ESTUScience

LIFE SCIENCES AND BIOTECHNOLOGY

VOLUME 15 - NUMBER 2 - JULY 2026

Estuscience-Life
Eskişehir Teknik Üniversitesi
Bilim ve Teknoloji Dergisi - C
Yaşam Bilimleri ve Biyoteknoloji

e-ISSN: 2667-4203

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C- YAŞAM BİLİMLERİ VE BİYOTEKNOLOJİ

Eskişehir Technical University Journal of Science and Technology
C -Life Sciences and Biotechnology

Estuscience – Life

Volume: 15 / Number: 2 / July - 2026

Eskişehir Technical University Journal of Science and Technology C – Life Sciences and Biotechnology (formerly Anadolu University Journal of Science and Technology C – Life Sciences and Biotechnology) is an **peer-reviewed** and **refereed international journal** by Eskişehir Technical University. Since 2010, it has been regularly published and distributed biannually and it has been published biannually and **electronically only since 2016**.

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Indexed by **ULAKBIM TR Dizin, EBSCO**

ISSN: 2667-4203

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Volume: 15 / Number: 2 / July – 2026

Owner / Publisher: Prof. Dr. Adnan ÖZCAN (Rector) for Eskişehir Technical University

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Books

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Kennedy J, Eberhart R. Swarm Intelligence. San Diego, CA, USA: Academic Press, 2001.

Chapters in books

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Estuscience - Life
Volume / Cilt**: 15 / Number /** Sayı**: 2 / July / Temmuz- 2026**

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Eskişehir Technical University Journal of Science and Technology
C -Life Sciences and Biotechnology

Estuscience – Life, 2026, 15 (2) pp. 83-96, DOI: 10.18036/estubtdc.1785751

RESEARCH ARTICLE

EFFECT OF NATURAL DEEP EUTECTIC SOLVENTS ON ANTIOXIDANT AND PHENOLIC PROFILES OF TURKISH COFFEE

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Abstract

Natural deep eutectic solvents (NADES), developed as alternatives to traditional solvents, are attracting attention as environmentally friendly and effective solvents. This study aimed to investigate the effects of using deep eutectic solvents on antioxidant and phenolic compound levels in Turkish coffee. Turkish coffee samples were extracted using ultrasonic-assisted extraction with NADES solvents prepared from betaine, glycerin, and water. Acidic, neutral, and alkaline NADES solvents were compared. Total antioxidant capacity (TAC) was determined using the ABTS method, and total phenolic content (TPC) was determined using the Folin-Ciocalteu method. The highest TAC values were found in the pure NADES (8.90 ± 0.14 mmol TE/g) and 1/10 diluted NADES (8.79 ± 0.13 mmol TE/g) groups ($p<0.05$). The acidic NADES (5.24 ± 0.20 mmol TE/g) group showed significantly lower antioxidant activity compared to the neutral NADES (6.28 ± 0.25 mmol TE/g) and alkaline NADES (7.02 ± 0.17 mmol TE/g) groups ($p<0.05$). The neutral and alkaline NADES groups exhibited similar values ($p>0.05$). Total phenolic compound content was highest in neutral (665.76 ± 19.46 mg/L), alkaline (654.05 ± 11.38 mg/L), and acidic NADES (652.35 ± 8.27 mg/L) groups ($p>0.05$). The extract obtained with distilled water had the lowest values for both parameters (TAC and TPC) ($p<0.05$). This study demonstrates that NADES solvents constitute an effective alternative for extracting phenolic compounds and antioxidants from foods rich in bioactive compounds, such as coffee, due to their environmentally friendly and highly efficient structures.

Keywords

NADES,
Total antioxidant capacity,
Total phenolic content,
Turkish coffee

Time Scale of Article

Received : 17 September 2025
Accepted : 05 March 2026
Online date : 27 July 2026

1. INTRODUCTION

Coffee is known as an essential part of life for many people and, as such, accounts for approximately 75% of non-alcoholic beverage consumption [1]. With consumption reaching approximately 500 billion cups worldwide, coffee is one of the most preferred beverages. Rich in bioactive substances, including antioxidants and phenolic compounds, it offers various health benefits by neutralizing free radicals [1, 2]. The high presence of compounds with antioxidant capacity in coffee stems from both the plant's natural structure and new compounds formed during processing, such as roasting [3, 4]. The types of coffee, its processing, and brewing affect its components [4, 5]. Turkish coffee is an integral part of Turkish culture and cuisine, and the main characteristic that distinguishes it from other types of coffee is its unique preparation technique. No specific type of coffee bean is used in this coffee; the Coffea arabica variety is generally preferred [6, 7]. Turkish coffee is made by grinding the coffee beans very

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finely [8]. Turkish coffee contains various antioxidant compounds such as chlorogenic acid, caffeine, trigonelline, kahveol, and cafestol, and is also rich in phenolic compounds [9, 10].

Due to the intake of antioxidants through food consumption, the development of measurement techniques to assess the antioxidant content of foods is gaining importance. Antioxidants can act synergistically both in vitro and in vivo; therefore, the determination of isolated antioxidant compounds can lead to an underestimation or overestimation of antioxidant activity [11]. Thus, various analysis methods are used to assess antioxidant properties. Among these analysis methods, the (ABTS) method is generally used to determine antioxidants, and the Folin–Ciocalteu method is used to determine phenolic compounds [12]. The first step in studying antioxidants and phenolic compounds is their extraction. Separation and extraction are essential aspects of the investigation of phenolic compounds and compounds that exhibit antioxidant activity. The type of extraction system used is crucial in determining the process's applicability and achieving the desired results. The extraction method should be carefully selected to ensure maximum yield and high selectivity [13, 14]. Although solvents such as methanol and its aqueous solutions, ethyl acetate, and acetone are widely used, these traditional extraction methods mainly rely on organic solvents that are polluting or flammable [15, 16]. Furthermore, they are inadequate in terms of extracting extracts with different polarities and preserving bioactivities [17, 18]. In this context, the search for new solvents that can serve as alternatives to traditional organic solvents or ionic liquids for the preparation of food ingredients and nutraceuticals has brought the use of deep eutectic solvents (DES) to the forefront [19].

DESs are classified as green solvents widely applied in dissolution and separation processes due to their high dissolving power and diversity in physicochemical properties [20]. DESs have several advantages, including biodegradability, recyclability, economic benefits, and low toxicity of their components [21]. These solvents, which are generally prepared from widely used natural chemicals, are referred to as "natural deep eutectic solvents" (NADES) due to their natural origin [22]. In this context, NADES are attracting attention as an effective and environmentally friendly non-toxic alternative for hydroalcoholic extraction [23]. The main characteristic of NADES and DES systems is the presence of strong hydrogen bonds between the hydrogen bond donor and acceptor [22]. NADESs are formed by combining a hydrogen bond donor (HBD) compound with a quaternary ammonium salt, which acts as a hydrogen bond acceptor (HBA). Hydrogen bonding occurs via a halide ion and the hydrogen donor moiety, and charge delocalization across the hydrogen bond between HBD and HBA lowers the melting point of the mixture compared to its pure components [24]. These bonds result in eutectic mixtures with lower melting points and higher vapor pressures than their components [25]. NADES systems are suitable for food applications because they can be considered GRAS (generally recognized as safe) solvents and can be composed of quaternary ammonium salts such as choline chloride and betaine, as well as commonly used HBD compounds such as alcohols, polyols, amides, amino acids, sugars, carboxylic acids, glycerol, citric acid, and water [26-28]. NADESs have been demonstrated to be compatible with various applications, including extraction media, nanomaterial synthesis, pharmaceuticals, and medical products, due to their ability to extract both polar and non-polar compounds from natural sources and solubilize macromolecular compounds [29]. In this context, NADESs are attracting increasing interest in the recovery of bioactive compounds from plant-based by-products because they can form hydrogen bonds with plant cell walls, leading to structural weakening of these bonds and thus increasing extraction efficiency [30, 31]

In light of this information, the aim was to investigate the effects of using deep eutectic solvents on the antioxidant and phenolic compound levels in Turkish coffee.

2. MATERIALS AND METHODS

2.1. Study Materials

The study was conducted at the laboratory of the Food and Drug Administration of Parma, Italy, and used Turkish coffee as the material. Turkish coffee samples were obtained from a commercial coffee producer, Arabağa Türk Kahvesi (a traditional Turkish coffee brand produced and sold in Mersin, Türkiye), which contains medium-roasted Arabica coffee beans. Two grams of each sample were weighed, and all samples were stored in the refrigerator at 4 °C for the extraction process. All chemicals used in the study were of analytical grade.

2.2. Preparation of DES

In this study, NADES was prepared using betaine as the hydrogen bond acceptor (HBA), glycerin as the hydrogen bond donor (HBD), and water as the cosolvent. The solvents used were in different molar ratios (1:8:2), forming deep eutectic solvents. The mixture was then stirred at 30 °C and 180 rpm using a shaking incubator until a homogeneous colorless liquid was obtained [32]. After a homogeneous mixture was obtained, the solvent was maintained at room temperature until it became stable and was then used in the extraction process.

2.3. Ultrasonic-Assisted Extraction (UAE)

Extracts were prepared using the ultrasonic-assisted extraction (UE) method. For comparison, three different deep eutectic solvents were used in the study: acidic (by adding citric acid), neutral, and basic (by adding ethanolamine). The solvents used in the research, along with their properties, are shown in Figure 1.

Acidic NADES was prepared by adding citric acid to pure NADES to reach a pH of approximately 3–4, while alkaline NADES was obtained by adding ethanolamine to reach a pH of approximately 9–10. Neutral NADES was achieved by diluting pure NADES with distilled water at a 1:10 (v/v) ratio, resulting in a neutral pH of approximately 7.0. The pH of all solutions was measured using a calibrated pH meter. For Turkish coffee, samples were prepared at a sample/solvent ratio of 1/5 g/mL. The sample and solvent were placed in a beaker, and the surrounding area was surrounded by ice to prevent heating during sonication. The extraction process was carried out using an ultrasonic bath at 100% power and 40 °C for 30 minutes. At the end of the sonication period, the samples were passed through filter paper and filtered through a 45 µm membrane filter. Supernatants were collected and stored in dark glass bottles in the refrigerator at +4 °C before analysis.

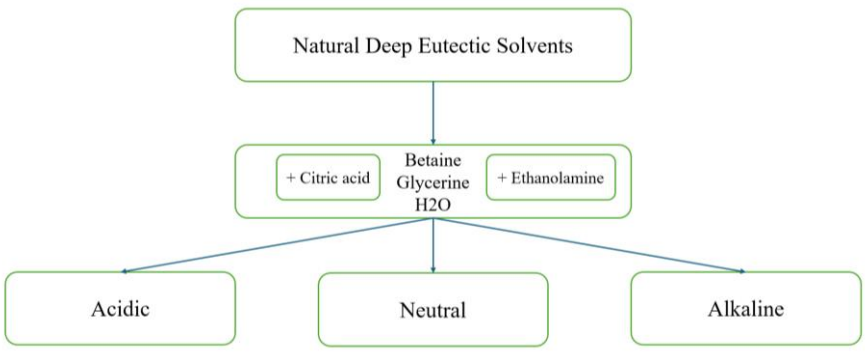

Figure 1. Solvents used in the study and their properties.

2.4. Total Antioxidant Capacity (TAC)

The total antioxidant capacity (TAC) of the coffee samples was determined using the ABTS method. ABTS cation radical scavenging activity was determined using 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) in the study [33]. In the next step, 30 µL of coffee sample was diluted with ethanol and distilled water, completed with a buffer solution prepared from the extract, mixed, and left in the dark for 5 minutes before absorbance was measured on a spectrophotometer at a wavelength of 734 nm. This procedure follows the standard ABTS radical scavenging assay protocol, where dilution with ethanol–water facilitates optimal radical formation and stabilizes the chromophore, while the extract-based buffer ensures appropriate pH and ionic strength for the reaction. Keeping the mixture in the dark for 5 minutes allows the $ABTS^{\bullet+}$ radical to react fully with antioxidant compounds present in the sample, preventing photodegradation and ensuring measurement accuracy. Finally, the absorbance at 734 nm corresponds to the maximum absorption peak of the $ABTS^{\bullet+}$ radical, enabling precise quantification of radical scavenging activity. The absorbance values obtained were quantified using a Trolox calibration curve prepared in the range of 10–100 µmol/L. The antioxidant capacity of each coffee extract was calculated from this curve and converted to mmol Trolox equivalents per gram of coffee (mmol TE/g) based on the sample mass used in extraction.

2.5. Total Phenolic Content (TPC)

Total phenolic content (TPC) analysis of coffee samples was performed using the Folin-Ciocalteu method [34]. For this purpose, 30 µL of sample extract, 150 µL of Folin-Ciocalteu (10-fold diluted), and 120 µL of Na2CO3 were placed on a microreader plate. Sample absorbance was measured at 765 nm after a 60-minute waiting period in the microreader. Results were calculated as mg gallic acid equivalent (GAE)/L. The unit mg GAE/L was used because the Folin–Ciocalteu assay quantifies phenolics based on the volume of the extract analyzed, whereas antioxidant capacity results were normalized to coffee mass (mmol TE/g). Therefore, different units were applied in accordance with standard conventions for each analytical method. Analyses were performed in duplicate. In this assay, the ‘sample extract’ refers to the clear supernatant obtained after centrifugation of the coffee–solvent mixtures, which is commonly used for Folin–Ciocalteu analysis.

2.6. Statistical Analysis

All analyses performed in the study were replicated in duplicate. In the statistical evaluation of the study, the Shapiro-Wilk test was used to assess the normality of the distribution of the samples used and the appropriateness of the sample size. A one-way ANOVA test was used to compare the groups, and a post-hoc Tukey test was used for pairwise comparisons. Quantitative variables were presented as mean and standard deviation (SD) values in the analyses. The obtained data were analyzed using SPSS (Statistical Package for the Social Sciences) 26. A p value of <0.05 indicates statistical significance.

3. RESULTS

Definitions of the samples are summarized in Table 1. Turkish coffees were extracted with pure NADES, diluted NADES (1:10 ratio), neutral, alkaline, and acidic NADES diluted at a 1:10 ratio, in the study.

Table 1. Description of samples

Samples
Pure NADES
Diluted NADES (1/10 Ratio)
Turkish Coffee + Distilled Water (1/10 Ratio)
Turkish Coffee + Acidic NADES
Turkish Coffee + Neutral NADES
Turkish Coffee + Alkaline NADES

NADES: Natural deep eutectic solvents. Pure NADES refers only to the solvent itself without coffee. It was included to characterise the solvent background and is not a coffee–solvent extraction sample. All Turkish coffee samples were prepared separately by mixing coffee with distilled water (neutral), with acidic, or alkaline NADES formulations.

Table 2 shows the TAC levels of the samples. According to the table, a significant difference was found between all groups ($p<0.001$). The highest TAC values were observed in pure NADES (8.90 ± 0.14 mmol TE/g) and 1/10 diluted NADES (8.79 ± 0.13 mmol TE/g), and no significant difference was found between these two groups ($p>0.05$). The extract containing only Turkish coffee and distilled water showed the lowest TAC value (3.66 ± 0.23 mmol TE/g) and was found to be significantly lower compared to all NADES groups ($p<0.05$). The antioxidant activities of the extracts obtained with neutral NADES (6.28 ± 0.25 mmol TE/g) and alkaline NADES (7.02 ± 0.17 mmol TE/g) were found to be similar to each other ($p>0.05$), but both were significantly higher than the extract obtained with acidic NADES (5.24 ± 0.20 mmol TE/g) ($p<0.05$). These findings indicate that the antioxidant capacity of the extract varies depending on the type of NADES.

Table 2. Total antioxidant activity levels of the samples

Samples	TAC (mmol TE/g)	p
Pure NADES	8.9 ± 0.14^{a}	**<0.001**
Diluted NADES (1/10 ratio)	8.79 ± 0.13^{a}	
Turkish Coffee + Distilled Water (1/10 Ratio)	3.66 ± 0.23^{b}	
Turkish Coffee + Neutral NADES	6.28 ± 0.25^{c}	
Turkish Coffee + Alkaline NADES	7.02 ± 0.17^{c}	
Turkish Coffee + Acidic NADES	5.24 ± 0.20^{d}	

NADES: Natural deep eutectic solvents, Values of different letters (a, b, c, d) in the same column are statistically different from each other ($p<0.05$). Bold font indicates statistical significance.

Table 3 shows the TPC values of the samples. According to the table, a significant difference was determined between all groups ($p<0.001$). Pure NADES (570.00 ± 12.73 mg/L) and 1/10 diluted NADES (550.82 ± 9.64 mg/L) groups showed similar TPC values and no significant difference was found between these two groups ($p>0.05$). The TPC value of the extract obtained with Turkish coffee and distilled water was found to be the lowest (134.52 ± 6.25 mg/L) ($p<0.05$). Extracts prepared with neutral, alkaline and acidic NADES had TPC levels of 665.76 ± 19.46 mg/L, 654.05 ± 11.38 mg/L and 652.35 ± 8.27 mg/L, respectively; No significant difference was observed between these three groups

($p>0.05$). These results show that different types of NADES solvents significantly increase the extraction of phenolic compounds from coffee and are much more efficient than the classical solvent, water.

Table 3. Total phenolic substance levels of the samples

Samples	TPC (mg/L)	p
Pure NADES	570.00 ± 12.73^{a}	**<0.001**
Diluted NADES (1/10 Ratio)	550.82 ± 9.64^{a}	
Turkish Coffee + Distilled Water (1/10 Ratio)	134.52 ± 6.25^{b}	
Turkish Coffee + Neutral NADES	665.76 ± 19.46^{c}	
Turkish Coffee + Alkaline NADES	654.05 ± 11.38^{c}	
Turkish Coffee + Acidic NADES	652.35 ± 8.27^{c}	

NADES: Natural deep eutectic solvents, Values of different letters (a, b, c) in the same column are statistically different from each other ($p<0.05$). Bold font indicates statistical significance.

4. DISCUSSION

The use of NADESs for bioactive extraction has been increasing in recent years. Chemical and thermal stability, low melting point, polarity, and miscibility are important factors in the increasing use of these solvents. In addition, they reduce the economic and ecological burden and offer ease of production by using natural and environmentally friendly materials [35]. Polarity is a fundamental property of solvating compounds, and the HBAs and HBDs used in NADESs have a decisive effect on the final polarity of the solvent [36]. Many researchers have reported that deep eutectic solvents (DESs), regardless of the extraction technique, have several advantages over traditional organic solvents (e.g., methanol, ethanol, acetone, etc.) [23, 37]. In this study, it was observed that NADES solvents increased the antioxidant capacity and total phenolic substance levels by extracting antioxidant components and phenolic compounds in Turkish coffee more effectively compared to distilled water.

Water plays a decisive role in the physicochemical properties of NADES. Water can enter the solvent system from the external environment via moisture or be added in a controlled manner. Water can be a structural component of NADES, acting as the HBD or its acceptor, HBA, or it can act as a co-solvent added solely to reduce the viscosity and density of the system [38, 39]. In a study conducted on coffee grounds, it was observed that the TPC value increased as the dilution rate of NADES increased, depending on the amount of dilution with water [40]. Although comparing the dilution ratios of NADES was not the primary objective of the present study, only the extracts obtained with pure NADES and 1/10 diluted NADES solvents were compared. The lack of a significant difference between these two groups suggests that the NADES system used does not lose its solubilizing capacity when diluted to a certain ratio and is effective in the extraction of antioxidant compounds. However, considering the increase in TPC with dilution ratio reported in the literature, more comprehensive comparisons with different dilution ratios may be guiding for future studies. Data in the literature indicate that the physicochemical properties of NADES can be controlled by the addition of water, and generally report positive benefits [38, 40]. Since viscosity decreases with the addition of water, substance transfer from solid samples to solvent is facilitated, and extraction efficiency increases. Therefore, increasing the water content may increase the polarity of NADES, facilitating the dissolution of more polar compounds and thus increasing the solubility of some compounds in the samples. However, considering that

excessive water addition can disrupt the hydrogen bond network, which is the main feature of NADES, and transform the system into an aqueous solvent mixture, this may be the source of different results.

One of the most important advantages of NADESs is that their components can be selected and combined according to preference and can be designed as special solvents. This helps the extraction of different bioactive compounds [41]. In studies using different samples and coffee types, it has been reported that NADESs play a better role in the recovery of phenolic compounds compared to conventional organic solvents and that alkaline NADESs are more selective towards phenolic compounds [23, 42, 43]. The findings obtained in this study are largely parallel to previous studies in the literature. It has been determined that NADES solvents provide much higher efficiency in the extraction of phenolic compounds and antioxidants from Turkish coffee compared to distilled water. Indeed, Phaisan et al. (2021) reported that extracts prepared using NADES had higher total antioxidant capacity (TAC) values than water-based extracts; this was also observed in our study [44]. The fact that the pure NADES and 1/10 diluted NADES groups exhibited the highest antioxidant capacity in this study supports the effectiveness of NADES solvents in solubilizing antioxidant compounds. Furthermore, the higher TAC value of the alkaline NADES group compared to both the neutral and acidic NADES groups suggests that an alkaline environment may be more effective in extracting antioxidant compounds.

When the TPC results in this study were examined, it was observed that the neutral, alkaline, and acidic NADES groups exhibited similar values, with the alkaline group being relatively higher than the acidic group, but this difference was not statistically significant. The pure and diluted NADES groups were determined to have lower TPC values compared to these three groups. This suggests that different NADES compositions may affect the solubility of phenolic compounds in the coffee matrix to different extents. Some studies in the literature are consistent with these findings. For example, Wang et al. (2025) reported that the acidic NADES group exhibited lower TPC values compared to neutral NADES [45]. In a study by Taweekayujan et al. (2023), the TPC value of the extract prepared with pure NADES was reported to be lower than that of the extract prepared with acidic NADES [46]. In this context, the selective solubilization capacity of NADES systems for phenolic compounds depends on the pH level of the system as well as the specific chemical interactions they establish with the desired compounds. The findings of this study also agree with the fact that acidic NADESs provide lower yields due to their inability to stabilize some phenolic compounds, whereas neutral or slightly alkaline NADESs provide higher solubility and phenolic recovery. Despite the general expectation that higher total phenolic content corresponds to higher antioxidant activity, this relationship was not strictly linear in the present study. Antioxidant capacity in coffee does not originate solely from phenolic compounds, as coffee contains various non-phenolic constituents with strong radical-scavenging potential, including caffeine, chlorogenic acid derivatives, and melanoidins formed during roasting, which have been reported to contribute to antioxidant effects in coffee brews and extracts [47]. These non-phenolic compounds may maintain or enhance total antioxidant capacity even when TPC shows limited variation, likely due to the complex matrix of coffee and the formation of Maillard reaction products that exert radical-scavenging activity [48]. Additionally, differences in NADES composition may influence the extraction efficiency of both phenolic and non-phenolic antioxidants in distinct ways. Therefore, a decrease or lack of proportional increase in TAC despite higher TPC values in certain groups likely reflects the multifaceted nature of antioxidant activity in coffee, arising from both phenolic and non-phenolic bioactive compounds.

Examples of extraction studies comparing antioxidant activity and total phenolic content (TPC) levels using different coffee sources and NADES compositions are summarized in Table 4. Furthermore, studies that included more comprehensive evaluations of NADES applications, such as toxicity profiles, environmental impacts, and potential formulation uses, are presented in Table 5.

Table 4. Evaluation of antioxidant activity and total phenolic content in extraction studies using coffee and deep eutectic solvents

Author (year)	Material	NADES Composition	Extraction Conditions	Antioxidant Activity / TPC or TFC
[49]	*Coffea arabica* beans	Lactic acid–sucrose (LA-Suc), Citric acid–glucose (CA-Glu)	Ultrasound-assisted extraction, 15–35 min, 10–30 mL/g liquid-to-solid ratio, various NADES concentrations	87.01 mg GAE/g (LA-Suc), 62.91 mg GAE/g (CA-Glu)
[50]	Orange by-product, coffee grounds	Citric acid:mannitol (CM-DES), Lactic acid:glucose (LG-DES)	10% water content, 80 °C, solid-to-liquid ratio 1:10–1:15 (w/w)	FRAP: 1.087–1.071 mol ascorbic acid/L; DPPH: 0.233–0.234 mol Trolox eq./L; ABTS: 0.284–0.319 mol Trolox eq./L 1782.92 ± 4.50 mg GAE/g (orange); 1620.71 ± 3.72 mg GAE/g (coffee)
[51]	Spent coffee grounds	Betaine:glycerol (Bet:Gly)	MAE (microwave-assisted extraction)	10.97±0.90 mg GAE/g - 20.55±0.29 mg GAE/g
[52]	Robusta coffee leaves	Choline chloride:citric acid (2:1), Choline chloride:lactic acid (1:1)	Maceration; Extraction times: 15, 30, 45, 60, 120, 240 min; Solvent: 96% ethanol and NADES	Max TPC: 29.42 mg GAE/g extract; Max TFC: 68.84 mg QE/g extract; Chlorogenic acid: 2.77 mg/g
[53]	Spent coffee grounds	1,6-Hexanediol:choline chloride (7:1) (DES)	Ultrasound-assisted extraction (UAE),	Significantly enhanced TPC and antioxidant activity; total phenolics >90% recovery by adsorption chromatography. The new NADES solvents showed higher antioxidant and polyphenol yields than classical solvents (ChCl:Urea and EtOH:H_2O).
[54]	Spent Coffee Grounds	Proline:glycerol:water (2:5:11.5 molar ratio) and 12 other NADES variants tested	Extraction with 13 NADES; cytotoxicity evaluation to select 5 best; stability assays; antioxidant capacity and elastase inhibition tested; incorporated into oil-in-water emulsions (Eco-Label standards)	Selected DES extract showed high total phenolic content, antioxidant capacity, elastase inhibition, and stability

Table 5. Toxicity profiles and safety assessments of deep eutectic solvents in coffee samples

Author (year)	Material	NADES Composition	Extraction/ Formulation Conditions	Key Results/Notes
[23]	Spent Coffee Grounds	Betaine:Triethylene glycol (Bet:TEG), Choline chloride:1,2-propanediol (Chol:Prop)	NADES aqueous solutions (25–50% water), mild temperatures	Extraction yield comparable to hydroalcoholic and hot water extracts; Antimicrobial activity ~10× higher than ethanolic and aqueous extracts NADES are green, nontoxic, sustainable solvents; operate at milder temperature; potential use as food ingredient formulation aids
[54]	Spent Coffee Grounds	Proline:glycerol:water (2:5:11.5 molar ratio) and 12 other NADES variants tested	Extraction with 13 NADES; cytotoxicity evaluation to select 5 best; stability assays; antioxidant capacity and elastase inhibition tested; incorporated into oil-in-	Sustainability: Use of biodegradable, natural metabolites; complies with Eco-Label standards; no toxic organic solvents used. Toxicity: Cytotoxicity screening eliminated unsafe DES; final extract and DES were biocompatible and safe for dermal use, supporting human and environmental safety.

			water emulsions (Eco-Label standards)	
[55]	Spent Coffee Grounds	Randomly methylated-β-cyclodextrin : propylene glycol - Hydroxypropyl-β-cyclodextrin : ethylene glycol	Microwave-assisted extraction 80 °C, 15 min, 10% water	Composition of one-step extract similar to Soxhlet; microwave-assisted NADES extraction is a greener alternative 5 times lower EC_{50} and 3 times lower GAE value than Soxhlet extract.
[56]	Coffee and cocoa byproducts	NADES, biobased solvents, supercritical fluids, supramolecular solvents, ionic liquids	Microwave-assisted, ultrasound-assisted, pulsed electric field-assisted, supercritical fluid, pressurized liquid extraction	NADES and other green solvents offer environmentally friendly extraction with lower energy consumption and reduced chemical waste compared to conventional solvents. Toxicity profiles are generally low for NADES, which improves safety and reduces environmental hazards; however, specific toxicity data for all solvents are limited and require further research.

5. CONCLUSION

NADES is considered an alternative to traditional organic solvents thanks to its environmentally friendly properties. Its use has increased due to its high biodegradability, which makes it a "green solvent". This study found higher TPC and TAC values in extracts prepared with NADES compared to extracts prepared with distilled water. Furthermore, it has been demonstrated that the pH level and components of NADES can significantly impact the stability and activity of the extracted compounds. The results indicate that the type and composition of NADES can significantly affect the antioxidant capacity and phenolic compound value of the product obtained during extraction processes. Therefore, the selection of the most suitable NADES type for extracting specific bioactive compounds should be carefully considered in relation to the targeted applications. These results indicate that NADES can efficiently extract phenolic compounds and antioxidants from coffee products such as Turkish coffee, and that this method may offer an environmentally friendly alternative to traditional solvents. To develop a more systematic and comprehensive approach to selecting the NADES system, it is essential to thoroughly examine the structure-activity relationships of NADES and investigate its use with various techniques. Studies on Turkish coffee on this subject are limited, and therefore, the findings of this study are believed to be instructive for future studies.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the staff of Parma Food and Drug Laboratory for their technical assistance during the analytical procedures.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

CRediT AUTHOR STATEMENT

Gül Eda Kılınç: Conceptualization, Methodology, Investigation, Formal analysis, Data curation, Writing – Original Draft, Writing – Review & Editing, Visualization. **Yeliz Vergi:** Conceptualization, Methodology, Investigation, Writing – Original Draft, Writing – Review & Editing.

FINANCIAL SUPPORT

No support was received from funding agencies in the public, commercial, or non-profit sectors during this study.

ETHICS STATEMENT

Ethical approval was not required because the study consisted exclusively of laboratory experiments and did not involve human participants or animals.

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ESKİŞEHİR TEKNİK ÜNİVERSİTESİ BİLİM VE TEKNOLOJİ DERGİSİ
C- YAŞAM BİLİMLERİ VE BİYOTEKNOLOJİ

Eskişehir Technical University Journal of Science and Technology
C -Life Sciences and Biotechnology

Estuscience – Life, 2026, 15 (2) pp. 97-110, DOI: 10.18036/estubtdc.1800160

RESEARCH ARTICLE

ASSESSMENT OF THE LEVEL OF KNOWLEDGE AND AWARENESS OF HOSPITAL EMPLOYEES ABOUT MEDICAL WASTE MANAGEMENT: THE CASE OF KÜTAHYA, TÜRKİYE

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Abstract

This study aims to assess the level of knowledge and awareness of employees of two hospitals in Kütahya regarding medical waste. The questionnaire conducted participants to indicate the extent to which they participated in the assessments by considering medical waste management practices in the facility where they work. The study population consisted of hospital employees working for two hospitals in the center of Kütahya, and the sample consisted of 516 hospital employees who participated in the survey, but some items weren't answered by the participants. The first 7 questions are demographic ones, the 8th question consists of a 5-point Likert scale with 16 items, and the last 7 questions consist of a total of 15 questions about whether the meanings of the symbols in the pictures are known or not, as well as the questionnaire to assess knowledge, attitudes, and perceptions. The data analyzed by SPSS V.26.

At the last part of the questionnaire, 96.7% of the respondents answered the question about "radioactive waste emblem correctly". Similarly, the 96.7% of the respondents answered correctly the question "What color are the bags used for medical waste in the healthcare facility where you work?", as "red". Based on the results of the research, it was found that the level of knowledge of healthcare professionals in the two hospitals was at a high level. Medical waste knowledge level varies significantly depending on the health institution where the individual works, age, and length of service. The level of medical waste knowledge does not differ significantly depending on the individual's gender, the unit the individual works in, the individual's position in the hospital, or the individual's education level. In the analysis results conducted to determine the relationship between participants' medical waste knowledge and awareness levels and gender, it was determined that there was no significant difference between the answers given to all statements and in terms of gender. Recommendations were made to help manage medical waste and improve the level of knowledge and awareness of medical waste. Informative work on the courses given at regular intervals to inform the employees about the activities of the medical waste sterilization facility by organizing a technical trip. As a result of the study, it is concluded that increasing the training of hospital employees on medical waste is of great importance in terms of public health, personnel health and environmental health.

Keywords

Health employees,
Knowledge and awareness level,
Kütahya,
Medical waste,
Waste management

Time Scale of Article

Received: 09 October 2025
Accepted: 13 May 2026
Online date: 27 July 2026

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1. INTRODUCTION

According to the United Nations Environment Programme (UNEP), healthcare (medical) waste comprises all waste generated by healthcare facilities, medical laboratories, and biomedical research institutions. [1]. Purnomo et al. [2] defined medical waste as waste generated in the diagnosis and treatment of diseases of humans and animals, as well as related research, biological production and testing. According to the US Environmental Protection Agency (EPA), medical waste is a subset of waste produced in healthcare facilities such as hospitals, doctors' offices, dental offices, blood banks, veterinary hospitals/clinics, as well as medical research facilities and laboratories. In general, medical waste is healthcare waste that may be contaminated with blood, body fluids, or other potentially infectious materials and is often referred to as regulated medical waste [3].

Medical waste can be considered a source of the greatest environmental concern as it can harbor potentially harmful microorganisms and carries the risk of transmitting infections from healthcare facilities to healthcare employees, patients, and the public. To prevent harmful consequences to human health, society, and the environment, there is a need for appropriate medical waste management, which involves managing waste from its generation to its final disposal through separation, collection, transportation, and treatment [4]. There has been an increase in people's demand for health services due to factors such as increasing population, industrialization, and urbanization. Indirectly, the amount of medical waste in healthcare institutions and organizations has also increased [5].

