant, antimicrobial and cytotoxic activities effects (Eruygur et al., 2018).

2.20. *A. spinulifolia* Fenzl ex Boiss.

In a study, 6-hydroxyflavonols and methyl ethers of 6-hydroxyflavones were isolated from *A. spinulifolia* (Wollenweber et al., 1986).

2.21. *A. schischkinii* Sosn. & *A. teretifolia* Willd.

Methanolic, aqueous and chloroform extracts of *A. schischkinii* and *A. teretifolia* showed strong antioxidant activity as shown by DPPH ABTS, hydrogen peroxide and superoxide anion scavenging, and metal chelating activities compared to reference. The results of the studies indicated that methanolic and aqueous extracts of *A. teretifolia* could be used as a source of natural antioxidant in pharmaceutical and food industry due to their high antioxidant and antimicrobial

properties. Furthermore, the data of the studies show that *A. schischkinii* extracts contain antioxidants but not antimicrobial compounds (Turkoglu et al., 2010).

1,8-cineole, artemisia ketone, camphor, caryophyllene oxide, *β*-eudesmol, *β*-pinene, piperitone, spathulenol, *p*-cymene, (E)-nerolidol were obtained as the main components on the essential oils of *A. schischkinii* species. Many authors have reported on the essential oil of the endemic species *A. teretifolia*. It has been reported that the main constituents of the essential oil showing antimicrobial, antioxidant and antiangiogenic activity are *α*-tujone (5 percent), terpinen-4-ol (8 percent) 1,8-cineole (34 percent), camphor (11 percent), and (Başer, 2016). It was revealed that the essential oils of *A. schischkinii* have antimicrobial, antiinflammatory and antinociceptive activities. However, the oil had shown lowactivity against (*Enterobacter aerogenes*, *Escherichia coli*, *Bacillus cereus*, *Salmonella typhimurium*, *Staphylococcus aureus*, and *Candida albicans*, while no remarkable *in vivo* antiinflammatory and antinociceptive activity was found (Tabanca et al., 2016).

3. Results and Conclusion

Biological activity studies have mostly focused on the antimicrobial, antioxidant and anticancer (cytotoxic) activities of *Achillea* species. Enzyme inhibition tests have been carried out in some *A. pseudoaleppica* and *A. sintenisii* endemic species through experimental studies and significant results have been revealed. Accordingly, essential oils and phenolic compounds were considered as potentially responsible compounds and studies were mostly conducted on the quantification of these compounds and their derivatives.

Phytochemical and biological activity studies on endemic *Achillea* species carried out to date have been quite limited and insufficient compared to the rich plant diversity of Türkiye. Designing and implementing subsequent studies on species that have not been studied will ensure the most efficient use of the existing rich plant diversity. So, the plant extracts from endemic *Achillea* species different parts may be a natural sources in both medicine/dermocosmetics and the food industry.

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Author Contribution

All authors declare equal contribution to the collect the literatures interpretation of the results and editing the manuscript.

Conflicts of Interest

The authors declare no conflicts of interest.

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