Today, changes in various areas, such as technological development, rapid population growth, and increased access to information, combined with advancements in social welfare, also lead to an increase in the number of applications to health services. Therefore, due to the increase in healthcare demand, it creates a medical waste problem that poses a risk to the environment [6]. The already unsustainable increase in medical waste production and management has been suddenly exacerbated by the COVID-19 pandemic; it has created a threat that, if not controlled safely and properly, will turn into environmental pollution and a public health crisis [7]. To prevent environmental pollution, precautions should be taken regarding the collection, transportation, and disposal of medical waste [8].

It is vital to encourage and raise awareness of the participation of healthcare professionals in the creation and implementation of policies and programs regarding waste management. Therefore, the management of healthcare waste needs to be embedded in a systematic, multifaceted framework and become an integral feature of healthcare services [9].

In studies conducted to evaluate the current situation of medical waste management in different countries, medical waste management methods in different countries were compared, and it was said that the medical waste situation is a worldwide public health problem, and choosing appropriate medical waste management is very important [10-27].

Studies conducted to measure the level of medical waste knowledge of healthcare professionals on medical waste should be repeated periodically. To minimize the negative effects of medical waste on the environment and public health, effective waste management practices supported by trained personnel are essential. Healthcare facilities should manage medical waste in compliance with national policies and legal regulations to ensure environmental and human health protection. Enhancing healthcare workers' awareness of medical waste management contributes to environmental sustainability and reduces the risk of infectious disease transmission. Therefore, the implementation of structured waste management plans, the establishment of responsible management units, and the provision of regular personnel training are critical for the safe disposal of medical waste [28-39].

For this reason, the study aimed to examine the medical waste management in Kütahya and to evaluate the knowledge and awareness of the employees of two hospitals in the Center regarding medical waste.

The online surveys were conducted by taking advantage of the knowledge and experience of all healthcare personnel working in two healthcare institutions and organizations. The gender, working period, age, working unit, educational status, and medical waste training status of the hospital personnel included in the research. Disruptions and other negative situations that occurred during the medical waste management phase were identified, and recommendations were made for preventive and corrective measures to be taken.

2. METHODOLOGY

The research is a descriptive and cross-sectional study in which the medical waste knowledge and awareness levels of hospital employees in two hospitals located in Kütahya Center are evaluated. Between 22nd February 2021 and 22nd March 2021, staff working in (A) health institution and (B) health institution in Kütahya participated in the survey online. Hospital employees serving in two hospitals in Kütahya constituted the population of our study. The sample of the research consists of 516 hospital employees who participated in the study. Small-scale health institutions with a bed capacity of 10 will be referred to with the letter (A), and large-scale health institutions with a bed capacity of 950 will be referred to with the letter (B).

During the data collection phase, a ready questionnaire, by Akpolat et al. [30], was used by the researcher and conducted to the participants online. The first 7 questions are about demographic variables, the 8th question consists of a 16-item scale with a 5-point Likert type, and the last 7 questions consist of a total of 15 questions consisting of a survey that evaluates whether the meanings of the symbols in the images are known or not. The data were analyzed in the SPSS V.26 program. To measure the level of medical waste knowledge, participants were asked to indicate their level of participation in the judgments according to the stated statements.

In line with scientific and universal ethical principles in the conduct of research, necessary permissions were obtained from Eskişehir Technical University Ethics Committee and the Chief Physician's Offices of the relevant hospitals to conduct the study.

Research hypotheses about the hospital employees in two hospitals in Kütahya Center are listed as follows:

H1: Medical waste knowledge and awareness level varies depending on the health institution where they work.
H2: Medical waste knowledge and awareness level varies according to age.
H3: Medical waste knowledge and awareness level varies according to gender.
H4: Medical waste knowledge and awareness level varies depending on the unit worked.
H5: Medical waste knowledge and awareness level varies depending on the position in the hospital.
H6: Medical waste knowledge and awareness level varies according to education level.
H7: Medical waste knowledge and awareness level varies according to the study period.

The fact that the study was conducted in two hospitals in Kütahya Center and that the participation was less than expected since the study coincided with the Covid-19 epidemic period was accepted as a limitation of the study.

Questionnaire form A and B employed in health institutions; It was applied to nurses-midwives, doctors, data preparation control operator-civil servants, health technicians, health officers, permanent employees (service personnel), and dentists. The survey link was shared with the participants, and they participated in the survey form online.

3. RESULTS

In the study, the medical waste knowledge and awareness level score was calculated, and the Kolmogorov-Smirnov and Shapiro-Wilk tests, which were used to assess normality, were applied to determine whether the scores were normally distributed. Within the scope of the study, the data of 92 participants were deleted because they did not respond to the scale items; The data of 2 participants were excluded from the analysis because they were outliers. According to the Kolmogorov-Smirnov (K-S test statistic=0.069) and Shapiro-Wilk (S-W test statistic=0.959) test results, it is concluded that the medical waste knowledge and awareness level score was not suitable for normal distribution in both the Kolmogorov-Smirnov test and the Shapiro-Wilk test ($p<0.05$), so it was decided that non-parametric statistical techniques should be used in the study.

Cronbach's alpha value was found to be 0.883. According to the analysis results, it was concluded that the scale was highly reliable.

Demographic characteristics of healthcare employees, such as gender, age, the unit they work in, their position in the hospital they work in, education level, and length of service of healthcare employees are given in Table 1, distributed according to demographic variables.

According to the analysis results, when the distribution of individuals according to their demographic characteristics was examined; 82.2% worked in hospital B, 40.4% were 30-40 years old, 58.8% were women, 41.8% worked in other units, 32.9% worked as permanent employees (service personnel) in the hospital where they worked, % It was concluded that 38.3% of them had undergraduate education and 53.2% had been working in this position for 1-10 years.

Table 1. The distribution of demographic characteristics of the contributors

		n	%
Health institution where you work	Hospital A	91	17.8
	Hospital B	420	82.2
Age	18-30	145	28.2
	30-40	208	40.4
	40 +	162	31.4
Gender	Female	302	58.8
	Male	212	41.2
The unit you work in	Clinic	124	24.1
	Policlinic	101	19.6
	Emergency room	55	10.7
	Laboratory	19	3.7
	Other	215	41.8
Your role in the hospital you work in	Nurse-Midwife	158	30.7
	Doctor	67	13,0
	Officer	33	6.4
	Health Technician (Radiology, Laboratory, etc.)	21	4.1
	Health Officer (Technician etc.)	23	4.5
	Permanent Worker (Servant Personnel)	169	32.9
	Dentist	43	8.4
Education Status	Elementary school	33	6.4
	High school	115	22.3
	Associate degree	76	14.8
	Bachelor's degree	197	38.3
	Graduate	94	18.3
How long do you work on duty?	1-10 years	273	53.2
	11-20 years	176	34.3
	21 years and above	64	12.5

At the same time, individuals; 38% have a bachelor's degree, 22% have a high school education, 18% have a postgraduate degree, 15% have an associate degree, and 7% have an elementary school (Figure 1).

In the survey administered to the participants, a 16-item medical waste knowledge level scale in the form of a 5-point Likert was applied, and the descriptive findings obtained are summarized in Table 1.

In the question measuring the knowledge level that medical waste should be disposed of in red bags, 96.9% was observed in the Özgür [36] study. Akbolat et al. [29] study observed a value of 97.1%, like this rate. In our study, the rate was 96.1%, which supports the findings in the literature. According to the descriptive statistics analysis results of the scale score related to the level of medical waste knowledge and awareness, the average score of the medical waste knowledge and awareness level was obtained as 60.62, with the participants receiving a minimum of 22 and a maximum of 80 points.

Additionally, to examine the distribution of the medical waste level scale, participants were asked to indicate their level of agreement with statements such as strongly disagree, disagree, no opinion, agree, and strongly agree (Table 2).

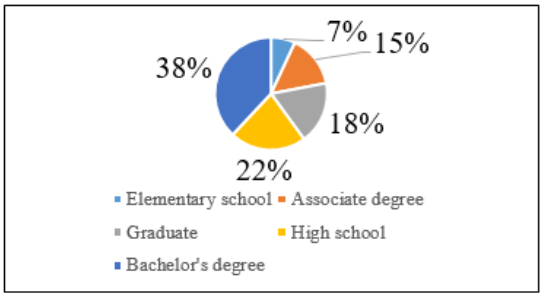

Figure 1. *Educational status pie chart*

According to the analysis results of the study conducted on the emblem information of professional groups, nurses and midwives, doctors, medical officers, health technicians (radiology, laboratory, etc.), health officers (technicians, etc.), permanent employees (service personnel), and dentists. When asked to examine the distribution of emblem information, the majority; Knowing the radioactive waste emblem, knowing that medical waste is thrown into red bags in the health institution where he works, knowing the biological risk emblem, knowing the corrosive emblem, providing the necessary training to increase the level of awareness about medical waste in the primary school period will enable us to become more conscious individuals about environmental problems and ecotoxic (environment and It was concluded that he knew the emblem (dangerous for nature) (Table 3). However, the risks posed by medical waste have already been addressed; nurses and midwives, medical personnel, health technicians (radiology, laboratory, etc.), health officers (technicians, etc.), permanent employees (service personnel) and dentists have never been exposed; It has been observed that doctors are exposed to it 1-2 times a year (Table 4).

According to the analysis results of the Mann Whitney U test, which was conducted to analyze whether the level of medical waste knowledge and awareness varies depending on the health institution where the individual works, the level of medical waste knowledge and awareness varies significantly depending on the health institution where the individual works (Mann Whitney U test Statistic= **-3.880,** $p<0.05$) [40]. Therefore, it was concluded that there is a difference in the level of medical waste knowledge and awareness of hospital employees working in two hospitals in the city center of Kütahya, depending on the health institution they work in. According to the average scores, it was observed that those working in hospital A had higher scores than those working in hospital B. Based on this, it is understood that the health institution where one works is an important factor in determining the level of medical waste knowledge and awareness.

H_0: There is no difference in the level of medical waste knowledge and awareness of hospital employees working in two hospitals in Kütahya city center, depending on the health institution they work in.
H_1: There is a difference in the level of medical waste knowledge and awareness of hospital employees working in two hospitals in Kütahya city center, depending on the health institution they work in.
H_0: There is no difference in the level of medical waste knowledge and awareness of hospital employees in Kütahya city center, depending on their age and the health institution they work in.
H_1: There is a difference in the age and level of medical waste knowledge and awareness of hospital employees in Kütahya city center, depending on the health institution they work in.

Table 2. Distribution for medical waste knowledge and awareness level scale

	I strongly disagree		I do not agree		No idea		I agree		I strongly agree	
	n	%	n	%	n	%	n	%	n	%
1. Medical waste collected from the units by the staff is stored in the temporary storage area.	14	2.7	9	1.7	80	15.5	293	**56.8**	120	23.3
2. Wastes are placed in separate-colored bags according to their source.	12	2.3	24	4.7	26	5.0	282	**54.7**	172	33.3
3. Medical waste is collected within a specified time.	7	1.4	25	4.8	65	12.6	272	**52.7**	147	28.5
4. The collection of waste is carried out by specially trained personnel.	16	3.1	29	5.6	82	15.9	250	**48.4**	139	26.9
5. An effective control is carried out regarding the management of waste.	18	3.5	64	12.4	162	31.4	187	**36.2**	85	16.5
6. In our hospital, personnel are given training on medical waste management at regular intervals.	9	1.7	30	5.8	90	17.4	267	**51.7**	120	23.3
7. There are enough bags and containers at medical waste production points	7	1.4	30	5.8	122	23.6	248	**48.1**	109	21.1
8. Cars and vehicles used for transportation to the temporary storage area are disinfected after the process.	19	3.7	60	11.6	221	**42.8**	145	28.1	71	13.8
9. To prevent medical waste from leaking out during transportation in our institution, 3/4 of the garbage collection boxes and bags should be filled.	12	2.3	28	5.4	113	21.9	256	**49.6**	107	20.7
10. During the Covid-19 Pandemic, an increase in the amount of Medical Waste is observed.	10	1.9	22	4.3	94	18.2	218	**42.2**	172	33.3
11. Necessary occupational health and safety measures are taken against any risks that may occur during the transportation of waste.	14	2.7	70	13,6	99	19.2	243	**47.1**	90	17.4
12. Medical Waste Management in Health Institutions in Kütahya is sufficient in terms of Environment and Public Health.	11	2.1	56	10.9	189	36.6	207	**40.1**	53	10.3
13. There is a high risk of contracting Covid-19 for personnel who encounter Medical Waste during the pandemic.	7	1.4	36	7.0	60	11.6	249	**48.3**	164	31.8
14. There is a Medical Waste Sterilization Facility in Kütahya.	10	1.9	22	4.3	359	**69.6**	99	19.2	26	5.0
15. Pathological waste is classified as medical waste.	12	2.3	18	3.5	113	21.9	276	**53.5**	97	18.8
16. The color of the medical waste transport vehicle is orange.	14	2.7	17	3.3	66	12.8	239	**46.3**	180	34.9

Table 3. Distribution for emblems related to medical waste

		n	%
 What do you think this emblem means?	Medical waste	61	11.9
	Radioactive Waste	449	87.7
	Domestic Waste	1	0.2
	Non-Hazardous Waste	1	0.2
In which color bags are Medical Wastes disposed of in the Healthcare Institution you work in?	Black	4	0.8
	Yellow	10	1.9
	Red	498	96.7
	Blue	3	0.6
 What do you think this emblem means?	Biological risk	259	51.1
	Dangerous waste	183	36.1
	Non-Hazardous Waste	3	0.6
	Radioactive Waste	62	12.2
Have you ever been exposed to the risks posed by medical waste?	Often	48	9.4
	1-2 per month	44	8.6
	1-2 per year	123	24.0
	Never	297	58.0
 What do you think this emblem means?	Caustic	371	72.7
	Flammable	90	17.6
	Ecotoxic (dangerous for the environment and nature)	49	9.6
	Radioactive	0	0.0
In what period do you think providing the necessary training to increase the level of awareness about Medical Wastes will enable us to become more conscious of environmental problems?	Pre-school	64	12.5
	Primary school	225	43.8
	Middle school	110	21.4
	High school	91	17.7
	University	24	4.7
 What do you think this emblem means?	Explosive	5	1.0
	Flammable	5	1.0
	Ecotoxic (dangerous for the environment and nature)	488	95.5
	Radioactive	13	2.5

Table 4. Distribution of professional groups' emblem information

		What is your role in the hospital you work in?													
		Nurse-Midwife		Doctor		Officer		Health Technician (Radiology, Laboratory, etc.)		Health Officer (Technician etc.)		Permanent Employee (Servant Personnel)		Dentist	
		n	%	n	%	n	%	n	%	n	%	n	%	n	%
 What do you think this emblem means?	Medical waste	18	11.4	6	9.0	5	15.6	3	15.0	1	4.5	24	14.3	4	9.3
	Radioactive Waste	140	**88.6**	61	**91.0**	27	**84.4**	17	**85.0**	21	**95.5**	142	**84.5**	39	**90.7**
	Domestic Waste	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	1	0.6	0	0.0
	Non-Hazardous Waste	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	1	0.6	0	0.0
In which color bags are Medical Wastes disposed of in the Healthcare Institution you work in?	Black	0	0.0	3	4.5	0	0.0	0	0.0	0	0.0	1	0.6	0	0.0
	Yellow	1	0.6	2	3.0	0	0.0	0	0.0	1	4.3	5	3.0	1	2.3
	Red	157	**99.4**	60	**89.6**	32	**97.0**	20	**100.0**	22	**95.7**	163	**96.4**	42	**97.7**
	Blue	0	0.0	2	3.0	1	3.0	0	0.0	0	0.0	0	0.0	0	0.0
 What do you think this emblem means?	Biological risk	72	**46.2**	42	**62.7**	15	**46.9**	10	**50.0**	15	**68.2**	82	**49.7**	22	**51.2**
	Dangerous waste	66	42.3	18	26.9	9	28.1	4	20.0	5	22.7	63	38.2	17	39.5
	Non-Hazardous Waste	1	0.6	0	0.0	0	0.0	0	0.0	1	4.5	1	0.6	0	0.0
	Radioactive Waste	17	10.9	7	10.4	8	25.0	6	30.0	1	4.5	19	11.5	4	9.3
Have you ever been exposed to the risks posed by medical waste?	Often	22	14.0	10	14.9	1	3.0	0	0.0	2	8.7	10	6.0	3	7.1
	1-2 per month	16	10.2	8	11.9	4	12.1	3	15.0	2	8.7	6	3.6	5	11.9
	1-2 per year	41	26.1	25	**37.3**	1	3.0	2	10.0	5	21.7	33	19.6	15	35.7
	Never	78	**49.7**	24	35.8	27	**81.8**	15	**75.0**	14	**60.9**	119	**70.8**	19	**45.2**
 What do you think this emblem means?	Caustic	118	**75.2**	58	**86.6**	23	**71.9**	13	**65.0**	15	**68.2**	106	**63.5**	37	**86.0**
	Flammable	19	12.1	7	10.4	7	21.9	5	25.0	4	18.2	45	26.9	3	7.0
	Ecotoxic (dangerous for the environment and nature)	20	12.7	2	3.0	2	6.3	2	10.0	3	13.6	16	9.6	3	7.0
	Radioactive	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
In what period do you think providing the necessary training to increase the level of awareness about Medical Wastes will enable us to become more conscious about environmental problems?	Pre-school	29	18.5	2	3.0	5	15.2	2	10.0	3	13.0	18	10.7	5	11.6
	Primary school	64	**40.8**	24	**35.8**	16	**48.5**	7	**35.0**	9	**39.1**	85	**50.3**	18	**41.9**
	Middle school	26	16.6	13	19.4	7	21.2	5	25.0	5	21.7	43	25.4	11	25.6
	High school	29	18.5	19	28.4	5	15.2	5	25.0	5	21.7	22	13.0	6	14.0
	University	9	5.7	9	13.4	0	0.0	1	5.0	1	4.3	1	0.6	3	7.0
 What do you think this emblem means?	Explosive	1	0.6	0	0.0	2	6.1	0	0.0	0	0.0	2	1.2	0	0.0
	Flammable	1	0.6	1	1.5	1	3.0	1	5.0	0	0.0	1	0.6	0	0.0
	Ecotoxic (dangerous for the environment and nature)	151	**96.8**	66	**98.5**	27	**81.8**	19	**95.0**	21	**95.5**	159	**94.6**	43	**100.0**
	Radioactive	3	1.9	0	0.0	3	9.1	0	0.0	1	4.5	6	3.6	0	0.0

According to the analysis results of the Kruskal-Wallis H test, which was conducted to analyze the level of medical waste knowledge and awareness in terms of the age of the individual, the level of medical waste knowledge and awareness differs significantly in terms of the age of the individual (H=**12.836**; $p<0.05$). As a result of the analyses carried out to observe which groups this difference originates from, it is observed that the 1st group stands out. In other words, according to the average scores, those between the ages of 18-30; It was concluded that those aged 30-40 and older than 40 had lower scores. Therefore, the H_0 null hypothesis is rejected; It was concluded that there is a difference in the age of hospital employees in Kütahya city center and the level of medical waste knowledge and awareness depending on the health institution they work in. It can be said that as we get older, the level of knowledge increases accordingly.

According to the results of the Mann-Whitney U test analysis, which was conducted to analyze the level of medical waste knowledge and awareness in terms of the gender of the individual, the level of medical waste knowledge and awareness does not differ significantly in terms of the gender of the individual (U=**-0.998**; $p>0.05$). Therefore, the H_0 null hypothesis is accepted at alpha significance level, that is; It was concluded that there is no difference in the level of medical waste knowledge and awareness of hospital employees in Kütahya city center, depending on their gender and the health institution they work in. There is no superiority or weakness in women over men in terms of medical waste knowledge and awareness level, and men have no superiority or weakness in terms of knowledge level over women.

H_0: There is no difference in the level of medical waste knowledge and awareness of hospital employees in Kütahya city center, depending on their gender and the health institution they work in.
H_1: There is a difference in the level of medical waste knowledge and awareness of hospital employees in Kütahya city center, depending on their gender and the health institution they work in.

According to the analysis results of the Kruskal-Wallis H test, which was conducted to analyze the level of medical waste knowledge and awareness in terms of the unit where the individual works, it was determined that the level of medical waste knowledge and awareness did not differ significantly according to the unit where the individual worked (H=**6.915**; $p>0.05$). Therefore, the H_0 null hypothesis is accepted at an alpha significance level, that is, it was concluded that there is no difference in the level of medical waste knowledge and awareness of hospital employees in Kütahya city center, depending on the unit they work in and the health institution they work in. It has been understood that no matter which unit the hospital staff works in, this does not create a significant difference in the level of medical waste knowledge and awareness.

H_0: There is no difference in the level of medical waste knowledge and awareness of hospital employees in Kütahya city center, depending on the unit they work in or the health institution they work in.
H_1: There is a difference in the level of medical waste knowledge and awareness of hospital employees in Kütahya city center, depending on the unit they work in and the health institution they work in.

According to the analysis results of the Kruskal-Wallis H test, which was conducted to analyze the level of medical waste knowledge and awareness in terms of the individual's position in the hospital, it was determined that the level of medical waste knowledge and awareness did not differ significantly according to the individual's position in the hospital (H=**8.619**; $p>0.05$). Therefore, the H_0 null hypothesis cannot be rejected at an alpha significance level; that is; It was concluded that there is no difference in the level of medical waste knowledge and awareness of hospital employees in Kütahya city center, depending on their duties in the hospital and the health institution they work in.

Although the duties performed and undertaken by various professional groups in the hospital are different, it has been understood that this does not create a significant difference in determining the level of medical waste knowledge and awareness.

H_0: There is no difference in the level of medical waste knowledge and awareness of the hospital staff in Kütahya city center and the hospital staff, depending on the health institution they work in.

H_1: There is a difference in the level of medical waste knowledge and awareness of the hospital staff in Kütahya city center and the hospital staff depending on the health institution they work in.

According to the analysis results of the Kruskal-Wallis H test, which was conducted to analyze the level of medical waste knowledge and awareness in terms of the individual's educational level, it was determined that the level of medical waste knowledge and awareness did not differ significantly according to the educational level of the individual, (H= **7.403**; $p>0.05$). Therefore, it was concluded that there is no difference in the education level of healthcare professionals in Kütahya city center and the level of medical waste knowledge and awareness in terms of the healthcare institution they work in.

H_0: There is no difference in the education level of hospital employees in Kütahya city center and the level of medical waste knowledge and awareness depending on the health institution they work in.
H_1: There is a difference in the education level of hospital employees in Kütahya city center and the level of medical waste knowledge and awareness depending on the health institution they work in.

According to the analysis results of the Kruskal-Wallis H test, which was conducted to analyze the level of medical waste knowledge and awareness in terms of the individual's working time on the job, the level of medical waste knowledge and awareness varies significantly according to the individual's working time (H=**10.702**; $p<0.05$).

As a result of the analysis carried out to observe which groups this difference originates from, it is observed that the 1st group stands out. In other words, according to the average scores, employees with 1-10 years of experience; It was concluded that those who worked in this position for 11-20 years and more than 21 years had lower scores. Therefore, the H_0 null hypothesis was rejected at the alpha significance level; that is; It was concluded that the working hours of hospital employees in Kütahya city center differ in the level of medical waste knowledge and awareness depending on the health institution they work in. It has been understood that the longer you work in the position, the more significant the level of medical waste knowledge and awareness varies, meaning that years of professional experience are an important factor in determining the level of medical waste knowledge and awareness.

4. CONCLUSION AND RECOMMENDATIONS

This research was carried out to evaluate the knowledge and awareness levels of hospital employees in two hospitals in Kütahya Center about medical waste. When the gender distribution of the participants included in the research is examined, it is seen that women are at the level of 58.80% and men are at the level of 41.20%. When the age variables are examined, it is seen that 28.20% are 30 years old and under, 40.40% are between 30-40 years old, 40 years and over are 31.40%. It appears that there is a distribution.

Hospital employees are generally undergraduate (38.30%) and high school (22.30%) graduates. When we look at the distribution of duties in the hospital, the majority are as follows; permanent worker (service personnel) (32.90%), nurse-midwife (30.70%), doctor (13%), dentist (8.4%), data preparation control operator -civil servant (6.4%), health officer (4.5%), health technician (4.1%). More than half of the employees (53.20%) have been working for 1-10 years. In this context, the participants' answers to the statements were analyzed using non-parametric statistical techniques.

According to the research results, it was determined that the knowledge and awareness levels of hospital employees in two hospitals were high. Medical waste knowledge level varies significantly depending on the health institution where the individual works, age, and length of service.

The level of medical waste knowledge does not differ significantly depending on the individual's gender, the unit the individual works in, the individual's position in the hospital, or the individual's education level.

In our study, the results of the analysis conducted to determine the relationship between participants' medical waste knowledge and awareness levels and gender found that there was no significant difference between the answers given to all statements and gender.

Improper management of medical waste in healthcare settings poses significant risks to human health. While proper disposal of medical waste protects public health, recycling practices contribute to reducing economic losses in healthcare institutions and support the national economy. Therefore, healthcare facilities must comply with medical waste management regulations and provide adequate training for healthcare workers to ensure occupational safety and environmental protection. In addition to training, strict implementation, monitoring, and regular on-site supervision of biomedical waste management guidelines are essential to enhance awareness and promote correct practices.

ACKNOWLEDGEMENTS

We are very grateful to each participant in this research.

FUNDING INFORMATION

We have not required any funding to conduct this research.

CONFLICT OF INTEREST STATEMENT

The authors declare no potential conflicts of interest with respect to the research, authorship, and publication of this article.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

RESEARCH ETHICS COMMITTEE APPROVAL

This study was approved by Eskişehir Technical University Ethics Committee protocol number 2/2 on January 29th, 2021, and T.C. Kütahya Provincial Health Directorate Scientific Research and Application Review Commission with protocol number 2021/28 on February 15th, 2021.

CRediT AUTHOR STATEMENT

Ahmet Demirtaş: Conceptualization, Data Curation, Writing-Original Draft, Visualization, Supervision, **Semra Malkoç:** Writing-Review & Editing, Conceptualization, Investigation**, Berna Yazıcı:** Writing-Review & Editing, Conceptualization, Validation, Formal Analysis, Data Curation

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ESKİŞEHİR TEKNİK ÜNİVERSİTESİ BİLİM VE TEKNOLOJİ DERGİSİ
C- YAŞAM BİLİMLERİ VE BİYOTEKNOLOJİ

Eskişehir Technical University Journal of Science and Technology
C -Life Sciences and Biotechnology

Estuscience – Life, 2026, 15 (2) pp. 111-120, DOI: 10.18036/estubtdc.1800366

RESEARCH ARTICLE

ANTIMICROBIAL CONSTITUENTS OF THE ESSENTIAL OIL OF *ECHINOPHORA TENUIFOLIA* L. SUBSP. *SIBTHORPIANA* (GUSS.) TUTIN

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Abstract

The hydrodistilled essential oil of *Echinophora tenuifolia* L. subsp. *sibthorpiana* commonly known as "çörtük," "tarhana grass," or "pickle grass" was evaluated for its antimicrobial activity against 17 microorganisms (16 bacteria and *Candida albicans*) by using microdilution and bioautography methods. Methyl eugenol (38.8%), δ-3-carene (31.2%) and *p*-cymene (9.0%) were determined by GC-FID and GC/MS systems as main constituents of the essential oil. The oil showed strong inhibitory effects, particularly against the plant pathogens *Xanthomonas campestris* and *X. campestris* pv. *phaseoli* with minimum inhibitory concentrations (MIC) of 62.5 and 31.25 µg/mL, respectively. *Candida albicans* was inhibited by the oil at 500 µg/mL. According to the results of TLC-bioautography assay, methyl eugenol and δ-3-carene were identified as the compounds responsible for antimicrobial activity. These bioactive compounds were further tested with the essential oil at the same time against certain pathogens for the determination of possible synergistic effects.

Keywords

Antimicrobial,
GC/MS,
TLC-Bioautography,
Methyl eugenol,
Synergy

Time Scale of Article

Received :09 October 2025
Accepted : 16 February 2026
Online date :27 July 2026

1. INTRODUCTION

Essential oils and their individual components have widely been used as bioactive agents in many industries for many years. Apart from being a major tool of aromatherapy they are valuable substances for food, cosmetic, agricultural, livestock and pharmaceutical industries [1-2].

In fact, most well-known properties of essential oils are their volatility and antimicrobial activity. Owing to their unique compositions, these natural terpenoid mixtures can act as growth inhibitory agents against various pathogenic bacteria, fungi and viruses. Despite their compositional differences, most of them exhibit inhibitory effects at concentrations up to 5% (v/v), increasing in a dose-dependent manner. Due to their hydrophobic structures, they cause several damages on the membranes of target microorganisms, resulting in protoplasmic leakage [3-7]. Developing eco-friendly alternatives to synthetic products is

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increasingly important with the growing demand for organic farming. Essential oils (EOs) may alternative natural way to control weeds, pests, and microbial plant diseases without any impact on crop yields. Employing essential oils for this purpose offers an extra economic value and acceptability in the growing "natural product market" [8-9]. *Echinophora tenuifolia* L. subsp. *sibthorpiana* (Guss.) Tutin, which is widely found in Türkiye, is known as "çördük, çörtük, tarhana otu". It is a thornless biennial or perennial bush with a height of 20-50 cm. In some regions of Anatolia, dried aerial parts are used in soups, pickles and tarhana production for its aromatic properties. In folk medicine infusion and decoction of herbs are used frequently against common cold, wounds, indigestion, stomach ulcers, shortness of breath spasms and pain. Fresh or dried herbs are also utilized as a natural antifungal remedy [10-13].

The essential oil composition of *E. tenuifolia* subsp. *sibthorpiana* has been reported in several studies. Although different collection times and geographic regions and the presence of cultivated and wild forms, δ-3-carene, α-phellandrene, methyl eugenol, β-phellandrene and *p*-cymene were reported as the main compounds of the identified essential oils in all published reports (Table 1.).

Table 1. Major constituents of *E. tenuifolia* subsp. *sibthorpiana* essential oils

Main compound %	Other main compounds %	Reference
α-Phellandrene (13-55%)	δ-3-carene (4-49%), methyl eugenol (22-25%)	[13]
α-Phellandrene (51.0%)	methyl eugenol (24.8%), β-phelladrene (5.0%)	[14]
α-Phellandrene (51.5%)	methyl eugenol (17.5%), *p*-cymene (14.7%)	[15]
α-Phellandrene (43.8%)	methyleugenol (28.6%), *p*-cymene (9.5%)	[16]
α-Phellandrene (47-66%)	methyl eugenol (21.3-38.7%), *p*-cymene (0.7-3.3%)	[17]
δ-3-Carene (30-38.8%)	methyl eugenol (21-26%), α-phellandrene (15-29%)	[18]
δ-3-Carene (23.5%)	methyl eugenol (18.4%), α-phellandrene (11.4%)	[19]
δ-3-Carene (17.9%)	*p*-cymene (9.0%), methyl eugenol (16.4%)	[20]
δ-3-Carene (31.8%)	α-phellandrene (31.%), methyl eugenol (16.9%)	[21]
Methyl eugenol (42-63%)	α-phellandrene (6-30%), *p*-cymene (7-9%)	[22]
Methyl eugenol (36.6%)	δ-3-carene (36.6%), *p*-cymene (7.6%)	[23]
Methyl eugenol (60.4%)	*p*-cymene (11.2%), α-phellandrene (10.2%)	[24]
Methyl eugenol (58.7%)	α-phellandrene (15.5%), *p*-cymene (11.0%)	[25]
Methyl eugenol (48.1%),	α-phellandrene (20.5%), *p*-cymene (12.5%)	[26]
Methyl eugenol (50.4%),	α-phellandrene (16.3%), δ-3-carene (17.4%)	[27]
Methyl eugenol (9.6-80.7%)	δ-3-carene (2.3–61.6%), *p*-cymene (0.5-6.0%)	[28]
Methyl eugenol (25-90.2%)	δ-3-carene (2.6%-34.8%), *p*-cymene (1.2-9.8%)	[29]
Methyl eugenol (45.5%)	*p*-cymene (11.5%), *δ*-3-carene (11.2%)	[30]

Several biological activity studies on the EO of *E. tenuifolia* L. subsp. *sibthorpiana* have also been reported. Essential oils of the aerial parts have been evaluated for their antimicrobial [13, 20, 24, 31], anti-inflammatory [32], antioxidant [20, 24, 32], insecticidal and larvicidal activities [26]. The oil has been demonstrated remarkable growth inhibition on *Staphylococcus epidermidis*, *S. aureus*, *Rhizoctonia solani*, *Aspergillus ochraceus*, *Penicillium ochrochloron* and larvicidal effects on *Culex pipiens* and *Tribolium castaneum* [26, 33, 34].

The present study aimed to identify the antimicrobial constituents of *E. tenuifolia* L. subsp. *sibthorpiana* essential oil against a range of human and phytopathogenic microorganisms.

Previous studies have reported promising potential for use as a biopesticide and insect repellent. Therefore, phytopathogenic microorganisms were specifically included in the test panel in our study. Major components (>1.0%) of the essential oil were presented. The antimicrobial activity of the EO and its major constituents, δ-3-carene and methyl eugenol, was evaluated against 16 human and plant pathogenic bacteria and *Candida albicans*. The TLC-bioautography assay was performed with *C. albicans* and *X. campestris* for the determination of bioactive constituents of the oil.

2. MATERIAL and METHODS

2.1. Plant Material and Essential Oil

The collected and identified plant specimens (viii, 1999, Bademağacı district, 50th km of the Antalya-Burdur highway, Türkiye) were deposited at the Herbarium of the Faculty of Pharmacy, Anadolu University, under the herbarium record number ESSE-13039. The flowering air-dried aerial parts of the plants were used to obtain the essential oil. Hydro-distillation was performed using a Clevenger-type apparatus for 3 hours, resulting in an oil yield of 1.6% (w/w, dry weight). The main components of the oil, methyl eugenol (Robertet) and δ-3-carene (Sigma-Aldrich), were obtained from commercial sources as authentic samples. Anhydrous sodium sulphate was used for drying the oil, which was then kept at +4°C until antimicrobial tests.

2.2. Gas Chromatography and Gas Chromatography-Mass Spectrometry Analysis

Essential oil dissolved in *n*-hexane (4 mg/mL) for injection. Gas Chromatography analysis was performed using a Shimadzu GC-17A system equipped with a CP-Sil 5CB column (25m × 0.25mm, 0.4 µm film thickness) and nitrogen as the carrier gas at a flow rate of 1 mL/min. The oven temperature was initially set at 60°C, then programmed to increase to 260°C at a rate of 5°C/min and held constant at 260°C for 40 minutes. The split flow was set to 50 mL/min. The injector and FID detector temperatures were maintained at 250°C. For Gas Chromatography-Mass Spectrometry (GC-MS) analysis, a Shimadzu GCMS-QP5050A system with a CP-Sil 5CB column (25 m × 0.25 mm) was used, with helium as the carrier gas. The GC oven temperature followed the same program as described above: starting at 60°C, increasing to 260°C at 5°C/min, and held constant at 260°C for 40 minutes. The split flow was also set to 50 mL/min, and the injector temp. was maintained at 250°C. MS data were acquired at 70 eV, with a mass range of *m/z* 30 to 425. Component identification was carried out using the Wiley and Adams GC/MS Library and Baser *in house* Library of Essential Oil Constituents. The identified major components of the oils are listed in Table 2. Relative percentages of the components were obtained from GC-FID data.

2.3. Antimicrobial Evaluation

2.3.1. Broth microdilution

The microdilution method [35,36] was used to determine the antibacterial and anticandidal effects of the essential oil, methyl eugenol, δ-3-carene and standard antimicrobial agents. Stock solutions of the samples were prepared in sterile dimethyl sulfoxide (DMSO, Carlo-Erba, France). Double-fold dilution series were prepared with Mueller Hinton Broth (MHB) medium in 96 well microplates. Overnight cultures of bacteria (10^8 CFU/mL) and *Candida albicans* (10^6 CFU/ml) were adjusted in MHB medium according to the McFarland No:0.5 turbidity standard by using turbidity meter (Biosan, Latvia). An aliquot of 100 µL from microbial suspensions was pipetted into the wells containing test samples previously diluted in MHB medium (4000 to 7,8 µg/mL in DMSO). The column with MHB and microorganisms was separated as growth control, and the last column was used for checking sterility. After incubation period at 37°C for 24 h, the first well without growing was determined as minimum inhibitory concentration (MIC). For better visualisation, an aliquot of 20µl of Tetrazolium salt (1%, w/v, in EtOH) (TTC, Sigma) was pipetted into the wells and re-incubated for 3 hours at 37°C. Chloramphenicol (Sigma-Aldrich) and ketoconazole (Sigma-Aldrich) were used as the standard agents. All microdilution tests were performed in duplicate.

2.3.2. TLC-Bioautography method

TLC-Bioautography method [37] was used for the detection of antimicrobial constituents in the oil. TLC plates cut to 6x10 cm (Merck) coated with 0.2 mm silica gel 60 GF_{254} on aluminum support were used. *n*-hexane:ethyl acetate (9:1 v/v) solvent mixture was used as mobile phase. *C. albicans* and *X. campestris* were selected as test organisms. Microbial cultures were prepared according to McFarland No:0.5 standard in MHB. Suspensions were transferred into soft agar (0.7% agar agar in MHB) kept in a 45°C water bath to obtain a final concentration of 10^5 and 10^8 cfu/ml, respectively. Developed TLC plates were placed onto nutrient agar base dishes (13 cm diameter) aseptic conditions. Inoculated soft agar was poured onto the TLC plate gently and not exceeding 1 mm thickness. The *Candida* plate was incubated at 37°C for 24 hours, while the *X. campestris* plate was kept at 28 °C for 48h. After incubation, the plate was sprayed with TTC (Triphenyl Tetrazolium Chloride) solution and incubated for an additional 3 hours. The growth inhibition was determined as uncolored zones on the TLC plate. Retention factors of clear spots and the derivatized spots were compared before the isolation protocol. For semi-preparative isolation of active spots, 10x20 cm pre-coated TLC plates were used. Essential oil solution was applied on the plate as a thin start line and developed with *n*-hexane:ethyl acetate (9:1 v/v) solvent mixture. After development, dried TLC plates were examined under 254-364 nm UV light with the reference plate, by considering the location of spots, they were determined and scraped with a micro spatula. The scrapped silica gel with the substance was eluted with acetone through a Buchner funnel. Eluted fractions belonging to two spots were analyzed separately by using GC/MS for the identification of the substances responsible for the antimicrobial activity.

3. RESULTS

The chemical composition of the essential oil of *E. tenuifolia* subsp. *sibthorpiana* is presented as main compounds (>1%) in Table 2 according to the GC and GC/MS analysis results. Methyl eugenol, δ-3-carene, and *p*-cymene were found as major compounds of the oil 38.8%, 31.2% and 9.0%, respectively.

Table 2. Major constituents (≥1.0%) of *E. tenuifolia* subsp. *sibthorpiana* essential oil

Major Constituents	Type of Compound	% Relative	RI	RI^{Lit}
Methyl eugenol	PP	38.8	1374	1394[a]
δ-3-Carene	BMH	31.2	1005	1010[c]
p-Cymene	MH	9.0	1007	1020[a]
α-Phellandrene	MH	4.4	998	997[a]
β-Phellandrene	MH	1.8	1017	1025[a]
Myrcene	MH	1.2	983	990[b]
α-Phellandrene epoxide	MH	1.1	1180	1192[a]
Limonene	MH	1.0	1022	1030[b]

RI: Kovat's Retention Indices, PP: Phenylpropene, BMH: Bicyclic monoterpene hydrocarbone, MH: Monoterpene hydrocarbon, [a]:[16], [b][17], [c][38]

The major compounds were identified as phenylpropene methyl eugenol and bicyclic monoterpene hydrocarbon δ-3-carene, accounting for 70% of the total oil (≥1.0). The remaining part consisted of monoterpene hydrocarbons (18.5%). Previous works on *E. tenuifolia* subsp. *sibthorpiana* generally reported that the main compound of the oil was methyl eugenol, like our results. According to the TLC-bioautography assay, the bioactive spots were identified as methyl eugenol and δ-3-carene through the extraction and analysis of silica scraped from the plate. In the *C. albicans* plate both major compounds

showed inhibition zones, while in the *X. campestris* methyl eugenol demonstrated more clear and distinct inhibition zones (Figure).

Considering this finding, methyl eugenol and δ-3-carene were subjected to broth microdilution tests simultaneously with the essential oil for determination of Minimal Inhibitory Concentration (MIC) values (Table 3). Chloramphenicol and ketoconazole were used as standard antibiotics for bacterial strains and *C. albicans*, respectively.

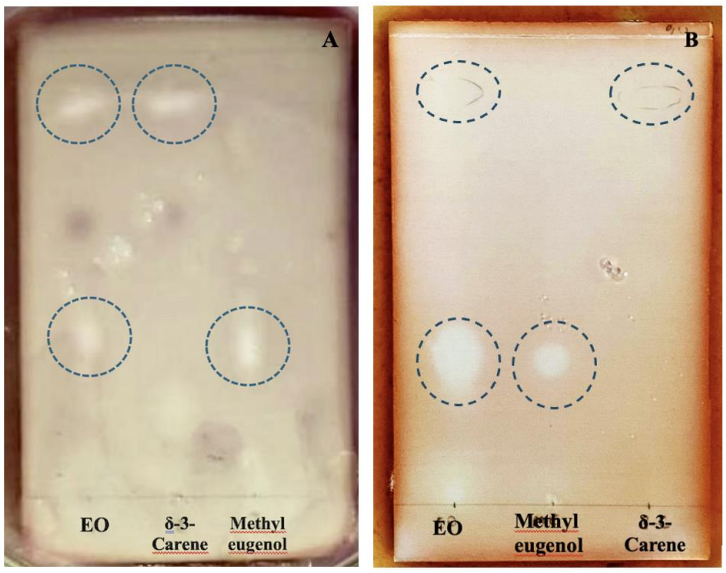

Figure TLC-bioautography assay (A: *C. albicans*, B: *X. campestris*)

Table 3. MIC values (µg/mL) of *E. teunifolia* subsp. *sibthorpiana* EO and its major constituents

Microorganisms	Source	EO	Methyl Eugenol	δ-3-Carene	Std
Gram-negative Bacteria					
X. campestris pv. *phaseoli*	TPPB 4212	31.25	500	1000	3.9
Xanthomonas campestris	TPPB 4250	62.5	125	62.5	3.9
P. syringae pv. *syringae*	TPPB 4101	125	1000	2000	15.6
Yersinia enterocolitica	AUBD	250	250	1000	1.9
Escherichia coli	NRRL B-3008	500	500	500	31.25
Enterobacter aerogenes	NRRL B-3567	500	500	2000	62.5
Proteus vulgaris	NRRL B-123	500	500	2000	15.6
P. syringae pv. *phaseolicola*	NRRL B-1459	500	125	2000	15.6
P. syringae pv. *tomato*	TPPB 5001	500	1000	125	1.95
Pseudomonas aeruginosa	ATCC 27853	1000	1000	2000	62.5
Salmonella typhimurium	NRRL B-4420	1000	500	2000	31.25
Klebsiella pneumoniae	AUBD	1000	1000	4000	1.9
Gram-positive Bacteria					
Staphylococcus epidermidis	ATCC 12228	250	125	500	3.9
Staphylococcus aureus	ATCC 6538	500	500	1000	3.9
Listeria monocytogenes	AUBD	500	250	500	1.95
Bacillus cereus	NRRL B-3711	500	1000	1000	15.6
Yeast					
Candida albicans	OUMD	500	500	1000	62.5[a]

Std: Chloramphenicol, [a]: Ketoconazole, AUBD: Ankara University, Department of Biology, OUMD: Eskisehir Osmangazi University, Department of Microbiology

The antimicrobial activity of EO and its major constituents, δ-3-carene and methyl eugenol, was assessed through the determination of Minimum Inhibitory Concentration (MIC) values against a panel of bacterial microorganisms and *Candida albicans*. Chloramphenicol and ketoconazole were used as standard antibiotics for bacterial and fungal strains, respectively. The EO exhibited broad-spectrum antimicrobial activity with MIC values ranging between 31.25 µg/mL and 1000 µg/mL. Remarkably, *Xanthomonas campestris* pv. *phaseoli* and *X. campestris* were inhibited by the oil, having MIC values of 31.25 and 62.5 µg/mL. With the MIC value of 62.5 µg/mL, δ-3-carene and the EO demonstrated similar effects on *Xanthomonas campestris*, while the oil showed synergistic activity on *Xanthomonas campestris* pv. *phaseoli* at the concentration of 31.25 µg/mL compared to MIC results of the main constituents (1000 and 500 µg/mL). Although the inhibition zone of δ-3-carene against *X. campestris* was not clear, according to broth microdilution test it was as effective as the essential oil and more effective than methyl eugenol, with a minimum inhibitory concentration (MIC) of 62.5 µg/mL. Among the major constituents, δ-3-carene displayed weaker antimicrobial activity with MIC values ranging from 62.5 µg/mL to 4000 µg/mL; *Klebsiella pneumoniae* (4000 µg/mL) was the least susceptible organism. Methyl eugenol demonstrated moderate activity, with MIC values ranging from 125 µg/mL to 1000 µg/mL. It showed the strongest activity against *Staphylococcus epidermidis* (125 µg/mL) and *Yersinia enterocolitica* (250 µg/mL). Overall, the antimicrobial activity of the essential oil (EO) and its major constituents were lower than that of the standard antibiotics.

4. DISCUSSION and CONCLUSION

According to previous studies, methyl eugenol, α-phellandrene and δ-3-carene were the major compounds of *E. tenuifolia* subsp. *sibthorpiana* essential oils. The EO composition varies with plant growth stage, drying methods, harvest time, storage conditions, and cultivation, affecting methyl eugenol, α-phellandrene, and δ-3-carene levels. Notably, methyl eugenol content is reported to be highest in fresh plant material, whereas phellandrene increases during the drying process [17].

According to antimicrobial activity results most potent effects of the EO observed against *Xanthomonas* species. These results highlight the oil's potential as a natural biocontrol agent against major agricultural pathogens, particularly those affecting bean and brassica crops. For the first time, TLC-bioautography assay demonstrated δ-3-carene and methyl eugenol may play a significant role in the antibacterial activity of the essential oil against *X. campestris*. Furthermore, the EO showed greater antimicrobial efficacy against some tested pathogens than its individual constituents, suggesting synergistic interactions; this effect was particularly evident against *Xanthomonas campestris* pv. *phaseoli* (MIC: 31.25 µg/mL). Although further studies are needed to clarify the exact mechanisms, the elevated susceptibility of the *Xanthomonas* species tested may be related to structural or metabolic properties that enhance their sensitivity to the tested essential oil and/or its constituents.

A previous work on *E. tenuifolia* essential oil showed inhibition on the phytopathogenic fungi *Rhizoctonia solani, Fusarium oxysporum,* and *Alternaria alternata* [13]. In another study *Echinophora tenuifolia* essential oil showed moderate to weak antibacterial effects between the MIC values of 62.5 and 1000 µg/mL similar to our results [20]. In another study, *E. tenuifolia* essential oil showed moderate antimicrobial activity against foodborne pathogens (MIC: 7.8–500 µg/mL) when compared with standard agents [31]. A recent study [39] reported that the 50% MeOH extract of the *E. tenuifolia* subsp. *sibthorpiana* showed potent antibacterial activity against *E. coli* and *S. aureus* (MICs of 1.85 and 3.7 mg/mL, respectively) but lacked antifungal efficacy against *C. albicans*. Only limited information is available in the literature regarding the antimicrobial activities of methyl eugenol and δ-3-carene.

Previous findings revealed that methyl eugenol showed antibacterial effects against *E. coli* and *Pseudomonas aeruginosa*, inducing disruption in the bacterial membrane [40]. In another work, methyl eugenol—the major constituent of essential oils from certain *Ocimum* species—was found to be

effective against *Pseudomonas aeruginosa* at a concentration of 0.72 µg/mL [41]. According to previous work, δ-3-carene has demonstrated remarkable antibacterial activity against *Pseudomonas* strains, which share a close genetic relationship with *Xanthomonas* [42]. The proposed mechanism of action includes membrane damage, disorder of amino acid metabolism, DNA disruption, and interference with other cellular functions, contributing to its antibacterial efficacy [43-44].

In conclusion, the essential oil of *E. tenuifolia* subsp. *sibthorpiana* and its major constituents exhibited promising antimicrobial activity synergistically, particularly against phytopathogenic bacteria. Although they may not be as effective as conventional antibiotics, their potential application as natural antimicrobials and biopesticides in sustainable agriculture highlights the need for further research and development.

ACKNOWLEDGEMENTS

This study was presented as a poster presentation at the 15th BİHAT symposium, held in Antalya-Belek, Turkey, between October 6-9, 2004.

CONFLICT OF INTEREST

The author(s) stated that there are no conflicts of interest regarding the publication of this article.

CRediT AUTHOR STATEMENT

Gökalp İşcan: Methodology, Investigation, Writing - original draft, Visualization, **Merih Kıvanç:** Supervision, Conceptualization, Resources, **Neşe Kırımer:** Conceptualization, **Kemal Hüsnü Can Başer**: Resources.

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ESKİŞEHİR TEKNİK ÜNİVERSİTESİ BİLİM VE TEKNOLOJİ DERGİSİ
C- YAŞAM BİLİMLERİ VE BİYOTEKNOLOJİ

Eskişehir Technical University Journal of Science and Technology
C -Life Sciences and Biotechnology

Estuscience – Life, 2026, 15 (2) pp. 121-129, DOI: 10.18036/estubtdc.1877891

RESEARCH ARTICLE

COMPARATIVE ANALYSIS OF ACUTE TOXICITY OF SODIUM HYPOCHLORITE ON *GAMBUSIA HOLBROOKI* (MOSQUITOFISH) IN DIFFERENT EXPERIMENTAL SYSTEMS

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Abstract

The aim of this study is to determine the acute toxicity of sodium hypochlorite (NaClO), which has become a common pollutant in aquatic ecosystems, on the model organism *Gambusia holbrooki* and to comparatively analyze the effect of three fundamental experimental systems (Static, Renewal, and Flow-through) used in ecotoxicology on LC_{50} values. Of the study, *G. holbrooki* individuals obtained from inland waters of the Thrace Region were exposed to different concentrations of NaClO for 96 hours. Mortality data were evaluated using Probit Analysis with IBM SPSS Statistics (v.26.0). According to the research findings, the 96-hour LC_{50} values were determined as 0.804 ppm in the static system, 0.404 ppm in the flow-through system, and 0.342 ppm in the renewal system. The results indicate that sodium hypochlorite falls into the "Highly Toxic" category for *G. holbrooki*. Moreover, it was determined that the choice of experimental system statistically significantly alters toxicity data; the renewal system, where the chemical is constantly refreshed and organisms are exposed to manipulation stress, yielded the most sensitive results. This study highlights the importance of experimental design selection in risk assessment protocols.

Keywords

Gambusia holbrooki,
Sodium hypochlorite,
Acute toxicity,
LC_{50},
Experimental design

Time Scale of Article

Received :02 February 2026
Accepted : 06 July 2026
Online date : 27 July 2026

1. INTRODUCTION

With the increase in anthropogenic activities, aquatic ecosystems are under intense pressure from chemical pollutants of agricultural, industrial, and domestic origin. Among these pollutants, disinfectants constitute one of the most widely used chemical groups globally due to their critical role in the elimination of pathogenic microorganisms. Sodium hypochlorite (NaClO), in particular, a potent oxidizing agent, is used in a wide range of applications, from water treatment plants to medical waste disposal. The COVID-19 pandemic in recent years has led to stricter hygiene protocols and a dramatic increase in the use of chlorine-based disinfectants. Reaching aquatic environments through sewage discharges and surface runoff, these chemicals can cause serious toxic effects on non-target organisms, such as oxidative stress, gill damage, and acute mortality [1-3].

Ecotoxicological risk assessments, determining the "Lethal Concentration 50" (LC_{50}) value of a chemical forms the basis for establishing legal regulations and safe limits. However, in the literature,

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the variations caused by the experimental systems used in toxicity tests (Static, Static-Renewal, Flow-through) on the results are often overlooked. Degradation of the chemical in static systems, manipulation stress in Static-Renewal systems, or hydrodynamic effects in flow-through systems can directly alter the obtained toxicity data[4].

Gambusia holbrooki (Mosquito fish), selected as the model organism in this study, belongs to the Poeciliidae family and is an ideal organism for biotoxicity tests due to its high tolerance range and ability to adapt to laboratory conditions [6]. The main aim of the study was to investigate the biotoxicity of sodium hypochlorite, the use of which increased during the pandemic. The aim is to determine the acute toxicity of *G. holbrooki* and to comparatively present the effect of three different experimental setups (Static, Static-Renewal, Flow-through) on the toxicity data [5].

2. MATERIALS AND METHODS

2.1. Live Materials and Chemicals

The *Gambusia holbrooki* individuals used in this study were obtained from the Güllapoğlu Pond in Edirne. This study was conducted in accordance with the ethical guidelines for vertebrate animal experimentation. All procedures were approved by the Local Ethics Committee for Aquatic Vertebrate Experimental Animals (SOÇYEK) and collection permits were obtained from the Provincial Directorate of Agriculture and Forestry (Republic of Turkey Ministry of Agriculture and Forestry). Adult individuals, weighing an average of 1 gram, were adapted in 100-liter stock aquariums in a laboratory environment for 15 days. During the adaptation and experimental period, the photoperiod was fixed at 12:12 (light/dark), and the water temperature was fixed at 20±1°C. The fish were not fed during the experiment [6].

Commercial sodium hypochlorite (NaClO, CAS No: 7681-52-9) with 5% purity was used as the test chemical. Stock solutions used in the experiments were prepared by dissolving the chemical in 1 L of distilled water to obtain a 50 ppm parent stock solution.

2.2. Experimental Systems

Test water of predetermined chemical composition was utilized for the LC_{50} assays (Table 4). Toxicity tests were performed in three different setups for 96 hours. The exposure doses employed for the LC_{50} determinations were designated based on the outcomes of preliminary screening tests. The experimental groups were established by randomly selecting fish with respect to their sex and size. Each system included a control group and 5 dose groups with increasing NaClO concentrations (0.3, 0.4, 0.5, 0.6, 0.7, 0.8, and 1.0 ppm), as determined from preliminary range-finding tests. Seven fish were used per group in each replicate (n=7), a sample size validated by post-hoc power analysis and consistent with established ecotoxicological bioassay standards [8, 9].

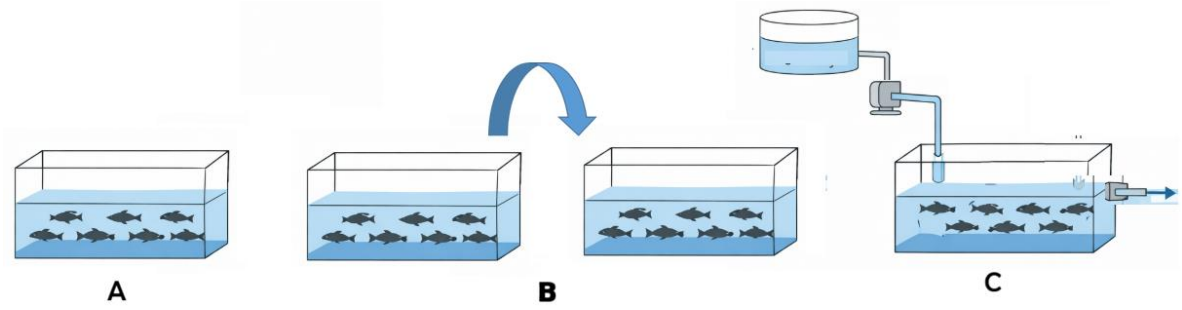

Figure 1. Different LC_{50} experimental systems used in the study (A: Static, B: Static-Renewal, C: Flow-through)

1. Static Experimental System: The experimental water and chemical were not changed for 96 hours. This system carries the risk of degradation or evaporation of the chemical over time (Figure 1A).
2. Renewal Experimental System: The experimental solution was completely renewed with fresh stock solution every 24 hours. During this process**,** fish were transferred and subjected to physical manipulation (Figure 1B).
3. Flow Experimental System: This is a dynamic system where the chemical and water continuously flow through the experimental vessels at a constant rate. Using a dosing set **,** a continuous influx of fresh solution was provided at flow rates of 50, 100, and 150 ml/hour, and metabolic wastes were removed from the environment (Figure 1C) [7].

2.3. Data Analysis

Mortality data were recorded at 24-hour intervals. LC_{50} values and 95% confidence intervals were calculated using **Probit Analysis with IBM SPSS Statistics (v.26.0)**[14]. To evaluate the relationship between the independent variables and the probability of the binary outcome, a Probit Analysis was conducted. All statistical procedures were performed using IBM SPSS Statistics (Version 26.0). The probit model was selected due to the assumption of a normally distributed error term, where the inverse standard normal cumulative distribution function (quantile function) was modeled as a linear combination of the predictors. Morphometric differences between groups (height/weight) were checked using a t-test. Post-hoc power analysis confirmed that a sample size of n=7 per group had sufficient statistical power within a 95% confidence interval [8].

3. FINDINGS

When evaluating the post-hoc power analysis performed within the framework of the G* Power approach, it is known that the required sample size decreases as the effect size (variance) between groups increases. In this study, the observation of 0% mortality at the low dose (0.3 ppm) and 100% at the high dose (1.0 ppm) indicates an extremely high effect size between the doses. Due to this significant difference, it is understood that the 7 fish used per group provide sufficient and high statistical power for calculating the LC_{50} value at a 95% confidence interval. In addition, repeating each experimental system three times (Replications 1, 2, and 3) increased the total sample size to 126, thus significantly increasing the statistical power and reproducibility of the experiment compared to a single experiment. In conclusion, the post-hoc evaluation performed for the experimental design applied in this study revealed that the sample size of n=7 groups has a statistical power above 95% at the α=0.05 significance level and considering the large differences between mortality rates; It was concluded that this design is adequate and scientifically appropriate in terms of LC_{50} calculations [9].

3.1. Comparison of LC_{50} Values

The study revealed that the toxicity of sodium hypochlorite showed notable differences depending on the experimental system. LC_{50} values across systems ranged from 0.342 to 0.804 ppm, representing approximately a 2.4-fold difference in acute toxicity estimation. In all systems, a dose-dependent increase in mortality rates was observed as the dose and exposure time increased. The non-overlapping 95% confidence intervals of the Probit-derived LC_{50} values indicate that these differences are statistically meaningful. The 96-hour LC_{50} values obtained from three different experimental systems are presented in Table 3.

Table 1. Average weight and length of fish used in the experiment.

Experimental System	Parameter	Repeat 1 (Mean ± SD)	Repeat 2 (Mean ± SD)	Repeat 3 (Mean ± SD)	General Min - Maximum Range
Static	**Length (mm)**	24.50 ± 3.15	22.82 ± 2.73	25.07 ± 3.18	18.79 – 31.99
	Weight (g)	0.18 ± 0.08	0.12 ± 0.05	0.18 ± 0.08	0.05 – 0.37
Renewable	**Length (mm)**	24.19 ± 3.11	25.50 ± 4.16	23.80 ± 2.76	18.49 – 42.03
	Weight (g)	0.14 ± 0.07	0.18 ± 0.14	0.14 ± 0.07	0.07 – 0.36
Flow	**Length (mm)**	23.81 ± 2.22	24.53 ± 3.07	26.20 ± 3.65	18.71 – 30.76
	Weight (g)	0.14 ± 0.06	0.14 ± 0.07	0.19 ± 0.08	0.06 – 0.33

Table 2. Comparison of fish length and weight among experimental systems using independent samples t-test.

Parameter	Groups Compared	t	sd (df)	p
Height (mm)	**Static – Recyclable**	-0.21	40	0.834
	Static – Flow	-0.34	40	0.735
	Renewable – Flow	0.18	40	0.858
Weight (g)	**Static – Recyclable**	0.42	40	0.676
	Static – Flow	-0.27	40	0.788
	Renewable – Flow	-0.19	40	0.850

No statistically significant difference was found in the length and weight distributions of the fish across experimental groups (independent samples t-test; all $p>0.05$), confirming that the differences in toxicity were due to the experimental system design, not biological variations.

Table 3. 96-h LC_{50} Values of Sodium Hypochlorite (NaClO) for *G. holbrooki* in Different Experimental Systems (95% Confidence Intervals)

Experimental System	1. Repeat LC_{50} (ppm)	2. Repeat LC_{50} (ppm)	3. Repeat LC_{50} (ppm)	Min - Maximum Value (ppm)
Static	0.804	1,085	0.588	0.588 – 1.085
Renewable	0.342	0.439	0.325	0.325 – 0.439
Flow	0.702	0.388	0.404	0.388 – 0.702

Note: The first replicate (0.702 ppm) in the Flow system was performed at a low flow rate of 50 ml/hour, therefore it was significantly higher than the other replicates (100 and 150 ml/hour).

According to the findings, sodium hypochlorite showed the highest toxic effect **in the Static-Renewal System (0.342 ppm)**. This was followed by the Flow System (0.404 ppm) and the Static System (0.804 ppm), respectively. The higher LC_{50} value in the static system indicates that the survival rate of the fish is higher in this system, and therefore the toxicity is measured to be lower.

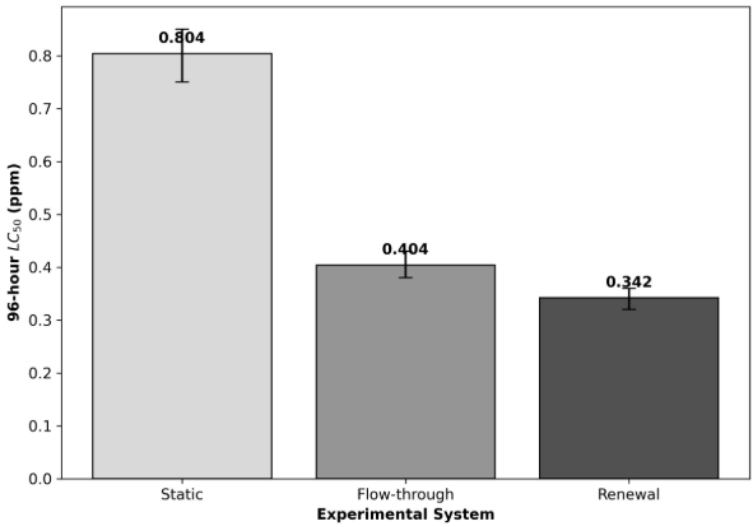

Figure 1 Comparation of NaClO 96 hours acute toxicity (LC_{50})

3.2. Physicochemical and Morphometric Data

Water temperature, pH, and electrical conductivity values measured during the experiments showed minimal variation between systems. Morphometric measurements (length and weight) of the fish showed a statistically homogeneous distribution among the groups, confirming that differences in toxicity were not due to biological variations.

Table 4 Physicochemical Parameters Measured in Different Experimental Systems (Min - Max)

Experimental System	Water Temperature (°C)	pH Value	Electrical Conductivity (EC) (µS)
Static System	21.05 – 22.6	7.04 – 7.59	176.0 – 187.6
Renewal System	22.6 – 25.3	7.20 – 7.63	170.5 – 181.1
Flow System	21.7 – 25.3	7.14 – 7.63	173.9 – 178.3

The physicochemical parameters measured during the study period (96 hours) (Temperature: 21.05–25.3 ° C, pH: 7.04–7.63, Conductivity: 170.5–187.6 µS) remained within the viable limits of the fish.

4. DISCUSSION AND CONCLUSION

The morphometric homogeneity of experimental groups (Table 2) confirms that all inter-group variability in toxicity endpoints was attributable solely to the experimental design rather than biological factors. This design control is a prerequisite for valid comparative ecotoxicology [15]. The comparable length and weight distributions across Static, Static-Renewal, and Flow-through groups validate that the observed LC_{50} differences (0.342–0.804 ppm, a 2.4-fold range) are methodologically driven. This finding is particularly significant given the growing recognition that test system selection is one of the principal sources of variability in fish acute toxicity databases, where LC_{50} values for the same compound across different test systems and species can span multiple orders of magnitude [17].

The 96-h LC_{50} value of 0.804 ppm obtained from the Static system firmly places NaClO in the “Highly Toxic” category (<1 mg/L) under standard ecotoxicological classification criteria [10–12]. This classification is consistent with findings from comparative cross-species studies: Wilde et al. [13] reported 96-h LC_{50} values of 0.20–0.58 mg/L for rainbow trout (*Oncorhynchus mykiss*) and bluegill

(*Lepomis macrochirus* = 0.58 mg/L) under chlorine exposure, values that are directly comparable to those obtained in this study. Broader cross-taxa ecotoxicological assessments further document that sodium hypochlorite is orders of magnitude more acutely toxic than common organic pollutants such as acetone (LC_{50} ~13,000 mg/L) and phenol (LC_{50} ~56 mg/L), underscoring its particular ecological significance as a reactive oxidant [11–13]. Notably, Brito et al. [17] recently documented that the 24-h LC_{50} of bleach (NaClO) was as low as 0.025 mg/L for the freshwater cladoceran *Ceriodaphnia dubia*, confirming that invertebrates are particularly susceptible and that the ecotoxicological risk of NaClO cannot be assessed using a single taxonomic endpoint.

4.1. Mechanisms Underlying Inter-System Toxicity Differences

Static-Renewal System (LC_{50} = 0.342 ppm): This system yielded the most sensitive toxicity estimate. The mechanism is twofold: (1) daily 24-h solution renewal maintains nominally undiminished chemical concentrations throughout the 96-h exposure window, preventing the degradation-related attenuation characteristic of static conditions; and (2) the repeated physical transfer of fish during renewal introduces cumulative handling stress, which physiologically compromises the organism's adaptive capacity and lowers resistance thresholds [16]. The interaction between sustained chemical exposure and handling-induced physiological stress represents a worst-case ecological scenario. This dual stressor effect parallels the concept of "multiple stressor" toxicity widely discussed in the recent ecotoxicological literature, whereby the combination of physicochemical and procedural stressors compounds organismal vulnerability beyond that predicted by either factor alone [18].

Static System (LC_{50} = 0.804 ppm): This system generated the highest (least sensitive) LC_{50} value. NaClO is a highly reactive oxidant with well-documented instability in aqueous solution: it rapidly degrades through volatilization of chlorine gas, photolytic decomposition, and reactions with dissolved organic matter and ammonia [3, 4]. In a closed static system, this progressive concentration decline effectively reduces the biologically available dose over the 96-h test period, causing an underestimation of true acute toxicity. This phenomenon is especially pronounced for chlorine-based compounds, and it has direct implications for regulatory risk assessment: static-system data may systematically underreport the hazard potential of reactive, volatile disinfectants entering surface water [19]. Parveen et al. [19] documented that even low concentrations of residual chlorine from pandemic-period disinfection events measurably altered aquatic microbial community composition, underscoring that the ecologically relevant exposure window extends well beyond single acute-test scenarios.

Flow-Through System (LC_{50} = 0.404 ppm): The intermediate toxicity value of the Flow-through system reflects the balance between two opposing forces: (1) the continuous delivery of fresh toxicant maintains a more stable actual concentration than the static system, increasing effective exposure; and (2) continuous flushing removes metabolic waste products (ammonia, CO_2), mitigating secondary stressors [7]. It is notable that the first replicate of the Flow-through system (0.702 ppm) was conducted at 50 mL/h, whereas subsequent replicates at 100 and 150 mL/h yielded 0.388 and 0.404 ppm respectively. This flow-rate dependency illustrates that within the flow-through paradigm itself, the nominal vs. actual concentration relationship and associated toxicity estimates are sensitive to hydrodynamic parameters—a consideration not addressed by OECD TG 203 [20] or ASTM E729 [7] at the level of detail required for reactive compounds.

4.2. Ecotoxicological Context: NaClO in the Post-Pandemic Aquatic Environment

The ecotoxicological significance of this study is amplified by the extraordinary increase in NaClO use precipitated by the COVID-19 pandemic. Xiao et al. [18] documented that residual chlorine concentrations in Chinese surface waters reached up to 0.4 mg/L during the peak pandemic disinfection period of February–March 2020—a concentration that, based on our Static-Renewal LC_{50} result of 0.342 ppm, corresponds to approximately 117% of the acute lethal threshold for *G. holbrooki*. This comparison

illustrates the real-world relevance of our data: pandemic-era disinfection practices could generate surface water concentrations that are acutely lethal to fish even when evaluated against the most conservative (Static) test estimate. A parallel concern is raised by Narayanan et al. [21], whose assessment of eco-toxic effects of NaClO on zebrafish (*Danio rerio* swimming behavior demonstrated behavioral disruption at sublethal NaClO concentrations, highlighting that sub-LC_{50} exposures carry significant ecotoxicological risk, and that mortality-based LC_{50} values alone underestimate the full scope of ecological impact. At the molecular level, Wang et al. [22] demonstrated that NaClO exposure at 300 μg/L significantly disrupts lipid metabolism and global gene transcription in larval zebrafish, activating pathways related to oxidative stress, energy metabolism, and glycometabolism. These findings indicate that NaClO toxicity operates through a multi-target mechanism, with sub-acute exposures causing cryptic physiological impairment. Collectively, these data reinforce that the LC_{50} values obtained in this study should be considered conservative estimates of the true ecological risk that NaClO poses to freshwater communities.

4.3. Implications for Ecotoxicological Test Standardization and Regulatory Risk Assessment

The present findings carry direct implications for regulatory ecotoxicology and the harmonization of test protocols. Current internationally recognized guidelines, including OECD Test Guideline 203 [20] and ASTM E729 [7], permit the use of static, semi-static, or flow-through conditions for acute fish toxicity testing without mandating a specific system for reactive or volatile compounds. Our data demonstrate that this permissiveness introduces a systematic bias: static systems will consistently underestimate the acute toxicity of chemically unstable agents such as NaClO. As a consequence, environmental quality standards and predicted no-effect concentrations (PNECs) derived from static-test data may provide inadequate protection margins for freshwater ecosystems exposed to chlorine-based disinfectants. We therefore recommend that, for compounds with known hydrolytic or oxidative instability, toxicity classification and regulatory limit-setting should prioritize data from Static-Renewal or Flow-through systems, which preserve exposure integrity more reliably. This recommendation aligns with the broader argument advanced by Rand et al. [4] that experimental system selection is a first-order determinant of toxicological outcome, and with the recent call by Brito et al. [17] for ecotoxicological risk frameworks to adopt species-diverse, multi-system test batteries for pandemic-related disinfectants.

5. CONCLUSIONS

The principal conclusions of this study are as follows: (1) Sodium hypochlorite (NaClO) is definitively classified as a “Highly Toxic” acute pollutant to *Gambusia holbrooki* across all three experimental systems (Static: 0.804 ppm; Flow-through: 0.404 ppm; Static-Renewal: 0.342 ppm). (2) The choice of experimental system exerts a statistically meaningful and reproducible effect on LC_{50} estimation for reactive compounds, with the Static system underestimating toxicity by up to 2.4-fold relative to the Static-Renewal system. (3) Static test systems carry an inherent risk of masking true toxicity for volatile, reactive compounds such as NaClO, potentially generating non-protective regulatory limits. (4) In ecological risk assessments and the derivation of environmental quality standards, data from Static-Renewal or Flow-through systems should be prioritized for reactive disinfectants. (5) The acute LC_{50} values reported here must be interpreted as lower bounds of ecological risk, given that sublethal NaClO concentrations produce behavioral, metabolic, and molecular impairments at concentrations below the acute lethality threshold [21, 22]. Future studies should investigate chronic endpoints (NOEC, LOEC), sublethal biomarker responses (oxidative stress indices, histopathology), and multi-species community-level effects of NaClO to provide a comprehensive ecotoxicological risk profile for this globally significant disinfectant.

CONFLICT OF INTEREST

The authors stated that there are no conflicts of interest regarding the publication of this article.

CRediT AUTHOR STATEMENT

Utku Güner: Conceptualization, Data curation, Formal analysis, Writing – original draft. **Kamile Kökver:** Methodology, Visualization, Validation, Writing – review & editing.

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ESKİŞEHİR TEKNİK ÜNİVERSİTESİ BİLİM VE TEKNOLOJİ DERGİSİ
C- YAŞAM BİLİMLERİ VE BİYOTEKNOLOJİ

Eskişehir Technical University Journal of Science and Technology
C -Life Sciences and Biotechnology

Estuscience – Life, 2026, 15 (2) pp. 130-146, DOI: 10.18036/estubtdc.1892371

RESEARCH ARTICLE

In vitro/in silico ASSESSMENT OF *Symphytum officinale* L. EXTRACT ON A549 CELLS; NEUROKININ-1 RECEPTOR-MEDIATED INTERACTIONS OF MALAXINIC ACID AND GLOBOIDNAN A

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Abstract

Lung cancer is still one of the main causes of cancer-related mortality worldwide. Angiogenesis plays a vital role in tumor progression and metastasis. Natural products have acted as valuable resources in drug discovery due to their diverse biological activities and rich content of bioactive secondary metabolites. *Symphytum officinale* L. is a medicinal plant known for its high phenolic content and has attracted research interest for various biological properties. However, its angiogenesis-related effects have not yet been clearly characterized. In this study, the phenolic content and antioxidant capacity of *S. officinale* extracts were determined, and their effects on A549 lung cancer cell proliferation were evaluated using the MTT assay. The levels of substance P, vascular endothelial growth factor, and interleukin-6 released from A549 lung carcinoma cells were quantified by ELISA. In addition, molecular docking analyses were performed to investigate the interactions of malaxinic acid and globoidnan A with receptors associated with substance P, vascular endothelial growth factor, and interleukin-6 signaling pathways. The *S. officinale* extract significantly reduced the viability and proliferation of A549 lung cancer cells. ELISA assays revealed a significant reduction ($p<0.05$) in vascular endothelial growth factor and interleukin-6 levels at early time points, whereas substance P levels did not show a consistent or statistically significant change. Molecular docking analyses suggested favorable binding interactions of malaxinic acid and globoidnan A with NK-1, NRP-1, Tie-2, and VEGFR-1 receptors. Taken together, these findings indicate that *S. officinale* extract reduces A549 cell viability and may exert transient anti-angiogenic effects on A549 cells, while its phenolic constituents exhibit strong predicted binding affinities to receptors involved in inflammatory and angiogenic signaling, particularly the NK-1 receptor.

Keywords

Symphytum officinale,
Malaxinic acid,
Globoidnan A,
Substance P,
VEGF

Time Scale of Article

Received : 18 February 2026
Accepted : 15 June 2026
Online date : 27 July 2026

1. INTRODUCTION

Natural products play an important role in drug innovation due to their broad spectrum of biological activities and the presence of bioactive secondary metabolites [1]. The genus *Symphytum* (Boraginaceae), widely distributed across Western Asia, Europe, and North America, comprises several species in Turkey. Among these, *Symphytum officinale* L. (comfrey) is one of the most widely used medicinal plants [2, 3]. Preparations derived from the aerial parts (*Symphyti herba*), leaves (*Symphyti folium*), and roots (*Symphyti radix*) have traditionally been used both externally, such as in ointments and compresses, and internally in the form of infusions and tinctures. These preparations have been used in the treatment of various conditions, including inflammatory conditions, tonsillitis, and respiratory disorders [4-6].

Symphytum genus are known to contain diverse secondary metabolites, including naphthoquinones, flavonoids, terpenoids, and phenolic derivatives. Phytochemical investigations of this genus have led to the isolation and characterization of numerous compounds, such as phenolic acids, flavonoids, aliphatic ketones, naphthazarins, and naphthoquinones. Among these, caffeic acid, globoidnan A, globoidnan B, rabdosiin, malaxinic acid, and rosmarinic acid have been reported as major phenolic constituents of *S. officinale*. In addition, Boraginaceae family are known to contain pyrrolizidine alkaloids, which should be considered in terms of safety [6-8]. These phytochemical constituents have been reported to exhibit antioxidant, antitumor, and anti-inflammatory activities [9].

Lung cancer accounts for a significant proportion of malignant tumors worldwide. Non-small cell lung cancer (NSCLC), which represents approximately 85–90% of all cases, is associated with high morbidity and mortality [10]. Angiogenesis plays a central role in lung cancer progression by promoting tumor growth, invasion, metastasis, and modulation of the tumor microenvironment. The increasing development of resistance to conventional chemotherapy has highlighted the need for alternative therapeutic strategies, including approaches targeting angiogenesis. Among angiogenic mediators, the vascular endothelial growth factor (VEGF) family represents the primary target of anti-angiogenic therapies. In addition, interleukin-6 (IL-6), a pro-inflammatory cytokine that interacts with VEGF and other signaling molecules, has been shown to enhance angiogenic responses [11]. Substance P (SP), a neuropeptide involved in

inflammatory signaling, has also been reported to promote angiogenesis and to increase tumor cell invasion and metastasis within the lung tumor microenvironment [12]. Although studies have reported various biological effects of *S. officinale* extracts and their bioactive constituents, the effects of this plant extract and its phenolic components on angiogenesis-related factors released from cancer cells, as well as their potential interactions with angiogenic receptor proteins, remain largely unexplored.

The total phenolic content and antioxidant capacity of *S. officinale* were determined at the Food Safety and Agricultural Research Center of Akdeniz University. The effects of *S. officinale* extract on the proliferation of A549 lung cancer cells were evaluated. To explore the potential involvement of inflammatory and angiogenic pathways, the levels of SP, VEGF, and IL-6 were evaluated following treatment. In addition, molecular docking analyses were performed to investigate the potential interactions of malaxinic acid and globoidnan A, phenolic constituents of *S. officinale*, with angiogenesis-related receptors, including neurokinin-1 (NK-1), vascular endothelial growth factor receptor 1 (VEGFR-1), neuropilin-1 (NRP1), and Tie2.

2. MATERIALS AND METHODS

2.1. Plant Material and Extraction

Plant extract (10 g/25 ml water) was given as a kind gift from Kütahya Dumlupınar University, Hekim Sinan Medical and Aromatic Plants Research Center. In this study, 10 g of the plant's leaves were extracted using 25 mL of water.

2.2. Determination of Total Phenolic and Total Antioxidant Capacity of Plant Extract

The total phenolic and total antioxidant capacity of plant extract was determined by Akdeniz University, Food Safety and Agricultural Research Center with a report number 2023-355.

2.3. Cell Culture Conditions

A549 cells (ATCC®CCL-185 ™) were cultured in DMEM (Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS), 10 µg/mL gentamicin, and 5% sodium pyruvate. The cells were incubated under 5% CO_2 with 95% humidity at 37°C.

2.4. EC_{50} Analysis, and Cell Viability

The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was used to evaluate cell viability following treatment with the plant extract. The cells were seeded into 96-well plates at a density of 5×10^3 cells per well in 200 µL of culture medium and allowed to attach overnight. A stock solution of the plant extract (100 mg/mL) was serially diluted to obtain final concentrations of 1.87, 3.75, 12.5, 25, 50 and 100 µg/mL. Cells were treated with the extract and incubated for 24, 48, or 72 hours at 37°C in a humidified atmosphere containing 5% CO_2.

At the end of each incubation period, the culture medium was removed, and 90 µL of serum-free medium together with 10 µL of MTT reagent was added to each well. Following a 4-hour incubation, the MTT-containing medium was carefully removed, and 100 µL of dimethyl sulfoxide (DMSO) was added to each well to dissolve the formazan crystals. The plates were gently shaken for 10 minutes in the dark, and absorbance was measured at 570 nm using a microplate reader.

Cell viability was calculated as a percentage relative to untreated control cells (0 mg/mL) according to the formula:

$$Viability\ \% = \frac{Absorbance\ of\ treated\ cells}{Absorbance\ of\ control\ cells} \times 100$$

2.5. ELISA Assays for SP, VEGF, and IL-6

Cells were seeded at a density of 1×10^6 cells per well in 2 mL of complete culture medium in 6-well plates. After overnight attachment, cells were treated with *S. officinale* extract at two different concentrations (1 and 2 mg/mL). The levels of SP, VEGF, and IL-6 released into the culture supernatant were quantified using enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions (Invitrogen Corp., Camarillo, CA, USA) after 24 and 48 hours of treatment. Recombinant human SP, VEGF, and IL-6 standards provided with the kits were serially diluted to generate standard curves. Results obtained from treated samples were compared with those of untreated control cells. All experiments were performed in four independent replicates, and data are presented as mean ± standard error (SE).

2.6. *In Silico* Studies

2.6.1. Protein structure preparation

X-ray crystal structures were obtained from the Protein Data Bank (PDB). PDB file names are as follows: Human neurokinin-1 receptor, PDB ID: 6e59; Human vascular endothelial growth factor receptor-1, PDB ID: 1vr2; human Neuropilin-1 receptor, PDB ID: 1kex; and tyrosine kinase receptor, PDB ID: 1fvr. BIOVIA Discovery Studio was used for Protein Preparation.

2.6.2. Ligand structure preparation

3-D Structures of Malaxinic acid and Globoidnan A were obtained from PubChem. PubChem IDs of Malaxinic acid and Globoidnan A were 162641407 and 135263380 respectively. Conjugate Gradients and OpenBabel software in the PyRx program were used for energy minimization as an MMFF94 force field and optimization algorithm [13].

2.6.3. Molecular docking studies

CB-Dock-2 uses efficient quasi-Newton and Broyden-Fletcher-Goldfarb-Shanno methods for local optimization. Ligands were docked to the binding site cavity by the automated system in the program. Docking calculations were performed with an exhaustiveness option of 9 (average accuracy). The Vina gives docking scores as predicted inhibition constants (Ki), which is derived from Gibbs free energy of binding calculated by the following equations:

$G = -(\ln Ki)$ (1)

$Ki = e^{-\Delta GRT}$

where R (gas constant) is 1.98 cal/mol K, and T is 98.15 K. The BIOVIA Discovery Studio program was used to visualize the results.

2.7. Statistical Analysis

Cell viability was assessed based on the absorbance values obtained after the cells were treated with the drug. These values were normalized to the control group (0 μg/mL), and the percentage of viable cells was calculated. Bar graphs showed the were used to present the reduction in A549 cell viability, and the data were presented as the mean ± SD. Statistical differences between groups were evaluated using one-way ANOVA and the Dunnett's multiple comparison test with GraphPad Prism 8.0.2 software (GraphPad Software, San Diego, USA).

The half-maximal effective concentration (EC_{50}) of the extract and the EC_{50} values for 24, 48, and 72 hours were determined by plotting dose-response curves using non-linear regression. p-value of < 0.05 was considered statistically significant.

3. RESULTS

3.1. *In vitro* Studies

3.1.1. EC_{50} analysis

As shown in Figure 1, cells treated with *S. officinale* extract at concentrations differing from 1.87 to 100 µg/mL exhibited a decrease in viability compared with the control group. However, at 24 and 48 h, the response didn't reach the threshold required to reliably calculate an EC_{50} value. In contrast, after 72 h of exposure, a biologically meaningful EC_{50} of approximately 40 µg/mL was observed ($p < 0.05$), suggesting a response that varies as a function of both exposure time and concentration.

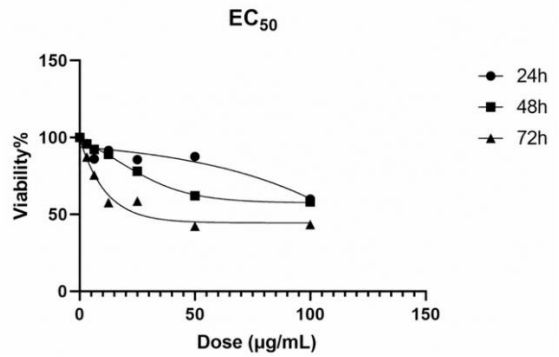

Figure 1. Dose-response curves and EC_{50} values for plant extract treatment at 24, 48, and 72 hours.

3.1.2. Cell viability

The effect of *S. officinale* plant extract (1.87-100 µg/mL) on the viability of A549 human lung carcinoma cells was assessed over 24, 48, and 72 h using the MTT assay, which measures mitochondrial activity as an indicator of metabolic function and cell survival. Cell viability was expressed as a percentage relative to untreated control cells. A dose-dependent decrease in cell viability was observed at higher concentrations across all incubation periods. At 24 h, *S. officinale* treatment demonstrated a concentration-dependent reduction in viability, with a statistically significant effect detected only at the highest dose (100 µg/mL), where viability declined to $59.88 \pm 4.53\%$ relative to control ($p < 0.001$). No significant change was observed at 1.87 µg/mL ($p = 0.1068$). After 48 h, statistically significant decreases in cell viability were observed at concentrations ≥12.5 µg/mL, with the strongest effect at 100 µg/mL, where viability decreased to $57.97 \pm 4.18\%$ ($p < 0.0001$). Similarly, at 72 h, *S. officinale* treatment was associated with a concentration-dependent reduction in A549 cell viability, with significant decreases at ≥12.5 µg/mL. The maximal effect was observed at 100 µg/mL, where viability declined to $41.27 \pm 5.88\%$ relative to control ($p < 0.0001$). Together, these data suggest that *S. officinale* progressively impairs A549 cell viability in a manner dependent on both exposure duration and concentration.

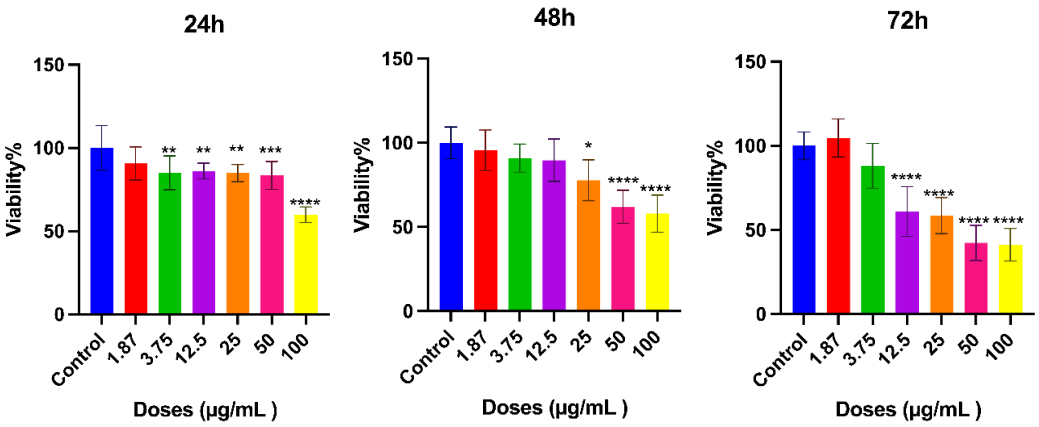

Figure 2. Concentration- and time-dependent effects of *S. officinale* extract treatment on A549 human lung carcinoma cell viability. The results are presented as the percentage change in viability relative to the control group and expressed as mean ± standard deviation (SD).

3.2. The Amount of SP Released from A549 Cells

The levels of SP released from A549 human lung carcinoma cells following treatment with 1 mg/mL and 2 mg/mL extract concentration doses of *S. officinale* extract were quantified at 24- and 48-hours using ELISA method. Untreated wells served as negative controls. After 24 hours, a slight increase in SP levels was observed in both treatment groups compared to the control group. Conversely, at the end of the 48-hour incubation period, a minimal decrease in SP levels was observed. Overall, there was no significant increase or decrease (Figure 3).

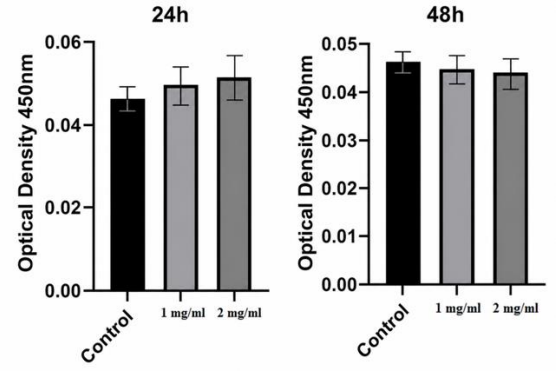

Figure 3. Quantification of SP levels released from A549 human lung carcinoma cells following treatment with *S. officinale* extract at 24 and 48 hours respectively. The results represent the mean standard deviation (SD).

3.3. The Amount of VEGF Released from A549 Cells

To evaluate the effect of *S. officinale* extract on angiogenic signaling, VEGF levels were measured in A549 lung carcinoma cell culture supernatants after 24 and 48 hours of treatment with ELISA methods. Two extract concentrations 1 mg/mL and 2 mg/mL were applied, and untreated wells served as controls. After 24 hours, both extract concentrations had dose-dependent decreased VEGF levels, but the 2 mg/ml concentration was found to be statistically significant compared to the control group. In contrast, after 48 hours of treatment, VEGF levels rebounded, showing no significant difference from control values. This may indicates that the anti-angiogenic effect of *S. officinale* extract is likely short-term and not sustained over extended exposure periods.

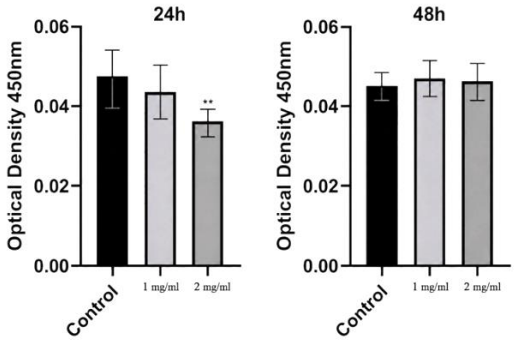

Figure 4. Quantification of VEGF levels released from A549 human lung carcinoma cells following treatment with *S. officinale* extract at 24 and 48 hours respectively. The results represent the mean ± standard deviation (SD).

3.4. The Amount of IL-6 Released from A549 Cells

The impact of *S. officinale* extract on IL-6 secretion by A549 human lung carcinoma cells was assessed after 24 and 48 hours of treatment. Cells were exposed to concentrations of 1 mg/mL and 2 mg/mL of the extract, and untreated cells served as controls. After 24 hours of treatment, no significant change in IL-6 levels was observed in either of the extract-treated groups compared to controls. However, at 48 hours, a time- and dose-dependent modulation of IL-6 secretion became apparent. Specifically, IL-6 levels were decreased at the 1 mg/mL dose relative to control, while a notable increase was observed at the 2 mg/mL dose. These findings suggest that *S. officinale* extract may exert a biphasic immunomodulatory effect on IL-6 release in A549 cells over prolonged exposure periods.

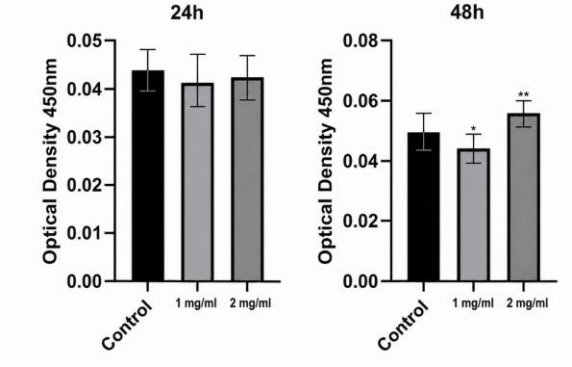

Figure 5. Quantification of IL-6 levels released from A549 human lung carcinoma cells following treatment with *S. officinale* extract at 24 and 48 hours respectively. The results represent the mean ± standard deviation (SD).

3.5. The Total Phenolic and Total Antioxidant Capacity of Plant Extract

The total phenolic and total antioxidant capacity of plant extract was determined as 84.57 mg Gallic acid equivalent /100 mL and 105.97 mg Trolox acid equivalent /100 mL, respectively.

3.6. *In silico* Results

Molecular docking simulations were performed to investigate the interactions between two phenolic compounds isolated from *S. officinale*, malaxinic acid and globoidnan A, and key receptor proteins involved in inflammation and angiogenesis. A clearer comparison of the *in silico* molecular docking data was achieved by presenting the binding profiles of malaxinic acid and Globoidnan A in two separate tables (Table 1 and Table 2). Each table presents the interacting target receptors, PDB IDs, number of

amino acid residues involved, key binding residues, and binding affinity. This structured presentation supports a better interpretation of the compounds predicted interactions with receptors implicated in angiogenic and inflammatory pathways.

Table 1. Molecular Docking Results: Malaxinic Acid

Ligand	Target Receptor	PDB ID	Number of Interacting Residues	Key Residues	Binding Affinity (kcal/mol)
Malaxinic acid	NK-1	6e59	19	THR86, PHE90, HIS108, ASN109, ILE113, TRP261	-8.5
	NRP-1	1kex	19	VAL90, SER91, ASP96, THR99, ARG130	-6.3
	Tie2	1fvr	17	LYS1059, CYS1063, ASP1065, TYR1102, PHE1111	-7.3
	VEGFR-1	1vr2	18	LEU840, VAL848, GLU917, LYS920, ASN923, ASP1046	-6.9

Malaxinic acid exhibited strong binding affinities with several targets. Among the four receptors, malaxinic acid showed the highest binding affinity to the NK-1 receptor.

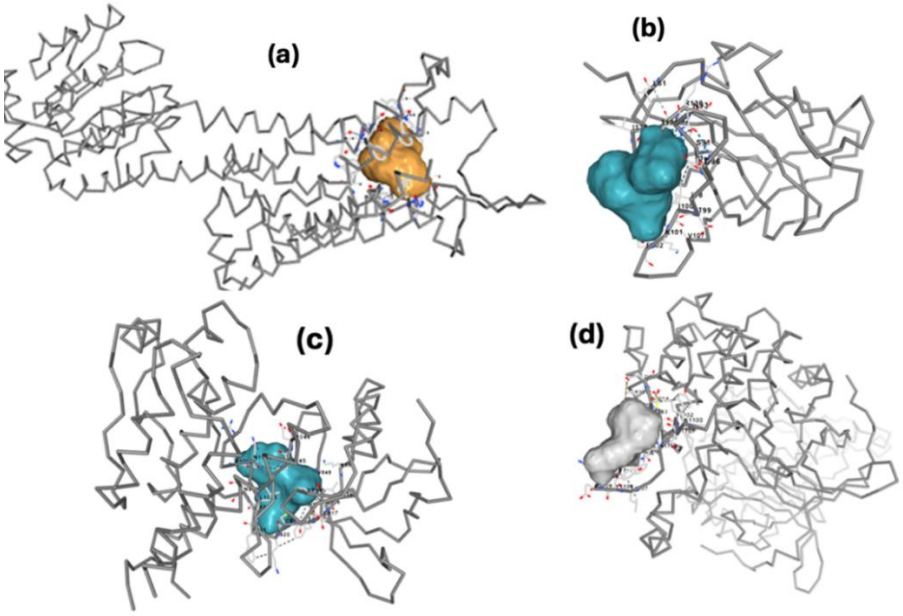

Figure 6. (a) Molecular docking interactions between malaxinic acid and the NK-1 (PDB ID: 6e59). **(b)** Molecular docking interactions between malaxinic acid and the human NRP-1 (PDB ID: 1kex). **(c)** Molecular docking interactions between malaxinic acid and Tie2 (PDB ID: 1fvr). **(d)** Molecular docking interactions between malaxinic acid and the VEGFR-1 (PDB ID: 1vr2).

SP inhibits cancer cells through the NK-1 receptor. Figure 6 illustrates the predicted binding interactions between malaxinic acid, a phenolic compound derived from *S. officinale*, and the active site of NK-1 receptor using molecular docking simulations. Malaxinic acid interacted with 19 active site amino acids of NK-1 namely THR86, PHE90, HIS108, ASN109, PRO112, ILE113, VAL116, GLN165, TYR196, HIS197, ILE198, VAL200, THR201, ILE204, TRP261, PHE264, HIS265, PHE268 and LEU269. These interactions suggest multiple binding modes: hydrogen bonding likely occurs with polar residues like THR86, HIS108, and ASN109; aromatic π–π stacking interactions are plausible between the aromatic rings of malaxinic acid and aromatic residues such as PHE90 and TRP261; and hydrophobic interactions may be established with non-polar residues such as ILE113. Consequently, the *in silico* binding energies point to a potentially stable interaction with the NK-1 receptor, which may underlie the compound's capacity to influence receptor-mediated signaling.

VEGF promotes tumor angiogenesis through its interaction with NRP-1, a co-receptor involved in angiogenic signaling. Therefore, NRP-1 has become a promising target in cancer therapy research. Figure 7 illustrates the binding interactions between malaxinic acid, a phenolic compound derived from *S. officinale*, and the NRP-1 receptor. Malaxinic acid interacted with 19 active site amino acids of NRP-1, namely LEU61, VAL90, SER91, SER92, ASN93, ASP96, ILE98, THR99, ILE100, LYS101, GLU102, VAL107, PRO124, LYS125, PRO126, LEU127, ILE128, THR129 and ARG130. Hydrogen bonding is likely formed with polar residues such as SER91, ASP96, and THR99, stabilizing the complex via polar contacts. Electrostatic interactions may also occur between the negatively charged groups of the ligand and the positively charged ARG130 side chain. Additionally, hydrophobic contacts with nonpolar residues like VAL90 may further enhance binding affinity. Accordingly, the favorable *in silico* binding profile of malaxinic acid with NRP-1 suggests that it may have the potential to modulate angiogenic signaling pathways associated with cancer progression.

The predicted binding interactions between malaxinic acid and Tie2, a key receptor involved in angiogenesis and vascular homeostasis. Malaxinic acid interacted with 17 active site amino acids of Tie-2 namely LYS1059, PRO1060, LEU1061, ASN1062, CYS1063, ASP1064, ASP1065, LYS1100, THR1101, TYR1102, ASN1104, LEU1107, TYR1108, GLU1109, LYS1110, PHE1111 and THR1112. These findings suggest several potential binding modes. Hydrogen bonds are likely formed with polar or charged residues such as ASP1065 and CYS1063, contributing to the specificity and orientation of the ligand. The presence of LYS1059, a positively charged residue, indicates possible electrostatic interactions with acidic groups of malaxinic acid. Furthermore, aromatic interactions such as π–π stacking with TYR1102 and PHE1111 may enhance binding affinity and stability. Overall, these interactions indicate a favorable binding profile, suggesting that malaxinic acid may influence angiogenesis through modulation of Tie2 receptor activity.

Figure 6d presents the molecular docking results illustrating the interaction between malaxinic acid and VEGFR-1, a key receptor involved in the regulation of angiogenesis. Malaxinic acid interacted with 18 active site amino acids of VEGF-1 namely LEU840, VAL848, ALA866, LYS868, VAL899, VAL916, GLU917, PHE918, CYS919, LYS920, PHE921, GLY922, ASN923, ARG1032, ASN1033, LEU1035, VYS1045 and ASP1046. These interactions suggest a combination of binding modes. Hydrogen bonding may occur through polar and charged residues such as GLU917, ASN923, and ASP1046, which helps stabilize the ligand in the binding pocket. Electrostatic interactions are likely between the negatively charged groups of malaxinic acid and the positively charged LYS920. Additionally, hydrophobic interactions with nonpolar residues such as LEU840 and VAL848 further contribute to the stability of the ligand–receptor complex. Collectively, these diverse molecular interactions highlight a favorable binding affinity, suggesting that malaxinic acid may inhibit VEGFR-1 signaling and thereby exert anti-angiogenic effects.

Globoidnan A also demonstrated notable receptor binding properties. Among the four receptors, globoidnan A has shown the highest affinity to the NK-1 receptor, like malaxinic acid.

Table 2. Molecular Docking Results: Globoidnan A

Ligand	Target Receptor	PDB ID	Number of Interacting Residues	Key Residues	Binding Affinity (cal/mol)
Globoidnan A	NK-1	6e59	21	PHE90, ASN109, VAL116, TYR196, HIS197, TRP261, PHE264, TYR272	-9.7
	NRP-1	1kex	18	VAL90, SER92, ASP96, ILE98, LYS101, GLU102, AERG130	-7.8
	Tie2	1fvr	16	LYS1059, ASP1064, TYR1102, GLU1109, PHE1111	-7.2
	VEGFR-1	1vr2	21	PRO839, LEU840, VAL848, GLU917, PHE918, ASN923, ASP1046, PHE1047	-8.4

Globoidnan A formed strong interactions with 21 residues at the NK-1 receptor, implying a high potential to modulate inflammation and tumor progression (Figure 7a). For the NRP-1 receptor, globoidnan A formed stable interactions with 18 residues, albeit suggesting a slightly weaker modulation compared to malaxinic acid (Figure 7b). Docking with the Tie2 receptor showed 16 key interactions, indicating the capacity to influence vascular pathways (Figure 7c). Lastly, globoidnan A exhibited strong and stable binding to VEGFR-1, interacting with 21 amino acid residues, reinforcing its potential to inhibit angiogenesis (Figure 7d).

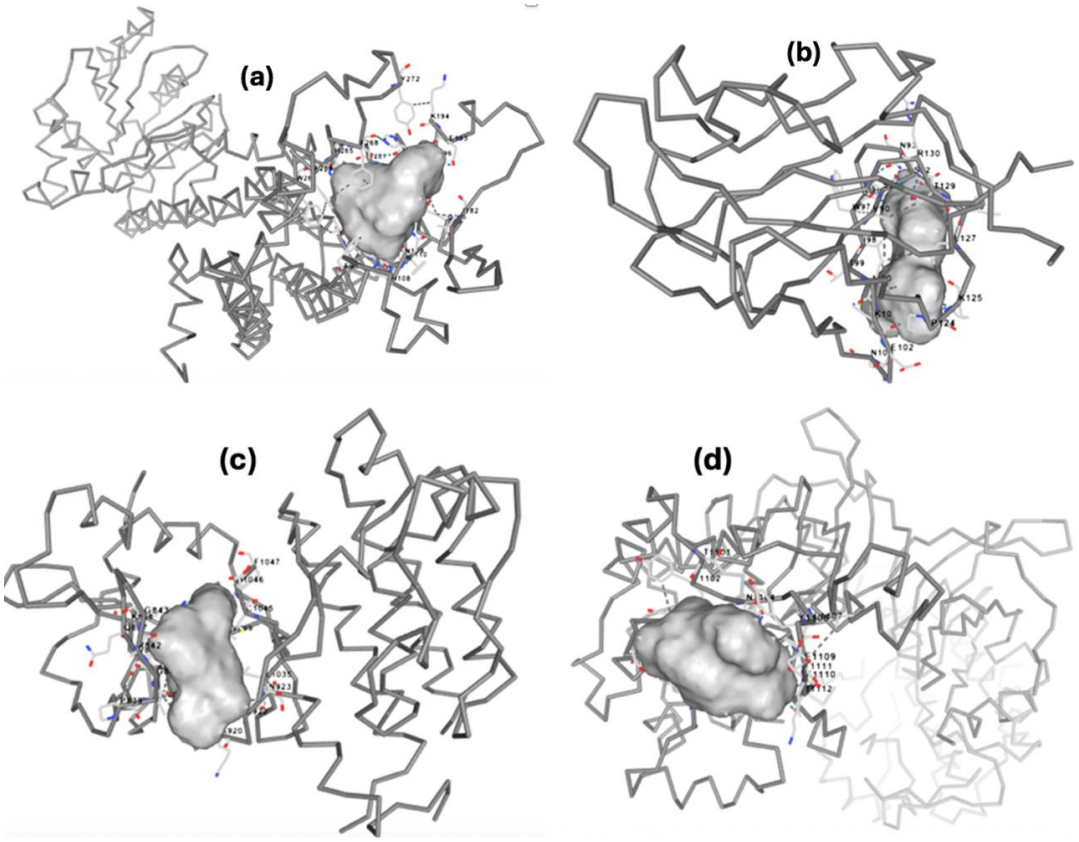

Figure 7. (a) Molecular docking interactions between globoidnan A and the NK-1 (PDB ID: 6e59). **(b)** Molecular docking interactions between globoidnan A and the NRP-1 (PDB ID: 1kex). **(c)** Molecular docking interactions between globoidnan A and the Tie2 (PDB ID: 1fvr). **(d)** Molecular docking interactions between globoidnan A and the VEGFR-1 (PDB ID: 1vr2).

Figure 7a illustrates the molecular docking results, showing the interaction of globoidnan with the NK-1 receptor, a G-protein-coupled receptor involved in inflammation, pain signaling, and cancer cell proliferation. Globoidnan A interacted with 21 active site amino acids of NK-1 namely PHE90, HIS108, ASN109, PHE110, PRO112, ILE113, VAL116, GLN165, ILE182, GLU193, LYS194, TYR196, HIS197, VAL200, THR201, ILE204, TRP261, PHE264, HIS265, PHE268 and TYR272. The binding interaction of the globoidnan A with the NK-1 is shown in Fig. 10. The binding profile suggests multiple interaction types contributing to a high binding affinity. Hydrogen bonds may be formed with polar residues such as ASN109 and HIS197, aiding in ligand stabilization. Aromatic residues including PHE90, TYR196, TRP261, PHE264, and TYR272 are likely to engage in π–π stacking interactions with the aromatic rings of globoidnan A. Additionally, hydrophobic interactions involving residues like VAL116 may further reinforce the binding. The predicted binding interactions suggest that globoidnan A may engage the NK-1 receptor and could potentially influence neuropeptide-mediated pathways involved in inflammation and tumor progression.

Figure 7b presents the molecular docking results of globoidnan A with the NRP-1 receptor, a co-receptor known to enhance VEGF signaling and facilitate tumor angiogenesis. Molecular docking analysis showed that globoidnan A interacts with 18 amino acid residues within the binding pocket of the NRP-1 receptor (PDB ID: 1kex). Key interacting residues include VAL90, SER92, ASP96, ILE98, LYS101, GLU102, and ARG130. These interactions suggest a combination of binding modes. Hydrogen bonding is likely between globoidnan A and polar residues such as SER92 and ASP96, while electrostatic interactions may occur between negatively charged functional groups of the ligand and the positively charged side chains of LYS101 and ARG130. Additionally, hydrophobic interactions with residues like

VAL90 and ILE98 could further stabilize the ligand within the receptor cavity. Although these interactions indicate a comparatively moderate binding affinity relative to NK-1, they suggest that the compound may modulate VEGF-related signaling via NRP-1

Figure 7c demonstrates the predicted binding interactions between globoidnan A and the Tie2 receptor, a tyrosine kinase involved in vascular development, endothelial cell survival, and angiogenesis. Molecular docking simulations revealed that globoidnan A interacts with 16 amino acid residues within the binding site of the Tie2 receptor (PDB ID: 1fvr), namely LYS1059, PRO1060, LEU1061, ASN1062, CYS1063, ASP1064, ASP1065, THR1101, TYR1102, ASN1104, LEU1107, TYR1108, GLU1109, LYS1110, PHE1111 and THR1112. These interactions suggest multiple binding modes. Hydrogen bonds may be formed with polar or charged residues such as ASP1064 and GLU1109, facilitating stable ligand orientation. The presence of LYS1059, a basic residue, suggests potential electrostatic interactions with acidic groups on the ligand. Additionally, aromatic interactions like π–π stacking between the aromatic rings of globoidnan A and residues such as TYR1102 and PHE1111 may enhance binding affinity. These combined binding modes support the hypothesis that globoidnan A may influence Tie2-mediated angiogenic signaling pathways relevant to tumor biology and inflammation.

Figure 7d presents the predicted molecular interactions between globoidnan A and VEGFR-1, a receptor tyrosine kinase involved in mediating angiogenesis and vascular permeability in cancer progression. Molecular docking analysis revealed that globoidnan A forms interactions with 21 amino acid residues within the binding site of the VEGFR-1 namely PRO839, LEU840, GLY841, ARG842, GLY843, GLN847, VAL848, ALA866, LYS868, VAL899, VAL916, GLU917, PHE918, CYS919, LYS920, GLY922, ASN923, LEU1035, CYS1045, ASP1046 and PHE1047. These residues suggest the presence of diverse binding interactions. Hydrogen bonds may occur with polar residues such as ASN923 and ASP1046, helping to anchor the ligand within the binding cavity. Electrostatic interactions are possible between the negatively charged GLU917 and acidic ligand moieties, while aromatic stacking may occur with residues like PHE918 and PHE1047, enhancing binding strength. Additionally, hydrophobic contacts with PRO839, LEU840, and VAL848 contribute to the overall stability of the complex. Collectively, these interactions support the hypothesis that globoidnan A has a high binding affinity for VEGFR-1 and may effectively modulate VEGF-mediated angiogenic signaling in tumor biology.

These molecular docking results suggest that both malaxinic acid and globoidnan A possess significant binding affinities toward multiple cancer- and inflammation-related targets, supporting the experimental observations of anti-inflammatory and anti-angiogenic effects of *S. officinale* extract (Figure 8).

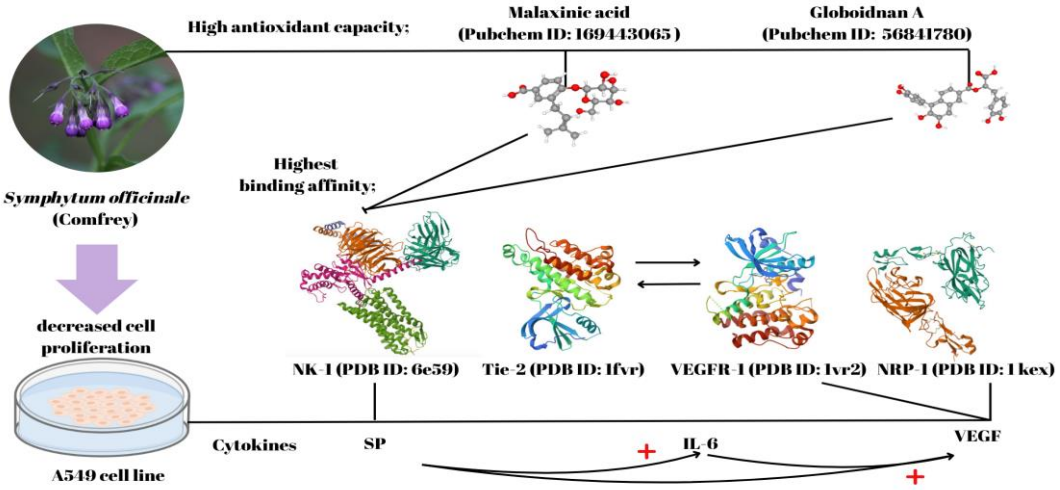

Figure 8. Illustration of the proposed mechanism by which *S. officinale* compounds (malaxinic acid and globoindnan A) may reduce A549 cell proliferation. These compounds show strong binding to NK-1, Tie-2, VEGFR-1, and NRP-1, key receptors in the SP–IL-6–VEGF inflammatory and angiogenic axis.

4. DISCUSSION

Medicinal plants have gained attention as potential sources of anticancer agents due to their bioactive compounds, which often exhibit selective antiproliferative activity against tumor cells while sparing normal tissues. Among them, *S. officinale* is a traditional medicinal plant historically used for inflammation reduction. Recent studies have expanded its known properties, highlighting its significant wound-healing, antioxidant, anti-inflammatory, and cytostatic activities [14,15]. Phytochemical analyses of *S. officinale* extracts have identified high concentrations of phenolic compounds, such as rosmarinic acid, caffeic acid, and chlorogenic acid, which contribute to its strong biological activities [16]. Concentrated extracts have demonstrated notable cytostatic effects against HeLa cancer cell line [17]. In addition, *S. officinale* has been reported to exert anticancer effects by inhibiting growth factor–related signaling pathways, including IGF-II, in hepatocellular carcinoma models [18]. Taken together, its rich phytochemical composition and promising biological activities position *S. officinale* as an attractive candidate for further exploration in cancer therapy.

Building on this evidence, the present study evaluated the *in vitro* and *in silico* effect of *S. officinale* on A549 human lung carcinoma cells. For the first time, the potential modulation of inflammation- and angiogenesis-related markers, including SP, VEGF, and IL-6, was examined at both the cellular and molecular levels. The present study demonstrates that *S. officinale* extract induces a time-dependent reduction in A549 cell viability, as reflected by the progressive shift in EC_{50} values over time. These findings are consistent with previous reports showing a significant (~57.6%) mitotic inhibitory effect of *S. officinale* on HeLa cells [17]. Although the extract displayed clear anticancer potential, the molecular mechanisms underlying these effects required further investigation. Accordingly, SP, IL-6, and VEGF levels were quantified by ELISA, and the two most abundant bioactive compounds in the extract were subjected to *in silico* analyses to explore their associations with angiogenesis and pain related signaling pathways. In parallel, the antioxidant capacity of the extract was assessed. Consistent with its high phenolic content, *S. officinale* extract demonstrated marked antioxidant activity, in agreement with previous studies employing DPPH, FRAP, and total phenolic content analyses, which reported particularly strong antioxidant effects in ethanol/water extracts [19]. Earlier work has also shown that *S. officinale* exhibiting minimal cytotoxicity toward normal human skin fibroblasts, even at concentrations up to 200 µg/mL [16,17].

SP is a neuropeptide known to play a role in tumor progression, angiogenesis, and inflammation [20,21]. In the present study, treatment of A549 cells with *S. officinale* extract for 24 and 48 hours did not result in statistically significant alterations in SP levels, despite previous reports demonstrating measurable SP release within 24-48 hours following stimulation in other cellular models [22,23]. Nonetheless, molecular docking analyses indicated that malaxinic acid and globoidnan A exhibit strong predicted binding affinities toward the NK-1 receptor. Among the three receptors examined, both compounds showed the highest predicted affinity for NK-1. Given the absence of statistically significant alterations in SP levels, the predicted NK-1 receptor interactions of malaxinic acid and globoidnan A are more likely to reflect SP-independent modulation of NK-1 associated signaling pathways. In this context, further studies should molecular dynamics simulations and functional assays are warranted to clarify the biological relevance of these interactions. While docking provides a rapid and cost-effective approach for screening ligand receptor interactions [24], its reliance on static protein structures limits its ability to capture protein flexibility, solvent effects, and entropic contributions to binding [25,26]. *S. officinale* and its bioactive constituents, particularly rosmarinic acid, can modulate cytokine production in immune cells, leading to reduced secretion of inflammatory mediators such as IL-6 [6]. Green silver nanoparticles synthesized from *S. officinale* extracts have been shown to suppress IL-6 production in UVB-irradiated HaCaT cells, further suggesting that the extract may influence inflammatory signaling [27]. In the present study, changes in IL-6 secretion were inconsistent across time points, representing a limitation that warrants further investigation.

Notably, the reduction in VEGF levels observed at 24 hours suggests a potential short-term modulation of angiogenesis-related markers of *S. officinale* extract. This observation aligns with previous reports indicating that plant-derived compounds can suppress VEGF production and thereby limit tumor-associated angiogenesis [18]. The attenuation of this effect at later time points may reflect degradation of bioactive compounds under culture conditions or the activation of compensatory angiogenic pathways within cancer cells, as reported for other botanical extracts [28]. The inhibition of VEGF signaling by natural compounds is frequently associated with suppression of regulatory pathways such as IGF-II and NF-κB, which are also implicated in the anticancer activity of *S. officinale* [29]. Collectively, these findings suggest that *S. officinale* extract exhibits a promising but transient anti-angiogenic effect *in vitro*, highlighting the importance of formulation stabilization for sustained activity. The anticancer, anti-inflammatory, and antioxidant effects of *S. officinale* are largely attributed to its polyphenolic constituents, including rosmarinic, chlorogenic, and caffeic acids [2]. In this study, malaxinic acid and globoidnan A were investigated for their potential interactions with apoptosis and angiogenesis related receptors *in silico*. Malaxinic acid demonstrated strong predicted binding affinities toward all tested receptors and formed interactions with key active-site residues, suggesting a potential to modulate receptor function. Its interactions with NRP-1 and VEGFR-1 support a possible anti-angiogenic role by interfering with VEGF-mediated signaling and tumor vascularization [28]. Globoidnan A exhibited comparatively lower affinity for NRP-1 but showed strong binding to NK-1 and VEGFR-1, indicating a potential capacity to suppress VEGF-driven angiogenic pathways. Both compounds also interacted with the Tie2 receptor, which plays a central role in vascular stability and tumor neovascularization, suggesting a broader impact on endothelial function. Previous studies have reported that globoidnan A exhibits pronounced antiproliferative effects in multiple cancer cell lines, including C6 (rat brain tumor), HeLa (human cervical carcinoma), and HT29 (human colon carcinoma) cells [30]. In the present context, the high predicted affinity of both malaxinic acid and globoidnan A for the NK-1 receptor indicates the need for further mechanistic studies to determine whether these interactions translate into functional modulation of neurogenic inflammation and tumor-associated signaling pathways.

Several limitations should be acknowledged. The present study focused exclusively on the A549 epithelial lung carcinoma cell line, and the inclusion of non-tumoral control cell lines, such as healthy fibroblasts or epithelial cells, would strengthen the interpretation of selectivity. Moreover, the observed inconsistencies in IL-6 and SP responses suggest that additional experimental validation, including expanded cytokine profiling and repeated ELISA measurements, is required. Finally, the *in silico* analyses were limited to molecular docking, and it should be emphasized that these results represent predictive interactions rather than direct evidence of biological activity; therefore, molecular dynamics simulations and in vivo studies are necessary to validate binding stability, receptor modulation, and pharmacological relevance.

5. CONCLUSION

Overall, the combined experimental and computational data support the hypothesis that phenolic constituents of *S. officinale* may exert anticancer effects through multi-target modulation of inflammatory and angiogenic pathways. These findings provide a rationale for further investigation into the extract's effects on cell viability, anti-inflammatory, and anti-angiogenic activities and highlight its potential as a promising source of therapeutic lead compounds.

CONFLICT OF INTEREST

The authors stated that there are no conflicts of interest regarding the publication of this article.

CRediT AUTHOR STATEMENT

Esra Tanyel Akçit: Software, Formal analysis, Investigation. **Nüket Akanıl Bingöl:** Conceptualization, Methodology. **Ece Şimşek:** Methodology, Validation, Writing – Review & Editing. **Altınay Altınkaynak:** Validation**,** Formal analysis, Writing – Original Draft. **Gizem Tutkun:** Data Curation, Visualization. **Zeynep Burcu Mekik:** Software. **Cemilenur Ataş:** Visualization **Nilüfer İmir:** Editing **İlker Çinbilgel:** Validation. **Kübra Yıldırım:** Methodology, **Emel Cengiz Kaynakcı:** Visualization. **Ahmet Yılmaz Çoban:** Supervision.

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ESKİŞEHİR TEKNİK ÜNİVERSİTESİ BİLİM VE TEKNOLOJİ DERGİSİ
C- YAŞAM BİLİMLERİ VE BİYOTEKNOLOJİ

Eskişehir Technical University Journal of Science and Technology
C -Life Sciences and Biotechnology

Estuscience – Life, 2026, 15 (2) pp. 147-162, DOI: 10.18036/estubtdc.1825882

RESEARCH ARTICLE

VITAMIN D_3 ENHANCES REPRODUCTIVE POPULATION EXPANSION AND DNA YIELD IN *CAENORHABDITIS ELEGANS*: A COMBINED EXPERIMENTAL AND COMPUTATIONAL STUDY

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Abstract

Vitamin D_3 is a multifunctional secosteroid regulating calcium homeostasis, immune regulation, and cellular metabolism; however, its reproductive and genomic roles in invertebrates remain largely unexplored. This study examined the effects of vitamin D_3 on population expansion and genomic DNA thermal behavior in *Caenorhabditis elegans* using integrated experimental and computational approaches. Synchronized L4-stage *C. elegans* were exposed to 1,000 IU, 2,000 IU, and 4,000 IU of vitamin D_3 per NGM plate, and population growth was monitored from Day 0 to Day 15, with image-based counts recorded during follow-up. Vitamin D_3 treatment significantly increased final worm numbers compared with controls, with the strongest population expansion observed in the 1,000 and 4,000 IU groups. Genomic DNA quantification showed the highest recovered DNA concentration at 4,000 IU (193.8 ± 102.9 µg/mL), under input-normalized extraction conditions. DSC analysis revealed treatment-dependent shifts in melting temperature and enthalpy, indicating altered thermal denaturation behavior of isolated genomic DNA rather than direct evidence of DNA damage protection. Molecular docking and 100 ns molecular dynamics simulations suggested DAF-12 as the most plausible candidate receptor for vitamin D_3 ($\Delta G \approx -87.67$ kcal/mol), followed by NHR-48 and NHR-8. Overall, vitamin D_3 enhanced *C. elegans* population expansion and altered recovered DNA yield and thermal stability-related parameters, possibly through sterol-responsive nuclear receptor pathways associated with lipid metabolism and germ-line regulation. These findings highlight a potential link between vitamin D_3 exposure, reproductive output, and DNA-associated biophysical responses in C. elegans.

Keywords

Caenorhabditis elegans,
Vitamin D_3 (cholecalciferol),
DAF-12,
Differential scanning calorimetry (DSC),
Molecular dynamics simulation

Time Scale of Article

Received :19 November 2025
Accepted : 21 July 2026
Online date : 27 July 2026

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1. INTRODUCTION

Vitamins are indispensable biomolecules that orchestrate a wide range of biochemical and physiological functions essential for maintaining life. Among them, vitamin D has attracted particular attention in recent decades, not only for its well-established role in skeletal development but also for its increasingly recognized functions in immune regulation, cellular differentiation, and disease prevention. As a fat-soluble secosteroid, vitamin D exists in two principal forms: ergocalciferol (vitamin D_2), primarily derived from plant sources and fortified foods, and cholecalciferol (vitamin D_3), obtained from animal products or synthesized in the skin upon exposure to ultraviolet B (UVB) radiation. Once ingested or synthesized, vitamin D_3 is biologically inert and requires successive hydroxylation in the liver and kidney to generate its hormonally active form, calcitriol (1,25-dihydroxyvitamin D_3) [1-3].

Vitamin D_3 has broad systemic effects that extend beyond its role in mineral metabolism. It contributes to neuromuscular function, epithelial homeostasis, immune regulation, and the control of cell proliferation and differentiation [4-6]. Mechanistically, its biological activity is mediated through binding to the vitamin D receptor (VDR), a nuclear receptor that heterodimerizes with the retinoid X receptor (RXR) to regulate transcription of genes involved in calcium homeostasis, cell cycle regulation, and tumor suppression. Dysregulation of vitamin D–VDR signaling has been implicated in diverse pathologies ranging from osteoporosis and autoimmune disorders to cancer, underscoring the pleiotropic nature of this molecule [7].

Beyond vertebrates, several invertebrate models provide useful systems for investigating sterol-responsive nuclear receptor biology. Although *Caenorhabditis elegans* lacks a direct ortholog of the human VDR, it possesses a large repertoire of nuclear hormone receptors (NHRs) with structural and functional similarities to mammalian steroid and lipid receptors. Among these, DAF-12, NHR-8, and NHR-48 are particularly noteworthy: DAF-12 responds to endogenous dafachronic acids—bile acid–like ligands derived from cholesterol—and governs key developmental and reproductive decisions [9,15]. Furthermore, NHR-8 and NHR-48 are involved in sterol metabolism, lipid homeostasis, and germ-line regulation [10]. This functional analogy suggests that vitamin D_3 and related sterol molecules may influence nematode NHR-associated pathways in comparable ways, at least in part, to secosteroid signaling in vertebrates. Hence, examining vitamin D_3's role in *C. elegans* provides a powerful evolutionary framework for understanding conserved aspects of steroid signaling, reproduction, and genomic stability across metazoans [9,10,32]. While the mammalian system has been extensively studied, the molecular underpinnings of vitamin D_3 activity remain incompletely understood.
Model organisms offer a framework to dissect these mechanisms in a controlled genetic and physiological context. *C. elegans*, a transparent, 1-mm nematode with a fully sequenced genome and conserved signaling pathways, has become a powerful system for such investigations [20]. Its short lifespan, well-defined developmental stages, and tractable genetics make it ideally suited to interrogate the interplay between micronutrients and cellular homeostasis. Previous studies, including the work of Messing et al. (2013), demonstrated that vitamin D can extend *C. elegans* lifespan, potentially involving nuclear hormone receptor-associated signaling pathways such as DAF-12 [11]. Additional studies have implicated related transcription factors and stress-response regulators, including DAF-16, SKN-1, HSF-1, and IRE-1, in mediating vitamin D-induced resilience to proteotoxic and oxidative stress [27]. Despite these advances, most research to date has focused on longevity and stress resistance. Far less attention has been paid to how vitamin D influences reproductive capacity, a critical determinant of species survival and evolutionary fitness.

Given that reproductive success is tightly linked to genomic integrity, evaluating vitamin D_3's effects on DNA stability offers further mechanistic insight. The maintenance of genome fidelity is a fundamental biological imperative; endogenous metabolic byproducts and exogenous stressors continually challenge DNA. In *C. elegans*, DNA-related alterations are commonly assessed in

toxicological and pharmacological assays, making it an excellent system to test micronutrient-DNA interactions [8].

Differential scanning calorimetry (DSC) has emerged as a robust technique for probing nucleic acid thermal behavior, drug-DNA interactions, and molecular stability. By measuring heat capacity changes during thermal denaturation, DSC provides quantitative information on melting transitions and enthalpy changes that reflect the thermal behavior of isolated DNA under defined experimental conditions [16-18,29]. When coupled with complementary methods such as UV absorbance spectroscopy, DSC enables a nuanced thermodynamic view of DNA structural alterations upon ligand interaction.

In the present study, we aimed to bridge this knowledge gap by examining, for the first time, the impact of vitamin D_3 on the reproductive output and DNA-associated biophysical parameters in *C. elegans.* Using randomized controlled trials with synchronized L4 larvae, we quantified reproductive outcomes under varying vitamin D_3 concentrations through image-based proliferation analysis. We further employed spectrophotometric DNA quantification and DSC to assess recovered genomic DNA yield and thermal denaturation behaviour, and performed in silico docking and molecular dynamics simulations with the nuclear receptors DAF-12, NHR-8, and NHR-48 to identify plausible receptor-associated mechanisms. By integrating experimental and computational approaches, this work introduces a new dimension to vitamin D research: its potential role in nematode population expansion and DNA thermal behavior and provides a framework for future mechanistic investigations across species.

2. METHODS

2.1. Reproduction Assay and Vitamin D3 Exposure

To examine the effects of vitamin D_3 on population expansion, synchronized L4-stage *C. elegans* were transferred to NGM agar plates seeded with E. coli OP50. Each treatment group consisted of five plates with 10 worms per plate (3 replicates). Vitamin D_3 was prepared as a stock solution of 200,000 IU in 10 mL carrier solvent (equivalent to 20,000 IU/mL). From this stock solution, 50, 100, and 200 µL aliquots were applied to OP50-seeded NGM plates, corresponding to 1,000, 2,000, and 4,000 IU vitamin D_3 per plate, respectively. Control plates received the same carrier solvent without vitamin D_3 under identical conditions. Parental worms were maintained on treatment plates, and total population expansion was monitored. To enable quantitative analysis, plates were imaged using a high-resolution flatbed scanner (Epson V800, Los Alamitos, CA, USA) at 24-hour intervals. Images were processed in Fiji/ImageJ using automated particle analysis to quantify the total number of worms and their distribution. The same thresholding and particle-size parameters were applied to all images. To validate automated counting, a randomly selected subset of images representing different treatment groups and population densities was manually counted and compared with the automated ImageJ output. Automated counts were also visually inspected, and images with obvious segmentation errors or overlapping particles were manually reviewed when necessary.

Each plate was seeded with 10 synchronized L4-stage worms at Day 0. Image-based population counts were subsequently recorded during follow-up, including the first post-seeding imaging time point at Day 3 and daily counts thereafter until Day 15. Day 0, therefore, represents the original number of synchronized L4 worms seeded per plate, whereas Day 3 and subsequent values represent image-based, quantified worm counts during population expansion.

To quantitatively evaluate the effect of vitamin D_3 on C. elegans population expansion, worm counts were analyzed using an exponential growth model:

$$N_t = N_0 \, e^{kt}$$

where Nt represents the final worm number, N0 represents the initial number of synchronized L4 worms seeded per plate at Day 0, t represents the observation interval in days, and k represents the specific growth rate constant (day−1).

Rearranging the equation gives:

$$k = \frac{ln\left(\frac{N_t}{N_0}\right)}{t}$$

The relative population increase (%) for each group was determined as:

$$Population\ \ Increase(\%) = \left(\frac{N_t - N_0}{N_0}\right) \times 100$$

This index represents the relative increase in population size compared with the initial number of synchronized L4 worms seeded per plate at Day 0. For the growth analysis, N0 was defined as 10 worms per plate and Nt was defined as the final mean worm count at Day 15.

All calculations were performed using the mean worm numbers from three independent replicate plates per condition. The resulting growth constants and relative population increase percentages were then compared among treatment groups to determine the dose-dependent influence of vitamin D_3 on *C. elegans* population expansion.

2.2. DNA Extraction and Purification

Following the imaging period, worms were harvested from NGM plates by washing with M9 buffer and transferred into 1.5 mL microcentrifuge tubes. Worm suspensions were pelleted by centrifugation at 14,000 rpm for 5 min, and the supernatants were carefully discarded. To reduce bacterial carryover, pellets were washed twice more with fresh M9 buffer before DNA extraction. For DNA extraction, samples were normalized using comparable worm biomass/pellet amount across groups before lysis.

For lysis, input-normalized worm pellets were resuspended in 200 µL tissue lysis buffer provided in a commercial genomic DNA extraction kit (e.g., Qiagen DNeasy Blood & Tissue Kit or equivalent). Proteinase K (20 µL) was added, and samples were incubated at 56 °C for 30–60 min with intermittent vortexing to ensure efficient cuticle disruption and protein digestion. For more complete lysis, brief mechanical agitation with zirconia/silica beads was used when necessary. RNA contamination was minimized by treating all samples with RNase A (10 µg per reaction, room temperature, 10 min).

Following lysis, genomic DNA was purified using silica-membrane spin-columns according to the manufacturer's instructions. Lysates were mixed with binding buffer and ethanol, transferred to spin columns, and centrifuged to allow selective binding of DNA. Columns were washed sequentially with proprietary wash buffers to remove proteins, salts, and residual inhibitors. A final dry spin at 12,000 × g ensured removal of ethanol. DNA was then eluted in the same fixed volume of pre-warmed Tris-HCl buffer (10 mM, pH 8.5) for all samples.

Extracted DNA was quantified and assessed for purity using a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA). Samples with A_{260}/A_{280} ratios between 1.8 and 2.0 and A_{260}/A_{230} ratios above 2.0 were considered suitable for downstream analyses. To minimize buffer-related effects on spectroscopic and DSC analysis, all DNA samples were eluted and stored under identical low-salt buffer conditions at −20 °C until use.

2.3. Differential Scanning Calorimetry (DSC) of Genomic DNA

Genomic DNA thermal denaturation behavior was evaluated by DSC to probe treatment-associated changes following vitamin D_3 exposure. Equal masses of DNA from each group (control; plates supplemented with 1,000, 2,000, or 4,000 IU of the 20,000 IU/mL vitamin D_3 stock) were adjusted to the same concentration in low-salt Tris–HCl (10 mM, pH 8.5) and brought to a final pan volume of ~35–40 µL. Samples were loaded into hermetically sealed aluminum pans, and a matched reference pan contained the identical buffer used for DNA dilution. To minimize evaporation and baseline drift, pans were sealed and briefly equilibrated in the autosampler before measurement.

DSC measurements were acquired on a Shimadzu DSC-60 Plus under a dry nitrogen purge (≈50 mL/min). Each sample was scanned from 25–110 ºC at 1 ºC/min (parameters within the recommended operating range for DNA duplex denaturation), with replicate runs performed to assess reproducibility. Instrument temperature and enthalpy scales were verified using standard metals before analysis.
To ensure interpretable thermodynamic comparisons, DNA concentration, buffer composition, and ionic strength were kept constant across groups, given their strong influence on DNA melting behavior. Equal amounts of DNA from each group were analyzed against the identical low salt Tris–HCl buffer used for sample preparation.

Thermograms were baseline-corrected using buffer-only scans and the instrument's analysis software. Melting temperature (Tm) was taken at the maximum of the endothermic transition (or from the first-derivative peak, where noted). Calorimetric enthalpy (ΔHcal) was obtained by integrating the transition area after baseline subtraction.

DSC measurements were used to compare melting temperature (T_m) and calorimetric enthalpy (ΔH_{cal}) among groups as thermal denaturation parameters of isolated genomic DNA.

2.4. In silico Studies

To prioritize the nuclear hormone receptor that may interact with vitamin D_3 in *C. elegans*, structure-based docking was followed by explicit-solvent molecular dynamics (MD) simulations and end-point binding free-energy estimation. These computations were designed to generate mechanistic hypotheses for future experimental validation.

2.5. Molecular Docking

The 3D structure of vitamin D_3 (cholecalciferol) was retrieved from PubChem and prepared with LigPrep using Epik protonation at pH 7.4 (default tautomer/protonation enumeration). Since experimental structures are not available for the *C. elegans* receptors of interest, AlphaFold models of DAF-12, NHR-8, and NHR-48 were obtained from the AlphaFold Protein Structure Database. Model confidence (pLDDT and PAE) was inspected; low-confidence termini and unresolved loops were trimmed only when clearly disordered (pLDDT < 50) and after confirming that truncation did not impact the predicted ligand-binding region. Protein structures were processed in Maestro's Protein Preparation Wizard (standard assignment of bond orders, addition of hydrogens, restrained minimization at pH 7.4).

For DAF-12, the binding site was defined by analogy to known endogenous ligands (dafachronic acids); the docking grid was centered by superposing the AlphaFold model with the DAF-12 ligand coordinates reported in the literature (Δ^7/Δ^4-dafachronic acid) and by mapping pocket residues described therein. In the absence of crystallographic *C. elegans* complexes for NHR-8 and NHR-48, pockets were identified using SiteMap and conserved NHR architecture, then cross-checked against AlphaFold confidence maps. Docking was performed in Glide (SP for screening, XP for final poses) with default van der Waals

scaling for nonpolar atoms; top-ranked poses were retained for MD. Docking into AI-predicted apo models can misplace side chains within flexible pockets; therefore, we used MD relaxation and ensemble MM/GBSA to mitigate receptor-conformation bias.

2.6. Molecular Dynamics Simulations

Protein–ligand complexes from docking were embedded in explicit solvent using Desmond's System Builder: orthorhombic box with TIP3P water, 0.15 M NaCl, and neutralizing counter-ions. Systems underwent the standard relaxation protocol and were simulated for 100 ns in the NPT ensemble at 310 K and 1.013 bar (Nosé–Hoover thermostat, Martyna–Tobias–Klein barostat; RESPA integration; recording interval 100 ps). The OPLS3e force field was used for proteins and ligand parameters (generated via the Force Field Builder). Trajectories were analyzed for RMSD, RMSF, hydrogen-bond persistence, and ligand–protein contact frequencies.
End-point binding free energies (ΔG_bind) were estimated with Prime MM/GBSA on MD snapshots (last 50 ns, every 10th frame), using the VSGB 2.0 solvent model with OPLS parameters. Reported MM/GBSA values represent the mean ± SEM across the snapshot ensemble and were used for comparative ranking among receptors rather than as absolute experimental binding affinities. Because conformational entropic contributions were not explicitly calculated in this protocol, the absolute magnitude of the MM/GBSA values may overestimate ligand affinity. Therefore, these values were used only to rank the tested receptors under the same computational workflow and were not converted into experimental affinity constants.

2.7. Statistical Analysis

For each treatment, three independent replicate plates were analyzed. Each plate was initially seeded with 10 synchronized L4 worms at Day 0, and image-based worm counts were recorded during follow-up until Day 15. Daily worm counts were summarized as mean ± SD. To quantify population expansion, an exponential growth model was applied, and the growth rate constant was computed as $k = ln(N_{15}/N_0)/15$, where N_0 represents the initial number of worms seeded per plate and N_{15} represents the final mean worm count at Day 15. The relative population increase was calculated as $[(N_{15} - N_0)/N_0] \times 100\%$. Group differences in final Day 15 worm counts were tested by one-way ANOVA followed by Tukey's HSD test for all pairwise comparisons. As a non-parametric sensitivity analysis for the small sample size, a Kruskal–Wallis test was also performed.

3. RESULTS

Figure 1 shows control and vitamin D_3-treated *C. elegans* groups, and the images provide a qualitative overview of worm distribution and population density across treatment groups.

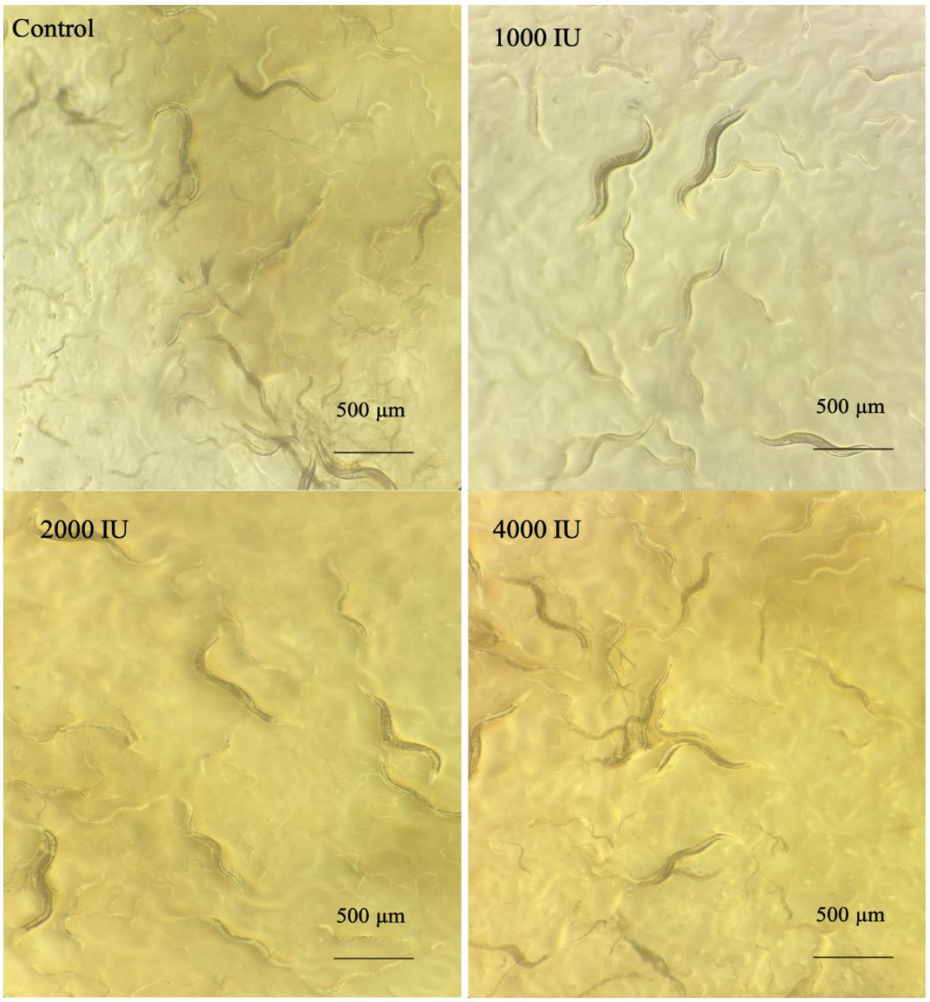

Figure 1. Microscopic observation of *C. elegans* exposed to different concentrations of the Vitamin D_3. (A) Control (B) 1,000 IU, (C) 2,000 IU, and (D) 4,000 IU treatment.

Table 1. NanoDrop spectrophotometric quantification of recovered genomic DNA from input-normalized *C. elegans* samples.

Vitamin D_3 exposure (IU/plate)	**DNA concentration (µg/mL)**			
	Rep 1	**Rep 2**	**Rep 3**	**Mean ± SD**
Control	99.3	102.4	87.6	96.4 ± 7.8
1,000	95.6	109.1	64.2	89.6 ± 23.1
2,000	169.7	165.7	80.5	138.6 ± 50.4
4,000	312.2	127.8	141.4	193.8 ± 102.9

Among vitamin D_3-treated groups, recovered genomic DNA concentration increased progressively between 1,000 and 4,000 IU, with the highest value observed at 4,000 IU (Table 1). Mean DNA concentrations increased progressively with higher vitamin D_3 doses, from 89.6 ± 23.1 µg mL^{-1} at 1,000

IU to 138.6 ± 50.4 µg/mL at 2,000 IU and 193.8 ± 102.9 µg mL^{-1} at 4,000 IU. The 2,000 IU group showed an intermediate recovered DNA concentration between the 1,000 and 4,000 IU groups. However, because of the marked inter-replicate variability, particularly in the 4,000 IU group, this finding should be interpreted as a descriptive trend rather than definitive evidence of a statistically robust increase in DNA yield.

DSC analysis showed treatment-dependent differences in the thermal denaturation profile of isolated genomic DNA (Table 2). The control group showed T_m of 58 ± 4.3 °C, whereas 1,000, 2,000, and 4,000 IU groups showed T_m values of 47 ± 2.2 °C, 52 ± 2.6 °C, and 50 ± 1.3 °C, respectively. The corresponding ΔHcal values were 51 ± 7, 130 ± 39, and 325 ± 25 J/g in the 1,000, 2,000, and 4,000 IU groups, respectively, compared with 408 ± 35 J/g in the control group.

Table 2. DSC-derived thermal denaturation parameters of genomic DNA isolated from *C. elegans* exposed to vehicle control or vitamin D_3.

Sample	**T_m (°C)**	ΔHcal (J/g)
Control	58 ± 4.3	408 ± 35
1,000 IU	47 ± 2.2	51 ± 7
2,000 IU	52 ± 2.6	130 ± 39
4,000 IU	50 ± 1.3	325 ± 25

3.1. Population Growth Analysis

Population growth analysis was performed using the seeding number of 10 L4 worms per plate as N_0 and the final Day 15 worm count as N_{15}. The control group reached 167.67 ± 5.03 worms per plate, whereas the 1,000 IU, 2,000 IU, and 4,000 IU groups reached 693.33 ± 10.79, 460.00 ± 13.45, and 648.00 ± 23.43 worms per plate, respectively. Growth rate constants were 0.188, 0.282, 0.255, and 0.278 day^{-1} for the control, 1,000 IU, 2,000 IU, and 4,000 IU groups, respectively. These results indicate that vitamin D_3-treated groups showed higher population expansion than the control group, with the greatest final worm count observed in the 1,000 IU group. Final Day 15 worm counts differed significantly among groups by one-way ANOVA ($F = 785.10$, $P = 3.23 \times 10^{-10}$), and Tukey's HSD test showed significant pairwise differences among all groups. A Kruskal–Wallis sensitivity analysis also supported a group effect ($P = 0.0156$).

Table 3. N_0 represents the initial number of synchronized L4 worms seeded per plate at Day 0, and N_{15} represents the final population count at Day 15.

Group	**N_0 (mean ± SD)**	**N_{15} (mean ± SD)**	**k (day^{-1})**	**Relative Population increase (%)**
Control	10	167.67 ± 5.03	0.188	1576.7
1,000 IU	10	693.33 ± 10.79	0.282	6833.3
2,000 IU	10	460.00 ± 13.45	0.255	4500.0
4,000 IU	10	648.00 ± 23.43	0.278	6380.0

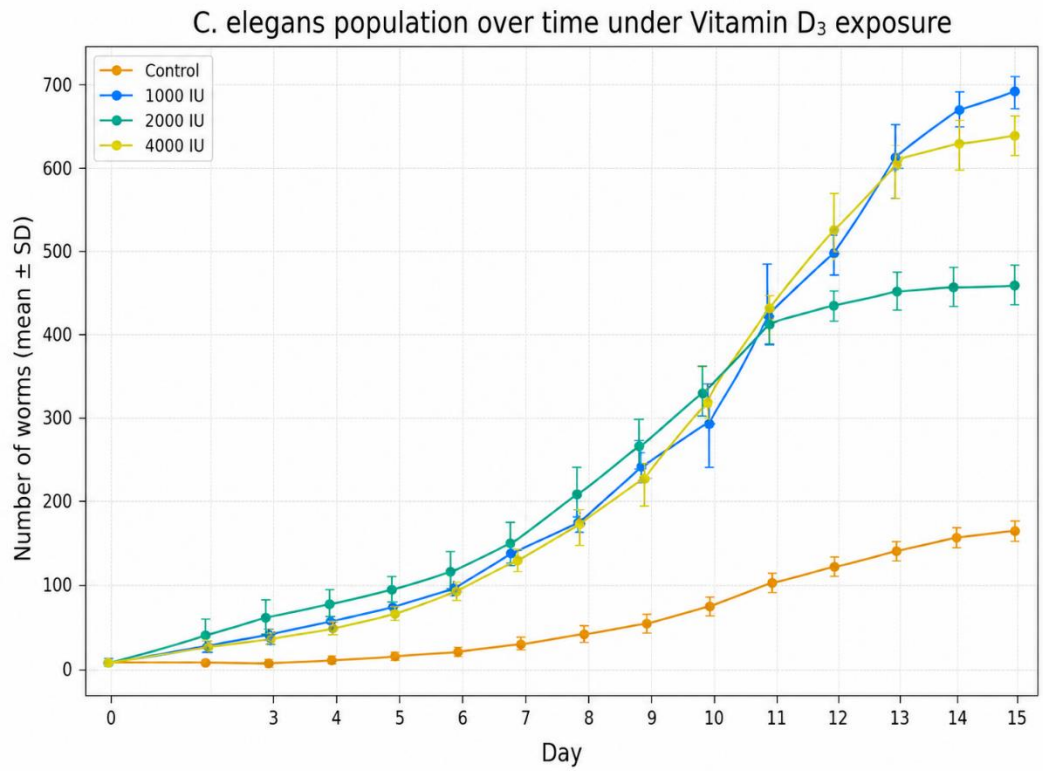

Figure 2. Population dynamics of C. elegans under vitamin D_3 exposure from Day 0 to Day 15. Day 0 represents the initial seeding of 10 synchronized L4 worms per plate. Values are shown as mean ± SD from three independent replicate plates.

The 1,000 and 4,000 IU groups showed higher final worm counts than the control group, whereas the 2,000 IU group showed a lower final count than the other vitamin D_3-treated groups (Figure 2).

Tukey post-hoc testing showed significant pairwise differences among all groups (all $P < 0.05$), with final worm counts ranked 1,000 IU > 4,000 IU > 2,000 IU > Control. A Kruskal–Wallis sensitivity analysis also supported a group effect ($P = 0.0156$). Growth constants computed from the first and final imaging time points were 0.188, 0.282, 0.255, 0.278 day^{-1} for 0; 1,000; 2,000; and 4,000 IU, respectively.

All experimental groups showed progressive population expansion throughout the 15-day observation period (Figure 2). The vitamin D_3-treated groups showed growth rate constants ranging from 0.20–0.28 day^{-1} and achieved higher final worm counts than the control group (Table 3). Among the vitamin D3-treated groups, the 2,000 IU group showed the lowest growth rate (0.255 day^{-1}) and the lowest final worm count (460.00 ±13.45 worms).

Overall, vitamin D_3-treated groups showed higher final population counts than the control group. Based on the original seeding number of 10 synchronized L4 worms per plate, the control group showed a 16.8-fold increase by Day 15, whereas the 1,000 IU, 2,000 IU, and 4,000 IU groups showed 69.3-fold, 46.0-fold, and 64.8-fold increases, respectively. Because these values were derived from total population counts rather than isolated brood-size measurements, they should be interpreted as population expansion metrics rather than direct per-worm fecundity measurements.

3.2. Molecular Docking and Molecular Dynamics Simulations

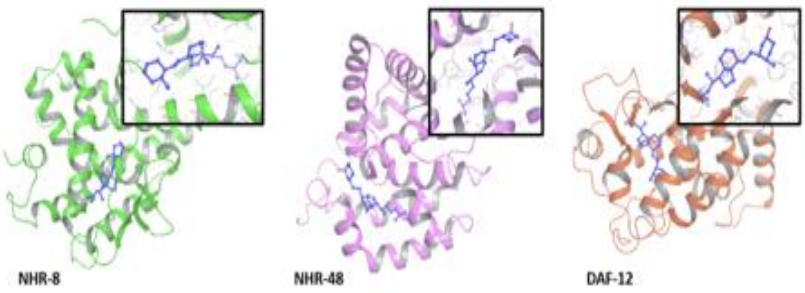

Figure 3. Binding poses obtained from molecular docking and molecular dynamics simulations

During the simulations, vitamin D_3 formed hydrogen bond interactions mainly through its hydroxyl group with receptor-specific residues, including Thr596 in DAF-12, Pro77 in NHR-48, and Leu351/Arg411 in NHR-8 (Figure 3, Figure 4).

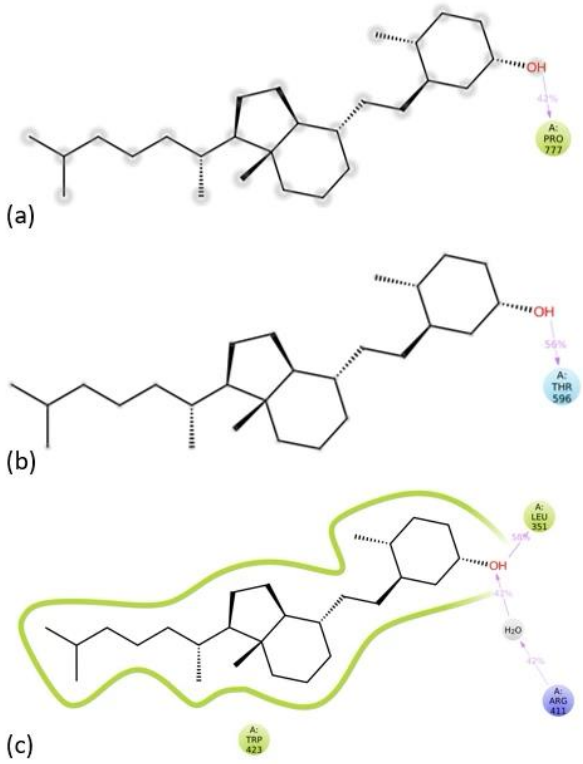

Figure 4. Ligand-contact residues observed in the receptor–vitamin D_3 complexes: (a) NHR-8, (b) NHR-48, and (c) DAF-12.

Table 4. Glide docking scores and MM/GBSA binding-energy estimates for vitamin D_3 interaction with NHR-8, NHR-48, and DAF-12. MM/GBSA values were used for comparative ranking among receptors and should not be interpreted as absolute experimental binding free energies.

Receptor	**Glide Score**	**MM/GBSA ΔG_{bind} (kcal/mol)**
NHR-8	-9.374	-82.7009
NHR-48	-4.240	-84.2746
DAF-12	-9.576	-87.6740

Molecular docking and molecular dynamics simulations suggested that vitamin D_3 can be accommodated within the predicted ligand-binding cavities of NHR-8, NHR-48, and DAF-12 through hydrogen bond and hydrophobic interactions (Figure 4, Table 4). The hydroxyl group of vitamin D_3 was involved in hydrogen-bond interactions with Thr596 in DAF-12, Pro77 in NHR-48, and Leu351/Arg411 in NHR-8. These interactions were maintained during the simulation trajectories, supporting the structural compatibility of vitamin D_3 with the predicted receptor pockets (Figure 3). Docking and MM/GBSA energy estimates ranked DAF-12 as the strongest computational candidate among the three tested receptors within the same workflow (Glide score = −9.576; MM/GBSA energy estimate = −87.67 kcal/mol), followed by NHR-48 and NHR-8 (Table 4).

4. DISCUSSION

The present study investigated the effects of vitamin D_3 on population expansion, recovered genomic DNA yield, DNA thermal denaturation behavior, and candidate nuclear receptor interactions in *C. elegans* using a combined experimental and computational approach. Population-based assays revealed treatment-associated differences in final worm counts, whereas DNA quantification, DSC, molecular docking, and molecular dynamics analyses provided complementary information on DNA recovery, thermal behavior, and possible receptor-associated mechanisms. The integration of in vivo, biothermal, and in silico data suggests that vitamin D_3 may influence nematode physiology through sterol-responsive nuclear receptor pathways that are partially comparable to vertebrate secosteroid signaling.

Vitamin D_3 is a secosteroid involved in cellular homeostasis, lipid metabolism, and stress-response pathways. Although *C. elegans* lacks a direct ortholog of the mammalian VDR, several nuclear hormone receptors, including DAF-12, NHR-8, and NHR-48, regulate sterol metabolism, developmental decisions, and reproductive pathways [28]. Previous studies have shown that DAF-12 ligands, particularly dafachronic acids, integrate metabolic and environmental cues that influence dauer formation, developmental progression, and reproductive growth [32]. Consistent with this framework, the higher final population counts observed in the 1,000 and 4,000 IU groups may reflect modulation of sterol-responsive pathways involved in reproductive and developmental regulation [19].

The lower final population count and growth constant observed in the 2,000 IU group suggest a non-linear dose–response pattern, although the underlying mechanism cannot be determined from the present data alone [22,32]. Non-linear or biphasic responses to micronutrients and sterol-related compounds have been described in *C. elegans* and other model systems [25]. Therefore, the present population data may be consistent with differential receptor engagement or feedback regulation, but this interpretation requires validation using receptor-specific genetic or functional assays.

The highest mean recovered DNA concentration was observed in the 4,000 IU group; however, this group also showed the largest inter-replicate variability. This finding should not be interpreted as direct evidence of improved DNA integrity without DNA damage-specific assays. Although vitamin D_3 has been associated with DNA repair, oxidative stress regulation, and cellular homeostasis in mammalian systems [6, 12], extrapolation to *C. elegans* should be made cautiously because direct VDR orthology is absent. The relatively large standard deviation in the 4,000 IU group indicates inter-replicate variability, which may reflect biological variation, differences in compound distribution on agar, or variability in DNA extraction efficiency.

The increased DNA concentration at higher vitamin D_3 exposure indicates higher recovered nucleic acid yield from input-normalized samples; however, DNA yield and DSC-derived thermal parameters should be interpreted as complementary but distinct outcomes. DSC analysis demonstrated treatment-dependent changes in the thermal denaturation behavior of isolated genomic DNA (Table 2). Under nitrogen purge and a heating rate of 1 °C/min, control DNA showed a Tm of 58 ± 4.3 °C, whereas vitamin D_3-exposed groups showed lower Tm values ranging from 47 ± 2.2 °C to 52 ± 2.6 °C, together with marked differences in calorimetric enthalpy. These findings indicate that vitamin D_3 exposure was associated with measurable changes in DNA thermal transition behavior under the experimental conditions used. The lower Tm values and variable ΔHcal values may reflect differences in denaturation energetics, transition cooperativity, DNA fragment distribution, and the known sensitivity of DNA melting to ionic strength and buffer conditions [16–18,23,24,29]. Therefore, the DSC findings support treatment-associated alterations in genomic DNA thermal behavior, but they should not be interpreted as direct proof of ligand-mediated DNA stabilization or DNA damage protection.

The population analysis showed a non-linear response pattern: the 1,000 and 4,000 IU groups reached higher final worm counts than the control, whereas the 2,000 IU group showed a lower final count and growth constant than the other vitamin D_3-treated groups. The higher final counts observed in the 1,000 and 4,000 IU groups suggest that vitamin D_3 may increase overall population expansion under the present experimental conditions. This pattern may be compatible with previous findings showing that sterol-derived ligands can regulate DAF-12-associated developmental and metabolic pathways in *C. elegans*; however, feedback inhibition or oxidative stress was not directly assessed in the present study [11,20]. Physiologically, these findings suggest that vitamin D_3 may influence *C. elegans* population dynamics through pathways related to sterol metabolism, energy balance, and nuclear hormone receptor signaling, including DAF-12-associated networks [11, 15, 21, 22, 25-28]. Because germ-line proliferation, egg viability, reproductive duration, oxidative stress, and feedback signaling were not directly measured, these mechanisms should be considered hypotheses for future studies rather than conclusions of the present work [28,30]. The highest final population count was observed in the 1,000 IU group, corresponding to 50 µL of the 20,000 IU/mL stock solution.

The lower response at 2,000 IU may reflect a non-linear biological response, but receptor saturation or energy reallocation remains speculative without direct mechanistic assays [21]. This pattern may be discussed in the context of nutrient-signaling trade-offs between fertility and somatic maintenance in nematodes [21,22,32].

Overall, the population, NanoDrop, and DSC findings collectively indicate that vitamin D_3 exposure was associated with increased final population counts, higher recovered DNA concentration, and altered DNA thermal denaturation parameters.

When integrated with the experimental data, the molecular simulation results provide a plausible mechanistic hypothesis rather than direct functional validation. The higher final population counts observed in vitamin D_3-treated groups may be related to modulation of DAF-12-associated pathways, but transcriptional activation, germ-line proliferation, and genomic protection were not directly

measured [28]. The non-linear response pattern may reflect receptor-level regulation or feedback mechanisms, but this interpretation requires confirmation using receptor mutants, RNAi, or gene-expression analyses. Moreover, the increased recovered DNA yield and the altered DSC profiles suggest treatment-associated changes in DNA recovery and thermal behavior, but they do not by themselves establish improved cellular integrity or activation of DNA repair pathways [31]. Although NHR-48 displayed a relatively modest docking score, molecular dynamics trajectories suggested stable ligand retention after equilibration, indicating that NHR-48 may also contribute to vitamin D_3-associated receptor interactions [19, 28].

These computational findings are compatible with the known physiological roles of the tested receptors. DAF-12 is a sterol-responsive nuclear hormone receptor that mediates dauer formation, developmental progression, reproduction, and longevity through dafachronic-acid signaling [28-30]. The predicted interactions with NHR-8 and NHR-48 may also be biologically relevant, given the reported roles of these receptors in cholesterol/fatty-acid metabolism, xenobiotic responses, and reproductive regulation [22]. These properties suggest that vitamin D_3 may influence energy-metabolic and reproductive pathways through multiple nuclear receptor targets, which could contribute to the population expansion observed at 1,000 and 4,000 IU exposures. All together, these findings suggest that DAF-12 is the strongest computational candidate among the tested receptors, with possible contributions from NHR-8 and NHR-48; however, receptor-specific experimental validation is required.

Our findings are broadly consistent with previous work showing that vitamin D can influence *C. elegans* lifespan and stress-response pathways, potentially involving nuclear hormone receptor-associated mechanisms [11,27]. Similar findings have been reported for NHR-8, where cholesterol and dafachronic acid availability were shown to regulate fertility and lipid homeostasis through nuclear receptor signaling [25]. The population expansion observed in the 1,000 and 4,000 IU groups may be interpreted in the context of reports linking lipid and energy metabolism to reproductive output in *C. elegans* [25]. The lower response at 2,000 IU may reflect a non-linear dose response, although adaptive energy trade-offs were not directly measured [21]. Sterol-related signaling pathways, including DAF-12-associated transcriptional programs, are known to integrate lipid metabolism, dauer signaling, and reproductive regulation in *C. elegans* [15]. Our current data extend this framework by showing treatment-associated differences in population expansion following vitamin D_3 exposure. Similar non-linear reproductive responses have been described under dietary and hormonal modulation in nematodes and other model systems, supporting the plausibility of a biphasic response pattern [31,32].

5. CONCLUSION

This study provides integrated experimental and computational evidence that vitamin D_3 modulates population expansion and is associated with changes in recovered genomic DNA concentration, DNA thermal denaturation behavior, and candidate nuclear receptor interactions in *C. elegans*. Population-level assays showed higher final worm counts in the 1,000 and 4,000 IU groups, whereas the 2,000 IU group showed a lower response than the other vitamin D_3-treated groups, suggesting a non-linear dose–response pattern. DSC analysis demonstrated treatment-dependent shifts in measured DNA melting temperature and calorimetric enthalpy, indicating that vitamin D_3 exposure was associated with altered thermal denaturation behavior of isolated genomic DNA. Molecular docking and 100 ns MD simulations ranked DAF-12 as the strongest computational candidate among the tested receptors (MM/GBSA $\Delta G_{bind} \approx -87.67$ kcal/mol), followed by NHR-48 and NHR-8. Collectively, these findings suggest that vitamin D_3 influences *C. elegans* population expansion and DNA-associated biophysical parameters, potentially through sterol-responsive nuclear receptor pathways. Future studies using receptor-specific mutants, RNAi, gene-expression analysis, and DNA damage-specific assays are required to confirm direct DAF-12 involvement and genomic protection. However, this study has some limitations. NanoDrop quantification and DSC

analysis provide information on recovered genomic DNA concentration and thermal denaturation behavior, but they do not directly demonstrate DNA damage reduction or DNA repair activity. Therefore, future studies using DNA damage-specific assays, such as comet assay and repair-marker analyses, are required to confirm whether vitamin D_3 has a direct protective effect on genomic integrity in *C. elegans*.

ACKNOWLEDGEMENTS

This work has no support from any organization.

CONFLICT OF INTEREST

The authors stated that there are no conflicts of interest regarding the publication of this article.

CRediT AUTHOR STATEMENT

Neslihan Demirci: Investigation, Software, Formal analysis, Visualization; **Recep Üstünsoy:** Data curation, Visualization; **Seyda Ignak:** Writing – review & editing; **Bircan Dinç:** Conceptualization, Methodology, Investigation, Writing – original draft, Writing – review & editing.

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ESKİŞEHİR TEKNİK ÜNİVERSİTESİ BİLİM VE TEKNOLOJİ DERGİSİ
C- YAŞAM BİLİMLERİ VE BİYOTEKNOLOJİ

Eskişehir Technical University Journal of Science and Technology
C -Life Sciences and Biotechnology

Estuscience – Life, 2026, 15 (2) pp. 163-175, DOI: 10.18036/estubtdc.1904036

RESEARCH ARTICLE

MORPHOLOGY AND ECOLOGY OF THE ENDEMIC *CROCUS FLAVUS* SUBSP. *DISSECTUS* DISTRIBUTED IN ESKİŞEHİR, TÜRKİYE

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Abstract

This study provides a comprehensive analysis of the morphological variation, habitat preferences, and mineral content of *Crocus flavus* Weston subsp. *dissectus* T.Baytop & B.Mathew, an endemic and vulnerable (VU) taxon of the Turkish flora. Focusing on populations in the Eskişehir region, our research offers a current morphological characterization that refines the diagnostic boundaries provided in the Flora of Turkey. Statistical analysis of 35 representative specimens indicates that floral traits, specifically stigma, style (CV: 9.43%), and anther (CV: 10.43%) lengths, exhibit the highest taxonomic reliability due to their low phenotypic plasticity. Conversely, vegetative organs like pedicels and spathes show higher variability. Edaphic data reveal a preference for well-drained, non-saline, and slightly alkaline soils with low lime content. Furthermore, ash analysis demonstrates a significant mineral accumulation in the corm (69.70%), exceeding above-ground organs by approximately 2.5-fold. Consequently, this study fills gaps in the literature regarding the taxonomically complex genus *Crocus* and provides a novel dataset for conservation strategies and future taxonomic revisions.

Keywords

C. flavus subsp. *dissectus*,
Morphology,
Ecology,
Edaphic characteristics

Time Scale of Article

Received :10 March 2026
Accepted : 22 July 2026
Online date : 27 July 2026

1. INTRODUCTION

Türkiye is a country that has considerable floristic diversity, with an increasing number of new species. In the flora of Turkey, there are approximately 9.753 natural species, of which 3.035 are endemic. This situation indicates that the rate of endemic species is at a high level of 31.12% [1]. In addition to the richness of the Flora of Turkey, it is very important to increase the number of taxonomically significant studies that will add value to this richness. A total of 816 geophyte species belonging to 73 genera naturally occur in Türkiye [2]. Geophytes are plants that store nutrients in underground storage organs, such as bulbs, tubers, and rhizomes [2]. Because of their underground stems, geophyte plants can adapt better to unfavorable environmental conditions compared to other flowering plants. In addition, some geophyte species are used for medicinal and aromatic purposes and are also utilized as ornamental plants in parks and gardens [2]. Despite their potential value, geophytes are less widely utilized in Türkiye than many other plant groups. One possible reason for their limited use is the lack of comprehensive inventory data.

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Because of their medicinal properties, *Crocus* L. species are among the plant groups used in traditional practices by local communities. It is reported that the genus *Crocus* (Iridaceae) has approximately 252 species worldwide [3]. Türkiye contains approximately 103 *Crocus* species [4]. However, apart from floristic studies, the number of studies conducted on *Crocus* species distributed in Türkiye is limited. Species of *Crocus* have historically been used as spices and sources of natural dyes. A significant portion of the species belonging to the genus *Crocus* naturally occurs in the Mediterranean Basin and its surroundings. Members of the genus are perennial plants with underground corms, and the shape of the corm is quite variable. In some regions of Anatolia, the stigmas obtained from the flowers of *C. sativus* L. are dried or powdered and used as a spice; they are added to rice dishes, desserts, and various foods to provide aroma and color [5]. *C. sativus* is a species that is well known among the public and widely used due to its medicinal properties, and existing studies have focused on this species. Ethnobotanical field studies conducted in our country have determined that different *Crocus* species are used by the public in Anatolia for therapeutic purposes. It has been reported that *Crocus flavus* Weston subsp. *dissectus* T. Baytop & Mathew (syn. *Crocus mouradi* Whittall) is known among the public in Manisa and its surroundings by the names "Çiğdem, sarıçiğdem, dildombak," and that the corm and flower parts of the plant are used as food and animal feed [2].

Crocus flavus Weston has long been known and has been cultivated as an ornamental garden plant for approximately 400 years. The flower color varies from white to cream–yellow tones. In subsp. *dissectus*, the style is generally divided into several thinner and weaker branches, whereas in subsp. *flavus*, the style is divided into three main branches. In addition, subsp. *dissectus* is distinguished by its smaller flowers [6].

The taxonomic identification of *Crocus* species is complex because it requires the detailed examination of cataphylls, prophylls, bracts, and bracteoles. The complex structure of the genus arises from the fact that some important characters are located completely or partially underground due to the ovary being subterranean. Accurate identification requires the careful recording of the corm tunic type, leaf number and width, the presence or absence of bracts and bracteoles, their relative sizes, precise flower coloration, anther color, and the degree of style division. While preparing herbarium material, some flowers should be split longitudinally, opened, and dried flat, and the detailed flower color should be recorded. Corms are important for identification.

The traditional use of plants by local communities contributes not only to the preservation of cultural heritage and biological diversity but also to public health and the discovery of new bioactive compounds. Previous studies have reported that extracts obtained from various *Crocus* species exhibit antitumor, antimutagenic, and cytotoxic activities and may inhibit nucleic acid synthesis in human malignant cells [7]. The dried stigmas and portions of the style of *C. sativus* constitute the aromatic spice saffron and contain crocin, a bright yellow carotenoid pigment. It is observed that most of the studies worldwide have been conducted on *C. sativus* species.

Rudall and Mathew [8] examined *Crocus* in terms of leaf anatomy. Mathew [9] investigated the morphological characteristics of the genus *Crocus*. Akan and Eker [10] studied the morphological and anatomical characteristics of *C. pallasii* subsp. *turcicus* B. Mathew and *C. cancellatus* subsp. *damascenus* (Herb.) B. Mathew. The morphological and anatomical characters of the taxon *C. flavus* Weston subsp. *flavus* were examined in detail [11]. Özdemir and colleagues [12] conducted anatomical and morphological studies on two endemic species in Türkiye, *C. danfordiae* Maw. and *C. fleischeri J. Gay* Baker. Some morphological and anatomical characteristics of *Crocus leichtlinii* (Dewer) Bowles, an endemic species naturally distributed in Şanlıurfa province, were examined, and its habitat and population status were determined [13]. Morphological and anatomical studies were carried out on two endemic species distributed in Southern Anatolia, *C. cancellatus* subsp. *lycius* and *C. reticulatus* subsp. *hittiticus* [14]. In that study, Kandemir [14] compared 14 *Crocus* taxa in terms of leaf anatomy. Anatomical studies on *Crocus olivieri* J. Gay were carried out by Özdemir et al. [15]. In a different

study, the morphological and anatomical characteristics of *Crocus chrysanthus* (Herb.) Herb. were examined in detail [16]. In another study, the morphological characteristics and leaf anatomy of two yellow-flowered endemic *Crocus* taxa (*C. ancyrensis* (Herb.) Maw, *C. siehenaus* Barr ex B.L.Burtt) were investigated [17]. Raca et al. [18] studied three *Crocus* taxa in Serbia. Uslu and colleagues [1] presented in detail the leaf anatomical characteristics of two endemic species for Bolu province, *Crocus abantensis* T. Baytop & B. Mathew and *Crocus* × *paulineae* Pasche & Kerndorff (hybrid), together with *C. ancyrensis* (Herb.) Maw subsp. *ancyrensis* and *C. olivieri*. In light of these characteristics, it was aimed to determine the true parents of the hybrid and possible other hybrid taxa distributed along the south–southeastern shore of Lake Abant. Many species growing in Türkiye have been brought under cultivation at Kew and examined cytologically [9].

Unlike previous studies, this research aims not only to clarify the morphological boundaries of *C. flavus* subsp. *dissectus*, an endemic taxon categorized as Vulnerable (VU), but also to characterize the edaphic conditions of its natural habitats. By integrating morphological data with soil analyses, this research provides essential baseline data for the conservation and potential cultivation of this endemic taxon.

2. MATERIALS AND METHODS

2.1. Plant Material

Crocus flavus subsp. *dissectus* specimens were collected in March 2024 from the Sarıcakaya district of Eskişehir, Türkiye (39°55′26″ N, 30°37′56″ E) (Figure 1). According to Davis's grid system [19], the study area is situated within the B3 square. To evaluate the ecological characteristics of its habitat, soil samples were simultaneously collected from the rhizosphere (the immediate vicinity of the corms) for edaphic analysis.

Figure 1. *Crocus flavus* subsp. *dissectus* plant and its habitat

2.2. Morphological Studies

The taxonomic identification of the specimens were performed according to the Flora of Turkey and the East Aegean Islands [19]. For morphological characterization, the following traits were examined: leaf number; flower structure; stem length and width; the distances from the base of the corm to the tip of the longest leaf and to the tip of the longest flower; leaf length and width; the lengths and widths of the inner and outer tepals; and the dimensions of the anthers, filaments, and style. Measurements were obtained from 35 individuals selected from the collected specimens. Larger vegetative and floral structures were measured using millimetre paper and a ruler, whereas smaller floral structures were measured using a Carl Zeiss microscope equipped with an ocular micrometer. Leaf width was measured

from the midpoint of the visible part of the leaves. Style length was determined starting from the level where the filaments are attached to the tepals.

2.3. Soil Analyses

Soil samples (approx. 1 kg) were collected from a depth of 15 cm in the natural habitat. The samples were air-dried at room temperature for seven days with daily mixing, then ground using a porcelain mortar and sieved through a 2 mm mesh to obtain a homogeneous sample [20].

2.3.1. Determination of soil moisture content

Soil moisture was determined gravimetrically. Samples were weighed into pre-tared porcelain crucibles using a Precisa LS 3200C SCS precision balance and dried in an oven at 105 °C for 24 hours. Following the drying period, samples were cooled in a desiccator to room temperature and reweighed to calculate the percentage of moisture loss [20].

2.3.2. Measurement of physicochemical parameters (pH, Eh, and EC)

The pH, redox potential (Eh), and electrical conductivity (EC) of the soil were measured according to the Jackson method. The dried soil samples (25 g) were placed in glass beakers, mixed with 50 mL of distilled water, and allowed to stand for 1 hour. Measurements were subsequently recorded from the supernatant using an HQ40D digital dual-channel multimeter [20].

2.3.3. Determination of calcium carbonate ($CaCO_3$) content

Calcium carbonate content was determined by measuring the volume of CO_2 gas released as a result of the reaction of calcium carbonate with hydrochloric acid, using the volumetric method with a Scheibler calcimeter. To ensure analytical precision, atmospheric pressure and temperature data were obtained from the Eskişehir Regional Directorate of Meteorology (3rd Region) during the analysis. The recorded values were calculated to volume at 0 °C and 760 mm Hg pressure, and this value was then expressed relative to 100 g of soil. One gram of soil sample was weighed and transferred to a 250 mL Erlenmeyer flask containing a distilled water: HCl solution (1:3, v/v; prepared from 37% HCl). The amount of CO_2 evolved was measured volumetrically using a calcimeter, and the analysis was performed in duplicate [20].

2.3.4. Determination of ash content in aboveground and underground organs

To compare mineral accumulation patterns, plant specimens were separated into above-ground (floral and vegetative parts) and underground (corms) organs. The samples were placed into porcelain crucibles and incinerated in a Nüve MF120 muffle furnace at 550 °C for one hour to ensure complete oxidation of organic matter (Figure 2). Following incineration, the crucibles were cooled to room temperature in a desiccator and reweighed to determine the total ash content, expressed as a percentage of the initial dry weight.

Figure 2. Ash content determination in plant organs

2.4. Statistical Analysis

In order to determine the range of morphological variation of the examined taxon, measurements were carried out on 35 randomly selected individuals (N = 35) representing the population. The obtained data were subjected to descriptive statistical analyses, and for each character, minimum (min.), maximum (max.), arithmetic mean (X), and standard deviation (SD) values were calculated. To determine the stability of the characters within the population and their levels of variability, the coefficient of variation (CV %) was calculated using the following formula:

$$CV\ (\%) = (SD/X) \times 100\ [21]$$

Characters with low coefficients of variation were evaluated as potentially stable markers within the studied population, whereas those with high coefficients of variation were defined as highly variable or plastic under the specific sampling conditions. The normality of the data was analyzed using the Shapiro–Wilk test, and it was determined that all variables exhibited a normal distribution ($p > 0.05$). Accordingly, Pearson's correlation analysis was applied to determine the linear relationships among the characters, and significance levels were set at $p < 0.05$ and $p < 0.01$. Statistical calculations and visualizations were performed using MS Excel, SPSS 26.0, and Python (version 3.10.12).

3. RESULTS

3.1. Morphological Studies

As a result of morphological examinations conducted on 35 individuals collected from Eskişehir province within the scope of the study, the specimens were identified as *Crocus flavus* subsp. *dissectus* (Figure 3). The herbarium material prepared from the examined samples was deposited in the Herbarium of Anadolu University Faculty of Pharmacy (ESSE, voucher no: 16289).

Figure 3. Morphological appearance of *C. flavus* subsp. *Dissectus*

The results of the variation analysis of the morphological characters of the examined taxon are summarized in Table 1. When the coefficients of variation (CV) calculated to determine the stability of the characters were evaluated, it was determined that the lowest variation occurred in the reproductive organs.

Table 1. Morphological measurements of *C. flavus* subsp. *dissectus*

Morphological Character		Min.-max. (cm)	Mean ± SD (cm)	CV (%)	Variation Level
Plant height		10.7-19.7	15.19±2.15	14.15	Low
Number of leaves		3-7	4.16±1.12	26.92	Medium
Leaf	**width**	0.1-0.2	0.11±0.13	9.09	Low (stable)
	length	5.9-15.9	11.04±2.30	20.83	Medium
Spathe	**width**	0.2-1.9	1.03±0.38	36.89	High
	length	2.2-12.1	6.48±2.41	37.19	High
Pedicel		1.2-7.1	3.74±1.60	42.78	High
Anther		0.8-1.4	1.15±0.12	10.43	Low (Reliable)
Filament		0.4-0.9	0.58±0.09	15.51	Medium
Outer tepal	**width**	0.6-1.2	0.8±0.13	16.25	Medium
	length	1.9-3.2	2.42±0.30	12.40	Low
Inner tepal	**width**	0.5-1	0.73±0.12	16.44	Medium
	length	1.5-2.8	2.12±0.32	15.09	Low-Medium
Stigma		0.5-0.7	0.53±0.05	9.43	Low
Style		3.9-10.3	6.96±1.30	9.43	Low

The normality of the data was assessed using the Shapiro–Wilk test, and it was determined that all examined characters exhibited a normal distribution ($p > 0.05$). Parametric relationships were determined using the Pearson correlation coefficient (r).

Characters with a coefficient of variation (CV) below 15% are considered "low-variation," that is, "reliable" [21]. Notably, both stigma and style exhibited the same minimum variation coefficient (CV = 9.43%), reinforcing their role as stable diagnostic characters for the taxon. Along with these, anther length (CV = 10.43%) also stands out as a stable character, supporting the general observation that

generative organs tend to possess higher taxonomic reliability than vegetative ones. In contrast, among vegetative characters, pedicel length (CV = 42.78%) and spathe length (CV approximately 37.19%) exhibited relatively high variation. The wide ranges observed in these measurements suggest that pedicel and spathe lengths may possess noticeable phenotypic plasticity, potentially influenced by fluctuating environmental conditions, such as light, moisture, or soil structure, or the phenological stage of the plant. Regarding leaf dimensions, width values were relatively narrow and stable (0.1–0.2 cm), representing a characteristic morphological feature of the taxon, whereas leaf length showed a wider range of variation (5.9–15.9 cm). When overall vegetative traits were evaluated, plant height varied between 10.7 and 19.7 cm (mean 15.19 ± 2.15 cm), and the number of leaves ranged from 3 to 7. In terms of generative characters, morphometric measurements of floral parts indicated that reproductive organs generally have lower coefficients of variation (CV) than vegetative parts, which may therefore reflect higher taxonomic reliability. Within the perianth segments, a size hierarchy was observed: outer tepals (mean 2.42 ± 0.30 cm) were larger than inner tepals (2.12 ± 0.32 cm). Additionally, anthers (1.15 ± 0.12 cm) were determined to be approximately twice as long as filaments (0.58 ± 0.09 cm). Stigma, style, and anther length were identified as the characters with the lowest variation, suggesting a potential high canalization or relative evolutionary stability. This situation aligns with the general botanical principle in plant systematics that floral characters often possess higher diagnostic value than vegetative characters [22]. Consequently, these relatively stable floral traits specifically the stigma, style, and anther dimensions along with the tepal proportions, can be recommended as reliable morphological criteria to be utilized in the identification of this taxon.

When previous studies conducted on the genus *Crocus* are examined, it is seen that morphological investigations have generally been carried out on the basis of different species and subspecies [1, 8-10, 13]. However, it is noteworthy that quantitative data on the morphological characteristics of *Crocus flavus* subsp. *dissectus* are limited, and that detailed measurements allowing intraspecific comparisons are lacking. In this study, the morphological characters of *C. flavus* subsp. *dissectus* are presented as a detailed dataset. The corm measurements obtained show parallelism with the values reported by Candan [6] in terms of the depressed globose–subglobose form of the corm and its size ranges. This confirms that corm size and general morphological form are characteristic and reproducible features for *C. flavus* subsp. *dissectus*. Similarly, the values for leaf number (3–7) and leaf width (1–2 mm) largely overlap with the leaf number (3–6(8) and width range (0.9–1.5 mm) reported by Candan [6]. These results demonstrate that, although leaf morphology remains within certain limits, it is a distinctive parameter for the taxon. In terms of generative organ morphology, the measurements of outer and inner tepals, when compared with Candan [6], show that the values for outer tepals (0.6–1.2 cm in width; 1.9–3.2 cm in length) and inner tepals (0.5–1.0 cm in width; 1.5–2.8 cm in length) support the ranges given in the literature. The fact that tepal dimensions constitute a stable criterion in the identification of *C. flavus* subsp. *dissectus* is also supported by other studies in the literature [13, 17]. In addition, filament, anther, and style lengths are consistent with the existing literature. In particular, the style being shorter than the stamens stands out as a critical feature in the differential diagnosis of the taxon [9, 10].

In the Flora of Turkey, *Crocus flavus* is defined by its membranous corm tunic that splits into coarse parallel fibers at the base and by persistent cataphylls forming a brown neck at the apex of the corm. In subspecies delimitation, style morphology is presented as the main diagnostic character; the style being distinctly divided into six or more thin branches is indicated as a diagnostic feature for subsp. *dissectus*. In the literature, this taxon has been reported to have a leaf width of 2.5–4 mm, perianth segment length of 1.5–3.5 cm, and filament length of 3–7 mm. When the results obtained from our specimens collected from the study area and identified as subsp. *dissectus* are compared, it is observed that floral characters (tepal length 1.5–3.2 cm; anther length 0.8–1.4 cm) show complete agreement with the literature data. In contrast, our findings indicate that leaf width remained between 1–2 mm (0.1–0.2 cm), below the lower limit reported in the literature (2.5 mm), while filament length, ranging from 4–9 mm (0.4–0.9 cm), slightly exceeded the upper limit reported in previous studies.

In particular, the highly divided structure of the style confirms that our specimens typically reflect the characters of subsp. *dissectus*. These morphological data contribute to the understanding of the variation limits of the taxon and could serve as supplementary local data for the description of the species in the Flora of Turkey. In conclusion, this study presents a comprehensive dataset by revealing the morphological limits of *C. flavus* subsp. *dissectus* with quantitative data. The obtained findings not only confirm the stability of the diagnostic characters of the taxon but also provide a valuable reference for future taxonomic studies.

Table 2. Pearson correlation coefficients between selected morphological characters of *C. flavus* subsp. *dissectus*

Character Relationship	Correlation Coefficient (r)	Significance (p)	Result
Outer Tepal Length & Inner Tepal Length	0.75	$p < 0.001$	Strong Positive Relationship
Spathe Length & Outer Tepal Length	-0.68	$p < 0.001$	Strong Negative Relationship
Leaf Number & Outer Tepal Length	-0.64	$p < 0.001$	Moderate-Strong Negative
Outer Tepal Width & Inner Tepal Width	0.40	$p < 0.001$	Moderate Positive Relationship

Pearson correlation analysis conducted to determine the interrelationships among characters revealed significant associations between the vegetative and generative organs of the taxon (Table 2). According to the analysis results, a strong and positive relationship was observed between the length of the outer and inner tepals ($r = 0.75$; $p < 0.001$). This indicates that the perianth segments develop in a synchronized manner. On the other hand, a significant negative correlation was found between the length of the spathe and the outer tepal length ($r = -0.68$; $p < 0.001$). These correlations among characters may reflect the plant's resource allocation patterns. The high positive correlation between outer and inner tepals can be considered taxonomic evidence supporting the consistency of flower morphology. The negative relationship between spathe length and tepal size suggests that excessive elongation of the spathe, a protective structure, may have a limiting effect on the size of floral organs. The negative association between leaf number and tepal size ($r = -0.64$; $p < 0.001$) suggests a potential resource allocation trade-off between vegetative growth and generative investment.

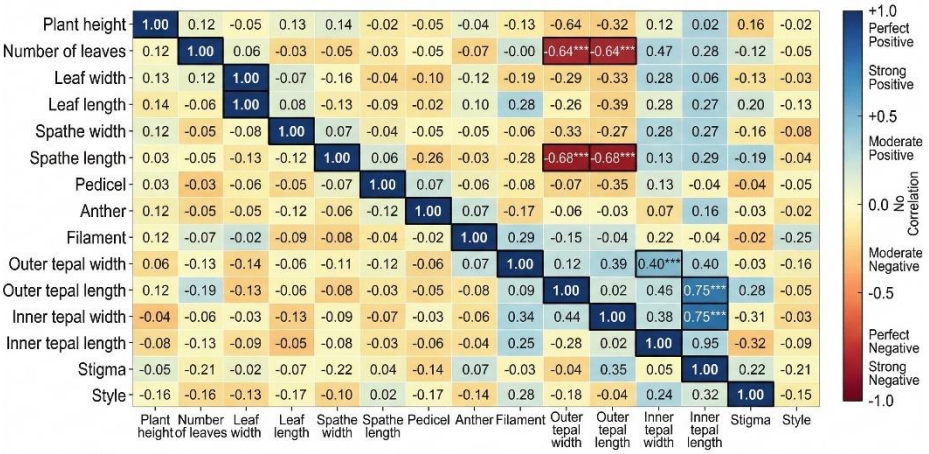

Figure 4. Heatmap of Pearson correlation coefficients (r) among morphological characters of *C. flavus* subsp. *dissectus* (N = 35). The color scale represents the degree of the relationship, ranging from -1 (perfect negative correlation) to +1 (perfect positive correlation). *** indicate significance at $p < 0.001$.

The patterns of morphological covariation observed in the heatmap provide insights into the morphological integrity of the taxon (Figure 4). The high correlation between the sizes of the outer and

inner tepals suggests that flower morphology is tightly coordinated. Furthermore, the tendency for tepal size to decrease as leaf number increases (negative correlation) is consistent with a potential growth-reproduction balance. Characters with low correlations with each other (e.g., leaf number and style length) exhibit a lack of strong linear relationship in the examined sample, suggesting they could potentially be evaluated together in future discriminatory analyses for taxonomic identification.

3.2. Ecological and Edaphic Findings

3.2.1 Soil moisture content

The soil samples collected from the natural habitat of *Crocus flavus* subsp. *dissectus* had a moisture content of 8.5%. Although Şik and Candan [23] did not report quantitative soil moisture values for the habitat of this taxon, they described its preferred ecological conditions as well-drained, loamy or clay-loam soils with moderate water retention capacity and without excessive moisture. The moisture content measured in the present study is generally consistent with these habitat characteristics. However, because soil moisture was measured only once during sampling, this value should be regarded as a site-specific observation rather than definitive evidence of the habitat's long-term drainage conditions or moisture regime.

3.2.2. Soil reaction (pH), redox potential, and electrical conductivity

The pH value of the soil samples collected from the study area was determined as 6.71, indicating that the soil reaction is close to neutral [20]. In the study conducted by Şik and Candan [23] on *Crocus flavus* subsp. *dissectus*, soil pH values were reported to range between 7.18 and 7.76 (slightly alkaline). Although the pH value obtained in our study is lower compared to the literature, its proximity to neutral indicates that the taxon can adapt not only to slightly alkaline but also to neutral–slightly alkaline soil conditions. This finding reveals that the species' ecological tolerance to soil reaction spans a broader spectrum than previously suggested in the literature.

The electrical conductivity (EC) of the soil sample was recorded as 167.3 µS/cm. Şik and Candan [23] reported EC values for the relevant taxon ranging from 210 to 1352 µS/cm, indicating that the species occurs in habitats with little to no salinity. Although the 167.3 µS/cm value we determined is slightly below the lower limit reported in the literature, it confirms that the soil is very low salinity. This low conductivity level supports the preference of *C. flavus* subsp. *dissectus* for non-saline soil conditions and can be considered a typical indicator of habitats that are undisturbed by anthropogenic effects and maintain their natural structure. Additionally, the redox potential (Eh) of the soil samples was measured as 130.4 mV. This value provides information on the soil's oxidation–reduction balance and aeration capacity and biogeochemically supports the "well-drained" soil structure noted in the previous sections.

3.2.3 Soil calcium carbonate ($CaCO_3$) levels

The calcium carbonate ($CaCO_3$) content in the soil samples was determined using the Scheibler calcimeter method. In this analysis, the volume of CO_2 gas released from 1 g of soil due to the acid reaction was recorded in two replicates (3 mL and 2 mL), and pressure correction factors to ensure analytical precision. Calculation of the lime percentage was based on the principle that CO_2 constitutes 44% of the $CaCO_3$ structure; thus, the measured CO_2 mass was multiplied by a constant factor of 2.273 to calculate the percentage of lime [20]. According to the standard soil classification based on lime content (Table 3), the study area falls within the Non-calcareous to Calcareous categories, supporting the taxon's ability to adapt to diverse edaphic conditions within the Turkish flora. In the study conducted by Şik and Candan [23], the lime content of soils where *Crocus flavus* subsp. *dissectus* occurs was reported to range between 0.23% and 12.87%. The $CaCO_3$ values determined in the present study are fully consistent with established literature ranges. These findings confirm that the taxon thrives within

its known ecological tolerance limits and supports its adaptation to calcareous or partially calcareous edaphic conditions typical of the Turkish flora.

Table 3. Soil Classifications Based on Lime Content [20]

% $CaCO_3$	Soil Class
0-2	Non-calcareous
2-4	Slightly calcareous
4-8	Moderately calcareous
8-15	Calcareous
15-50	Highly calcareous
Greater than 50	Very highly calcareous

3.2.4 Ash Analysıs of aboveground and underground plant organs

To determine the total ash content of *C. flavus* subsp. *dissectus*, ash analysis was performed in four replicates (n = 4) for both above-ground and below-ground organs. The samples were subjected to complete combustion (calcination) in a muffle furnace at 550 °C. The initial weights for the above-ground organs and the storage organs (corms) were recorded, and the resulting inorganic residue data are presented in Table 4.

Table 4. Ash Analysis Data of *C. flavus* subsp. *dissectus* Organs (n = 4)

Samples	Crucible No	Initial Weight (g)	Ash Weight (g)	Ash Content (%)
Aboveground Organs	1	0.8	0.2	25
	2	0.78	0.22	28.21
	3	0.77	0.23	29.87
	4	0.77	0.23	29.87
Mean				28.24 ± 2.3
Underground Organs	1	0.64	0.45	70.31
	2	0.66	0.43	65.15
	3	0.68	0.34	50
	4	0.6	0.56	93.33
Mean				69.70 ± 17.9

According to the analysis results, the average ash content in the above-ground organs was determined to be 28.24%. This suggests that the aerial parts of the plant are primarily composed of organic matter, with a relatively low proportion of inorganic minerals. In stark contrast, the ash content in the below-ground organs was remarkably high, averaging 69.70%. This significant difference (approximately 2.5-fold) indicates that the corms are highly enriched in mineral accumulation compared to the vegetative tissues.

The high ash percentage in the below-ground organs can be attributed to the corm's continuous contact with the soil and its physiological role as a specialized storage organ that sequesters minerals throughout the plant's life cycle. Furthermore, the morphological structure of the *Crocus* corm, which allows for high retention of edaphic (soil-related) minerals, further supports the elevated inorganic residue detected.

4. DISCUSSION AND CONCLUSION

Within the scope of this study, the morphological boundaries, habitat characteristics, and inorganic matter content of *Crocus flavus* subsp. *dissectus*, a taxon of critical importance for the flora of Türkiye,

have been comprehensively elucidated using quantitative data. Morphological measurements and coefficient of variation (CV%) analyses demonstrated that the floral organs (particularly the stigma and anther) possess significantly higher genetic stability compared to vegetative organs. The multi-part structure of the style confirms the identification of subsp. *dissectus*; however, variations observed in characters such as leaf width and plant height indicate morphological flexibility in response to environmental conditions. Notably, the fact that leaf width falls below the lower limit reported in the literature highlights the necessity for a revision of intraspecific variation limits.

Edaphic analysis results indicate that *C. flavus* subsp. *dissectus* exhibits optimal development in well-drained, non-saline soils with a pH range from neutral to slightly alkaline and low lime content. These findings align with the general ecological preferences of the genus *Crocus*; the majority of taxa distributed across the Mediterranean and Irano-Turanian regions thrive in well-drained, mineral-rich, and non-excessively calcareous soils [9,23]. The recorded moisture content of 8.5% and positive redox potential (130.4 mV) quantitatively support the taxon's affinity for habitats with high aeration capacity. Its broad tolerance to calcium carbonate content indicates the taxon's ability to occur in diverse geological formations.

Ash analysis revealed that the corm accumulates approximately 2.5 times more mineral matter (69,70%) than the aboveground organs. In soils with an alkaline but low-lime status, the availability of phosphorus and microelements is more balanced, supporting the mineral storage capacity of geophytes [24]. This suggests a strong relationship between soil properties and physiological adaptation, indicating that the corm is not only an energy reserve but also a strategic storage unit for critical inorganic elements. However, conditions leading to excessive moisture can increase the susceptibility of corms to rot and fungal infections [25]. Consequently, high drainage capacity is a significant ecological factor contributing to population stability.

Salt stress has been demonstrated to restrict growth through osmotic stress and ion toxicity [26]. The low coefficient of variation of the flower organs (stigma and anther lengths) in this study suggests that the taxon thrives in stable microhabitat conditions and maintains its morphological integrity in environments free from salinity stress. A considerable proportion of taxa of *Crocus* native to Anatolia are at risk of extinction due to habitat fragmentation and changes in land use [23]. The dependency of the studied population in Eskişehir on slightly alkaline and low-lime soils has been demonstrated to increase habitat specificity, which can enhance the species' sensitivity to environmental changes. Consequently, soil characteristics are not only an ecological parameter but also a fundamental factor to be considered in a conservation plan.

Although this study has delineated the morphological and edaphic framework of the taxon, it is recommended that its conservation status and resilience to climate change scenarios be supported with molecular marker studies and long-term phenological observations. In addition, analyzing the high mineral content detected in the corm on a specific element basis (using methods such as ICP-MS) will contribute to understanding the species' pharmaceutical potential.

ACKNOWLEDGEMENTS

This study was supported by the Scientific and Technological Research Council of Turkey (TÜBİTAK) under the 2209-A University Students Research Projects Support Program (Project ID: 1919B012424522)

CONFLICT OF INTEREST

The authors stated that there are no conflicts of interest regarding the publication of this article.

CRediT AUTHOR STATEMENT

İrem Bayram: Conceptualization, Investigation, Methodology, Writing - original draft**. Nagehan Saltan:** Supervision, Project administration, Formal analysis, Software, Validation, Funding acquisition, Writing - review & editing. **Gülçin Işık:** Investigation, Methodology, Resources, Data curation, Visualization, Writing - review & editing.

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ESKİŞEHİR TEKNİK ÜNİVERSİTESİ BİLİM VE TEKNOLOJİ DERGİSİ
C- YAŞAM BİLİMLERİ VE BİYOTEKNOLOJİ

Eskişehir Technical University Journal of Science and Technology
C -Life Sciences and Biotechnology

Estuscience – Life, 2026, 15 (2) pp. 176-188, DOI: 10.18036/estubtdc.1931129

RESEARCH ARTICLE

COMPARISON OF *IN VITRO* REGENERATION POTENTIALS OF COWPEA (*Vigna unguiculata* (L.) Walp) AND SELLUKA (*Vigna caracalla* (L.) Verdc.) SPECIES

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Abstract

Cowpea (*Vigna unguiculata* (L.) Walp), which belongs to the genus Vigna, has a very important role in both food and pharmaceutical uses in many countries of the world. Caracalla bean (*V. caracalla* (L.) Verdc.) whose flowers have volatile compounds is a perennial ornamental plant. The current study aimed to examine the differences between plant tissue culture stages and also regeneration potential in two different *Vigna* species, cowpea (*Vigna unguiculata* (L.) Walp) and caracalla bean (*Vigna caracalla* (L.) Verdc.). In order to determine the appropriate medium composition, callus cultures were established in MS and B5 media containing 2 mg/L 2,4-D with 0.2, 1 and 2 mg/L KIN concentrations. Then, the obtained calli were subcultured in media prepared by adding 10-50 g/L sucrose that gave the highest callus regeneration. The highest callus regeneration (76.66%) was obtained in MS medium containing 0.2 mg/L KIN in caracalla bean and 2 mg/L KIN in cowpea, while a higher regeneration rate was observed in MS nutrient medium than B5 for both species in all experiments. In addition, indirect root regeneration occurred in cowpea plant (46.67%), but a similar situation did not occur in caracalla bean. When the effect of sucrose was examined, the growth index tended to decrease as sucrose concentration increased in caracalla bean plants, whereas cowpea exhibited the opposite response. These results indicate that genotype plays a critical role in the *in vitro* culture response of *Vigna* species.

Keywords

Cowpea,
Caracalla bean,
Callus culture,
Regeneration potential,
Growth index,

Time Scale of Article

Received :15 April 2026
Accepted : 16 July 2026
Online date :27 July 2026

1. INTRODUCTION

Grain legumes are important sources of plant-based protein for human nutrition and are also valuable components of livestock feeding systems. They also contribute greatly to sustainable agriculture by fixing atmospheric nitrogen through their symbiotic relationship with rhizobial bacteria [1]. Despite this,

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climate change, loss of arable land, and unsustainable farming practices continue to undermine global food security, with disproportionate consequences for low-income and developing countries. Within this framework, alternative biotechnological methods, particularly plant tissue culture and *in vitro* regeneration systems, represent promising approaches to supporting genetic improvement, conservation efforts, and the sustainable cultivation of leguminous crops [2].

The genus *Vigna* in the family Fabaceae includes more than 100 wild species. This taxon is agriculturally important and includes 10 cultivated species, such as cowpea (*Vigna unguiculata (L.) Walp)*, mung bean (*Vigna radiata* (L.) Wilczek), and azuki bean *(*Vigna angularis (Willd.)). Since some of their wild relatives live in extreme environments such as arid lands, sandy areas, and limestone karsts, they are expected to harbor adaptive genes that can be used to develop stress-tolerant crops for agriculturally unsuitable lands. Furthermore, these legumes have the potential to contribute to low-input sustainable agriculture, as they have developed a symbiotic relationship with rhizobial bacteria that adapt to these extreme environments and contribute to nitrogen fixation [3]. Some wild species of *Vigna* are well known for various uses such as soil improvement , human food, medicinal plants, soil erosion prevention materials and animal feed. Legumes such as Bambara groundnut (*V. subterranea*), black lentil (*V. mungo*), pea (*Pisum sativum*), mung bean (*V. radiata*) and kidney bean (*Phaseolus vulgaris*) cover about 20-57% of the arable land in different countries because of their properties. Seeds of almost all species of *Vigna* have antioxidant properties and are used to treat different diseases such as rheumatism, liver diseases, diabetes, cough, cancer, fever, microbial infections, kidney disorders, stroke, hormonal disorders and for weight loss. Therefore, further research is needed to determine the pharmacognostic importance of individual components of these species. [4].

Cowpea (*Vigna unguiculata* (L.) Walp) is one of the most important grain legume, cultivated both as a food crop and animal feed in many regions, especially in sub-Saharan Africa [5,6]. Since it can be produced at low cost in arid and semi-arid regions, it is cultivated in low-income countries. It is also suitable for growing with different grains because it provides nitrogen fixation. It is estimated that 6.5 million tons of cowpea production is made on 14.5 million hectares of land worldwide [5]. It is mostly grown by farmers who sell the fresh or dried seeds, fresh pods and leaves as vegetables, the plant's green or dry residue (leaves and stems, even seeds) can be used as animal feed. Young cowpea leaves are eaten as a potherb and enjoyed in many parts of Africa. Cowpea shoots and leaves are rich sources of calcium, phosphorus and B vitamins [7].

Caracalla bean (*Vigna caracalla* (L.) Verdc.) is another species within the genus *Vigna* [8]. This tuberous, twining perennial, native to South and Central America, is widely recognized for its fragrant flowers and ornamental value, particularly for pergola decoration where its climbing habit is commonly utilized. In addition, its fresh fruits and dried grains are suitable for use as food. The important characteristics of the caracalla bean are its pleasant smell and its colorful flowers. However, caracalla bean is a plant species that is not very common in terms of usage areas compared to other plants of the genus *Vigna*, has not been studied much, and its production has decreased recently due to reasons such as low germination rate and difficulty in pollination [9]. It blooms during the rainy season from January through the end of March. Its flowers have volatile compounds and a total of 29 compounds have been identified [10].

Plant tissue culture applications are a technology that allows the production of higher quality and higher efficiency products [11]. In plant tissue culture, shoot and root regeneration in explants can be stimulated by various factors. Modifications to the nutrient medium composition or changes in environmental conditions are examples of these applications [12]. In plant tissue culture studies, the nitrogen source is one of the most important components affecting the morphogenic response. The regenerative capacity of explants generally depends on the ratio of nitrogenous compounds in the nutrient medium. In micropropagation studies, there are many different nutrient media for different plant species and desired response [13]. Sani et al., (2015) stated in their review studies that successful results were generally

obtained in studies conducted with MS nutrient medium in *Vigna* species [14]. In many studies in the literature similarly, MS nutrient medium was used [15-21]. Most researchers have noted the use of important MS mineral salts in combination with B5 vitamins [14,22,23]. In an experiment conducted by Diallo et al. (2008) in cowpea, B5 and MS nutrient media were tested for micropropagation purposes. As a result of the study, B5 nutrient medium was found to be more effective for multiple shoot formation, while MS nutrient medium was found to be more effective for shoot elongation [24]. In plant tissue culture applications, the ratio of auxins to cytokinins is also critical for ensuring successful plant regeneration. Different cytokinins such as BAP, KIN and zeatin have important effects on processes such as regulation of cell division and organ dedifferentiation [25]. Adenine-type cytokinins such as KIN and BAP play important roles in plant cell metabolism, including plant growth, development, and stimulation of anabolic and catabolic processes. KIN significantly affects cell proliferation and development [26]. Different auxins such as 2,4-D, NAA and IBA are effective on different regeneration processes such as cell elongation and root initiation [25]. Studies have shown that the morphogenic response in explants is related to components of the culture, such as nutrient salts and PGRs. Even within the same species, different salt requirements can be seen in the response to PGRs at different tissue culture stages [27].

Callus culture, one of the plant tissue culture techniques, is an extremely important source, especially to produce high-quality shoots and bioactive phytochemicals [28]. The most common problems encountered in callus cultures are slow growth and high biochemical variability. To prevent these problems, it is necessary to carry out studies on the optimization of the nutrient environment and culture conditions [29]. There are four critical steps in the process of ensuring callus regeneration in plant tissue culture studies. These can be listed as determining the basic nutrient medium composition, selecting the PGRs to be used, deciding on the correct genotype and determining the appropriate explant [1]. Callus formation can occur in the absence of exogenous factors and is usually associated with the balance of exogenously applied synthetic or organic auxins and cytokinins. The balance between these two PGRs has a decisive effect on the fate of the callus [30]. Many studies have achieved full plant regeneration through callus culture in legumes. Vats (2012) stated that the highest callus regeneration rate in cowpea was obtained as 90% in the nutrient medium containing 5 ppm KIN and 1 ppm NAA [20]. In the study conducted in *V. mungo* (L.) Hepper, it was determined that embryogenic calli from leaf explants were regenerated in MS medium containing 1.5 mg/L 2,4-D [31]. In the study conducted on mung bean, a 90% callus regeneration rate was observed in MS medium containing 3 mg/L 2,4-D. While the callus weight value per explant was determined as 1.11 g, it was reported that the addition of BAP made the calli harder and browner [32].

Sucrose, which consists of glucose and fructose, is an important energy and carbon source for plants [33]. Since sucrose supports the plant's photosynthetic activity, it is an essential carbon source for *in vitro* cultures. The carbohydrates provided by this carbon source maintain cellular osmotic potential and promote growth. In addition, sucrose added exogenously to callus cultures supports cellular respiration and therefore, increases cellular biomass. This situation is explained by a series of chain reactions. First, osmolarity increases and sucrose breaks down into its building blocks, glucose and fructose. Sucrose entry into the cell increases osmolarity and turgor pressure. This high turgor pressure causes the cell to elongate. Glucose and fructose undergo glycolysis and enter the Krebs cycle for ATP (energy) production. The ATP produced is also used for cell growth [34].

Cowpea has recently attracted increasing research attention due to its nutritional value and potential medicinal properties. Owing to its rich secondary metabolite and nutrient content, it is an important protein source in countries affected by famine. Like other legume plants, cowpea is also recalcitrant against *in vitro* regeneration and transformation, and this restricts tissue culture applications [30]. Although the caracalla bean, a plant of the same genus, is important in ornamental planting, research on it has only recently begun to intensify. In addition, being closely related to cowpea makes the caracalla bean valuable. Although developments in plant biotechnology have continued rapidly in the last 30

years, it is seen that more studies are needed on the *in vitro* propagation of cowpea rapid and space-efficient propagation. Therefore, these two plant species, *V. unguiculata* (L.) Walp and *V. caracalla* (L.) Verdc., were selected for this study to compare their responses under the same *in vitro* culture conditions. In this study, the callus initiation and regeneration potential of leaf-derived explants obtained through seed germination of two legume species were compared, and differences were identified.

2. MATERIAL AND METHODS

2.1. Material

In the study, leaves of young plants of young cowpea and caracalla bean plants, obtained by germinating seeds in pots containing peat, were used as starting material.

2.2. Explant Sterilization

Leaves from the caracalla bean plant were taken and shaken in distilled water with Tween-20 drops for 30 seconds. Then, they were shaken in 70% ethyl alcohol for 1 minute, in 0.1% $HgCl_2$ solution for 2 minutes, and finally, they were shaken in sterile water for 1 minute three times. In the case of the cowpea plant, the cut leaves were shaken in 70% ethyl alcohol for 1 minute, in 0.1% $HgCl_2$ for 3 minutes, and rinsed in sterile distilled water three times. The sterilized leaves were cut and wounded with a scalpel in 4 cm^2 areas so that their dimensions were 2 cm-2 cm.

2.3. Determination of Appropriate Basal Nutrient Medium and KIN Dose

In the study, B5 and MS based nutrient media were used for different doses of KIN. 2 mg/L 2,4-D was added to all experiments. 30 g/L sucrose and 3 g/L gelrite were added as the carbon source and gelling agent, respectively (pH: 5.8) (Table 1). Cut and wounded explants were transferred to culture vessels. All experiments were maintained at 24±2 °C under 3,500 lux light, 16 h light/8 h dark photoperiod.

Table 1. Compositions of MS and B5 nutrient media supplemented with different kinetin doses [1].

Basal Medium	**Doses of KIN (mg/L)**		
	0.2	1	2
B5	B5K1	B5K2	B5K3
MS	MK1	MK2	MK3

[1] In cowpea trials, codes were coded by adding "X".

2.4. Determination of the Effects of Different Doses of Carbon Source

In the first stage of the study, the most successful nutrient medium composition was determined to be MS medium containing 0.2 mg/L KIN and 2 mg/L 2,4-D and this composition was used in that stage. Calli without leaf tissue were weighed as 0.5 grams and transferred to nutrient media containing different doses of sucrose (10, 20, 30, 40 and 50 g/L). Finally, all established cultures were maintained at 24±2 °C under 3500 lux light, 16 h light/8 h dark photoperiod. Growth index values were determined at the end of 14 days of culture. For this;

$$\text{Growth index} = \frac{Biomass\ at\ the\ end\ of\ culture\ (g)}{Initial\ inoculum\ amount(g)} \quad (1)$$

formula was used. In addition, hardness values of the calli were calculated in the study. For this purpose, the soft ones were scored as "1", the medium-hard ones as "2" and the hard ones as "3" and the average score was calculated for each trial.

2.5. Statistical Evaluation

Throughout the study, experiments were conducted according to the randomized plot design with 10 explants per experiment and 3 replications. The Minitab 17 Statistical Software (Minitab Inc, PA, USA) program was used to evaluate the obtained data.

3. RESULTS

3.1. Callus Regeneration

In the study, observations were made in two-week periods and the data obtained as a result of 30-day observation were analyzed. Evaluation of the data indicated that the KIN dose alone was not an effective parameter, whereas the interaction between the species and the KIN dose was an influential factor. When this interaction was examined, the highest callus regeneration rate was obtained at 0.2 mg/L KIN for caracalla bean (58.83%) and at 2 mg/L KIN for cowpea (56.00%). In addition, these two values were in the same statistical group. Callus regeneration decreased with increasing KIN dose in caracalla bean but increased in cowpea. When the effect of the nutrient media was examined alone, a significant difference among plant species was observed (Table 2). As a result of the statistical analysis performed, a higher rate of callus regeneration was observed in MS nutrient medium for both species, while the regeneration rate in B5 nutrient medium remained low (Figure 1). No significant difference was observed among the species for the same nutrient medium.

Table 2. Callus regeneration ratio (%)±SE determined in explants in different nutrient medium compositions [1]

KIN (mg/L)	**0.2**		**1**		**2**		**Mean**
	Cb	**C**	**Cb**	**C**	**Cb**	**C**	
MS	76.67±6.44	60.67±8.17	62.00±2.00	59.00±13.1	48.33±6.69	76.67±1.67	63.89±3.53^{a}
Mean	68.67 ±5.87		60.50±5.97		62.50±7.05		
B5	41.00±17.9	5.33±0.67	35.00±12.5	36.00±9.50	32.00±10.4	35.33±8.11	30.78±4.75^{b}
Mean	23.2±11,3		35.50±7.04		33.67±5.94		
Mean	58.83±11.7^{a}	33.00±12.9^{b}	48.50±8.29^{ab}	47.50±8.88^{ab}	40.17±6.63^{ab}	56.00±9.96^{a}	

	p Value
KIN Doses (K)	0.935
Species (S)	0.508
Medium (M)	0.000
K*S	0.016
K*M	0.284
S*M	0.227
K*S*M	0.523

[1] The differences between the mean values shown with different letters in the same column are significant at the P≤0.01 level according to the Tukey multiple comparison test. SE: Standart error, (Cb: Caracalla bean, C: Cowpea)

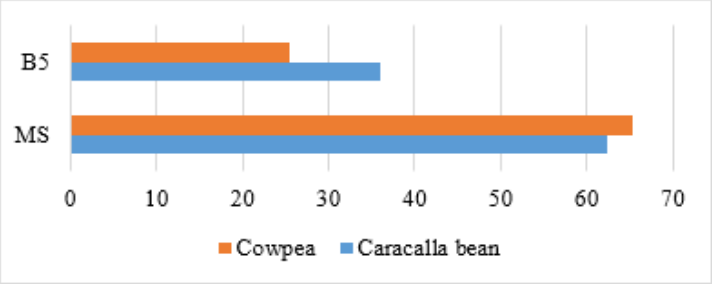

Figure 1. Comparison of mean callus regeneration percentage (%) in different basal nutrient media.

3.2. Color Change Rates and Textures in Regenerating Calli

In the study, calli from different species with varying colors and degrees of hardness were observed in different nutrient media (Fig. 2). It was observed that the proportion of green calli obtained in B5 nutrient medium was higher than that obtained in MS nutrient medium; however intensely darkened calli developed in cowpea explants cultured in B5K1 coded medium. In the caracalla bean plant, the calli were generally dark in color (blackening) (Fig. 3). Additionally, the calli obtained from the cowpea plant were generally soft whereas those from the caracalla bean plant were harder.

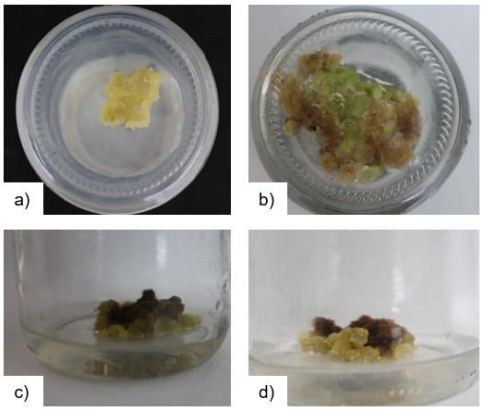

Figure 2. Callus formation in cowpea explants 8 days after culture: a) yellow callus; b) dark yellow-green callus; c) dark green callus; d) brown callus.

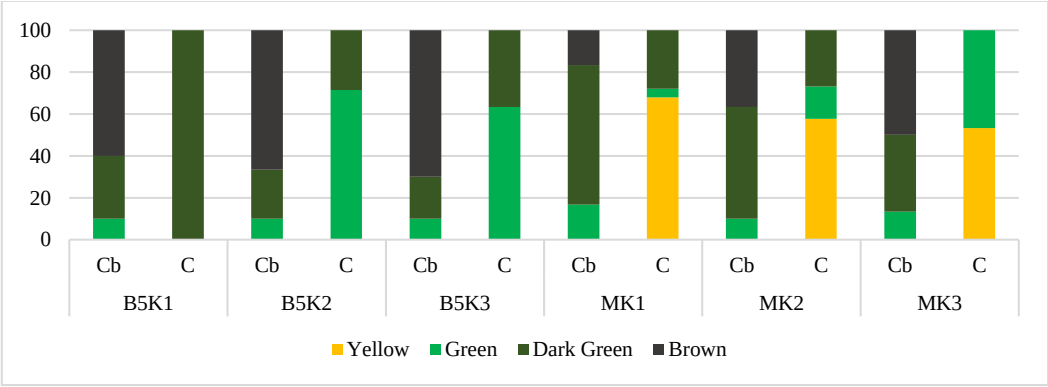

Figure 3. Color changes observed in regenerated calli under different kinetin doses and nutrient media in two plant species (Cb: Caracalla bean, C: Cowpea).

3.3. Root Regeneration from Calli

In the study, it was determined that root regeneration occurred from calli subcultured in the same nutrient medium (Fig. 4). That the roots were generally hairy and fibrous in appearance. When the two species were compared, rooting was observed in cowpea explants but not in caracalla bean explants. Although it was observed that the nutrient medium influenced root regeneration, KIN had a similar effect. Although the root regeneration percentage was higher in experiments using B5 nutrient medium, no statistically significant difference was found between the datasets. When the nutrient doses were examined, the highest root regeneration rate (46.67%) was obtained for cowpea explants cultured in B5 nutrient medium containing 0.2 mg/L KIN. Root regeneration decreased significantly with increasing KIN doses (Table 3).

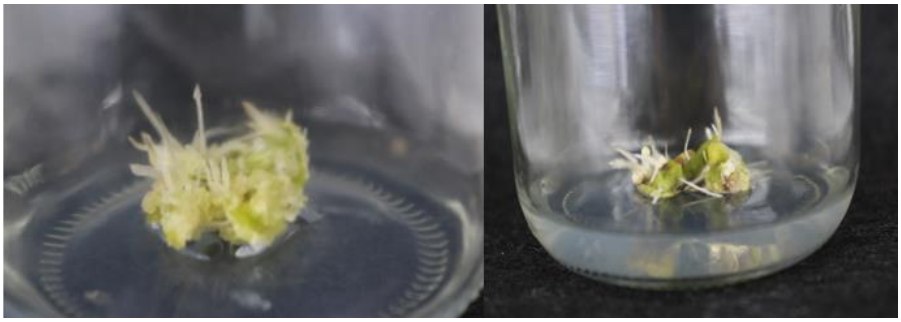

Figure 4. Root regeneration in cowpea explants

Table 3. Root regeneration ratio (%)±SE observed in calli in different nutrient medium compositions[1]

KIN (mg/L)	0.2		1		2		Mean
	Cb	C	Cb	C	Cb	C	
MS	0.00±0.00^{b}	16.67±8.82^{b}	0.00±0.00^{b}	13.33±8.82^{b}	0.00±0.00^{b}	0.00±0.00^{b}	5.00±2.46
Mean	8.33±5.43^{b}		6.67±4.94^{b}		0.00±0.00^{b}		
B5	0.00±0.00^{b}	46.67±6.67^{a}	0.00±0.00^{b}	6.67±3.33^{b}	0.00±0.00^{b}	0.00±0.00^{b}	8.89±4.17
Mean	23.3±10.9^{a}		3.33±2.11^{b}		0.00±0.00^{b}		
Mean	0.00±0.00^{b}	31.67±8.33^{a}	0.00±0.00^{b}	10.00±4.47^{b}	0.00±0.00^{b}	0.00±0.00^{b}	
			p Value				
KIN Doses (K)			0.000				
Species (S)			0.000				
Medium (M)			0.121				
K*S			0.000				
K*M			0.011				
S*M			0.121				
K*S*M			0.011				

[1] The differences between the mean values shown with different letters in the same column are significant at the P≤0.01 level according to the Tukey multiple comparison test. SE: Standart error, (Cb: Caracalla bean, C: Cowpea)

3.4. Determination of the Impact of the Carbon Source

In the initial stage of the study, callus regeneration experiments were conducted to compare the B5 and MS culture media. Based on the results, the MS medium exhibited a higher regeneration efficiency than the B5 medium. Therefore, the MS medium was selected for use in the subsequent stages of the study (Table 2). In this study, to determine the effect of sucrose on regenerated calli, experiments were initiated with 0.5 g of inoculum and growth index values were calculated at the end of the 14-day culture period. The highest growth index was obtained for caracalla bean plant in the medium containing 20 mg/L sucrose (Table 4). A lower growth index was observed in the cowpea plant in the same medium. The most suitable sucrose concentration was 20 mg/L for the caracalla bean plant and 50 mg/L for the

cowpea plant (Fig. 4). In the study, the calli obtained from caracalla bean plant were generally brown, while the calli obtained from the cowpea plant were more yellow. When the degrees of hardness of the calli were examined, were found to be caracalla bean calli were found to be structurally harder, similar to KIN experiments, whereas cowpea calli were softer. In caracalla bean plants, increasing the sucrose dose increased hardness but decreased the growth index. In the cowpea plant, the hardest calli were obtained in the MK1S3 trial containing 30 g/L sucrose. Although the softest calli were observed in the MK1S2 trial containing 20 g/L sucrose in the cowpea plant, the highest growth index value was obtained in the same trial but in the caracalla bean plant (Table 4, Fig. 5).

Table 4. Growth index values obtained at different sucrose doses[1]

	MK1S1	**MK1S2**	**MK1S3**	**MK1S4**	**MK1S5**
Caracalla bean	1.44^{ab}	1.50^{a}	1.07^{c}	1.22^{bc}	1.17^{c}
Cowpea	1.17^{c}	1.24^{abc}	1.22^{bc}	1.26^{abc}	1.45^{ab}
			p Value		
Species (S)	0.699				
Medium (M)	0.005				
S*M	0.000				

[1] The differences between the mean values shown with different letters in the same column are significant at the P≤0.01 level according to the Tukey multiple comparison test.

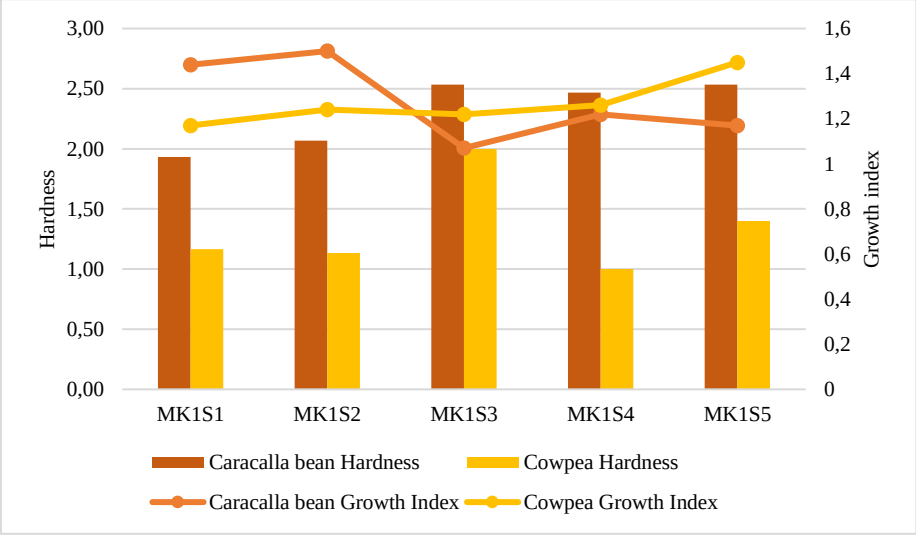

Figure 5. Determination of the effect of sucrose on the growth index and hardness of callus (calculated by coding as hard: 3, medium hardness: 2, soft: 1.)

4. DISCUSSION

Genotype is a very important factor affecting the regeneration potential in plant tissue culture. The ability of a plant to regenerate through tissue culture is largely determined by its genetic background, which governs cellular totipotency, hormone sensitivity, and the efficiency of organogenesis or somatic embryogenesis. Various plant species and even different varieties within a species exhibit varying levels of totipotency. Some genotypes readily produce shoots and roots, while others are resistant to tissue culture [35]. The data obtained in the study showed that the two species, regardless of how closely related they were, gave different responses. In the first stage of the study, the same sterilization method was applied to both plant species, but it was not suitable for cowpea. The reason for this is that cowpea

leaves are more sensitive than caracalla bean. Therefore, changes were required in the sterilization method for cowpea plants. Surface sterilization of the leaves of the caracalla bean was successful.

The composition of the nutrient medium is of great importance in micropropagation studies. For this reason, numerous studies are being carried out to determine the basic nutrient medium for plant tissue culture. All these studies have shown that the ionic composition of the nutrient medium and the ratio of major ionic elements are effective on biological processes such as growth, development and reproduction [26]. In the study, MS and B5 nutrient media were used to determine the basic nutrient medium. Although MS was used in previous years, studies in which B5 vitamins were added to the MS nutrient medium are also frequently encountered. For this reason, a similar path was followed in this study and the effects of MS and B5 nutrient media on the two species were compared. Analysis of the data showed that the highest callus regeneration percentage occurred in the MS nutrient medium for both species. Balapour (2019) stated in his study that the reason for this situation is related to the higher nitrogen (ammonium nitrate) content of the MS nutrient medium. [36].

In the study, the effect of KIN dose on callus regeneration investigated, and different results were obtained for both species. Increasing the KIN dose in the cowpea plant increased callus regeneration, whereas the opposite effect was observed in the caracalla bean plant. Although the result of KIN increasing cell proliferation in cowpea plant is related to Kaya et al., (2021), the completely different results of these species which are genetically very close to each other are explained by [27]. In the study, the highest callus regeneration rate obtained in cowpea plants was 76.67% in MS nutrient medium containing 2 mg/L KIN and 2 mg/L 2,4-D, and this value was below the study conducted by Vats (2012) (90%) [20]. It is thought that the differences between the studies are due to the explant source. While hypocotyl explants were used in the study of Vats (2012), leaf explants were used in this study .

Callus regenerating in the caracalla bean plant, exhibited intense browning in the B5 nutrient medium, whereas in the cowpea plant, the density of yellow callus was high, particularly in the MS nutrient medium. It is thought that browning may be due to various causes. Oxidative browning is a common and severe problem in plant tissue cultures including callus cultures of some plant species, where browning is caused by accumulation and oxidation of phenolic compounds (Taghizadeh and Dastjerdi, 2021). Browning of calli resulting from phenol accumulation affects their morphology and physiological properties and may cause cell death and tissue degeneration [38]. While the callus regenerating in the cowpea plant was softer and looser in structure, the callus in the caracalla bean plant was harder and more compact. Initially, slow growth is observed along with browning of the callus, followed by cell death [39]. In the studies conducted by Iqbal et al (2022) on mug bean, 80-90% soft and brown callus was obtained in the MS nutrient medium containing 3 mg/L 2,4-D. In the case where the rate of 2,4-D was reduced to 1 mg/L, the callus hardened and gained a more compact structure [32].

Root regeneration has been observed in cultured explants undergoing indirect organogenesis. Organogenesis is defined as the process of obtaining new plantlets or plant parts from a plant organ or seed. Organogenesis can occur through callus (indirect organogenesis) or directly from tissue (direct organogenesis) [30]. In the study, these root regenerations occurred only in the cowpea plant, not in the caracalla bean plant. It is thought that the reason for this difference is due to genetic differences. In the cowpea plant, root regeneration decreased in relation to the increasing KIN dose. Although no statistically significant differences were found when nutrient media were compared, a higher root regeneration rate (8.89%) was observed in the B5 medium. It is thought that the reason for this situation is related to the ion balance of the nutrient media, as stated by Vacu et al., (2025).

When the effect of sucrose on growth index values was examined, significant differences were seen between both species, and the responses given to increasing sucrose dose were different. The growth index tended to increase with increasing sucrose dose in the cowpea plant, but generally decreased in the caracalla bean. The hardness of the calli and the growth index values showed an inversely

proportional relationship. It is thought that nutrient transport cannot reach the inner parts of the tissue in hard, compact calli. In softer calli, nutrient transfer occurs easily, and growth is encouraged. The importance of sucrose uptake for cellular growth and development in plants was reported by [40]. Tripatyh (2021) explained the decrease in callus regeneration with increasing sucrose dose by cellular water content [33]. In their studies with *V. mungo* in Muruganantham et al., (2010), they determined that high glucose and fructose levels reduce embryogenesis. The results obtained in our study are also similar to those of these researchers.

5.CONCLUSION

Members of the Fabaceae family (Leguminosae) are critically important and should be produced for both human and animal nutrition because of their high protein content. In this study, cowpea (Vigna *unguiculata (L.) Walp), which* has been extensively studied but still requires further investigation, and caracalla *bean (Vigna caracalla* (L.) Verdc.), which has been studied only a few times, were compared with respect to their *in vitro* culture potential. Although these two species are genotypically similar, they exhibited different responses under the same culture conditions. This is the most important indicator that plant tissue culture is genotype-dependent. In breeding programs and biotechnology applications, selecting genotypes with superior regenerative ability is important for transformation efficiency. Integrating genetic analyses into future studies and elucidating the points at which these differences originate are of great importance for understanding plant metabolism.

CONFLICT OF INTEREST

The authors stated that there are no conflicts of interest regarding the publication of this article.

CRediT AUTHOR STATEMENT

Berfin Nazlıcan Kaman: Investigation, Formal analysis, Writing – Original Draft. **Ilyas Aidinov:** Investigation, Formal analysis, Writing – Original Draft. **Senem Şen:** Conceptualization, Methodology, Investigation, Writing – Review & Editing. **Begüm Güler:** Conceptualization, Methodology, Validation, Formal analysis, Data Curation, Writing – Review & Editing, Visualization. **Aynur Gürel:** Conceptualization, Methodology, Validation, Resources, Data Curation, Writing – Review & Editing, Supervision, Project administration, Funding acquisition.

ACKNOWLEDGEMENTS

This project was supported by the TUBITAK 2209-A University Students Research Projects Support Program with the project number 1919B012004932.

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ESKİŞEHİR TEKNİK ÜNİVERSİTESİ BİLİM VE TEKNOLOJİ DERGİSİ
C- YAŞAM BİLİMLERİ VE BİYOTEKNOLOJİ

Eskişehir Technical University Journal of Science and Technology
C -Life Sciences and Biotechnology

Estuscience – Life, 2026, 15 (2) pp. 189-199, DOI: 10.18036/estubtdc.1935710

RESEARCH ARTICLE

DOSE-DEPENDENT PROTECTIVE EFFECTS OF IRISIN AGAINST BISPHENOL A-INDUCED OXIDATIVE STRESS AND CASPASE-3-MEDIATED APOPTOSIS IN AML12 HEPATOCYTES

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Abstract

Bisphenol A (BPA) is a widespread environmental contaminant that causes hepatotoxicity through oxidative stress and apoptosis. In the present study, the dose-dependent protective effect of irisin against BPA-induced hepatotoxicity in AML12 hepatocytes was evaluated. AML12 hepatocytes were treated with BPA (100 µM) with or without irisin (50 and 100 nM). Cell viability was measured by MTT test and cytotoxicity was measured by lactate dehydrogenase (LDH) release. The oxidative status of the cells was characterized by evaluating malondialdehyde (MDA) levels, total oxidant status (TOS), total antioxidant status (TAS), and superoxide dismutase (SOD) activity. In addition, the oxidative stress index (OSI) and caspase-3 activity were assessed to provide insight into cellular redox balance and apoptotic responses. BPA significantly reduced cell viability and antioxidant capacity, and increased LDH release, lipid peroxidation and apoptotic activity. However, irisin significantly ameliorated these changes in a dose-dependent manner. More specifically, irisin significantly decreased MDA, TOS, OSI, and caspase-3 activity and increased SOD activity and TAS levels, and cell viability. The higher dose of irisin (100 nM) was more effective than the lower dose. This study found that irisin alleviated BPA-induced hepatotoxicity by mitigating oxidative stress and caspase-3-mediated apoptosis, implying that irisin may represent a potential therapeutic candidate for the treatment of toxin-induced liver injury.

Keywords

AML12,
Apoptosis,
Bisphenol A,
Irisin,
Oxidative stress

Time Scale of Article

Received :22 April 2026
Accepted : 24 June 2026
Online date :27 July 2026

1. INTRODUCTION

Bisphenol A (BPA) is among the most widely manufactured synthetic chemicals and serves as a major constituent of polycarbonate plastics and epoxy resins. Owing to its extensive industrial applications, human exposure to BPA occurs through numerous consumer products, particularly food packaging materials, drinking bottles, and thermal paper. Due to its widespread usage and environmental persistence it is considered a ubiquitous contaminant and exposure to humans can occur through dermal absorption, ingestion and inhalation. The potential effects of BPA on health have been extensively studied and this chemical has become well known as an endocrine-disrupting chemical that can affect the regulation of hormones and cellular homeostasis [1,2]. Among the various organs that are examined, the liver is one of the organs most susceptible to BPA exposure due to its central function in xenobiotic metabolism and detoxification. Recent data indicate that a primary mechanism behind BPA-induced hepatotoxicity is oxidative stress. Accumulating evidence indicates that BPA induces oxidative stress by enhancing ROS production and impairing antioxidant defenses [3,4]. The resulting disturbance in cellular redox homeostasis can contribute to a wide spectrum of detrimental effects, such as membrane lipid peroxidation, protein oxidation, and DNA lesions. An increase in lipid

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peroxidation and the resulting concentration of the mildly reactive aldehyde malondialdehyde (MDA) has been shown upon exposure to BPA, while the antioxidant enzymes superoxide dismutase (SOD) and catalase have been shown to decrease in several studies [5,6].

Evidence from earlier investigations suggests that BPA-induced oxidative stress is characterized by increases in both total oxidant status (TOS) and oxidative stress index (OSI), which collectively indicate impaired redox balance [7]. In addition to causing cellular damage, oxidative stress is also known to activate pro-apoptotic signalling pathways. In liver cells, the overproduction of ROS can lead to mitochondrial dysfunction, as pro-apoptotic factors are released. Activated caspases subsequently cleave various cellular substrates culminating in apoptotic cell death [8,9]. Recent data suggest that exposure to BPA can lead to a substantial elevation in caspase-3 activity and apoptotic cell death in hepatic cells [10]

In the past decade, irisin, a myokine released mainly during physical activity, has been proposed as a promising molecule with a wide array of biological functions. Although initially described as a pleiotropic regulator of energy metabolism, increasing evidence has indicated that irisin is involved in regulating several other physiological processes, such as oxidative stress, inflammatory responses, and apoptosis [11,12]. Several reports have shown that irisin has powerful antioxidant effects by upregulating endogenous antioxidant defenses and decreasing ROS production. Indeed, irisin was reported to increase the levels of SOD and glutathione peroxidase and to decrease lipid peroxidation [13,14]. Moreover, irisin could also exert anti-apoptotic effects by regulating intracellular signaling pathways, such as caspase-3 activation and AKT/mTOR signaling, leading to improved cell survival under oxidative stress conditions [15,16]. Nonetheless, limited information exists about the protective benefits of irisin against BPA-induced hepatotoxicity. Moreover, few studies have investigated the antioxidant and cytoprotective properties of irisin across various models, and the dose-dependent impact of irisin on oxidative stress and apoptosis in hepatocytes remains to be elucidated.

The objective of this study was to assess the dose-dependent protective effect of irisin against BPA-induced hepatotoxicity in AML12 hepatocytes by evaluating key oxidative stress biomarkers, including MDA, TOS, total antioxidant status (TAS), and SOD, along with apoptotic activity through caspase-3. This study utilized biochemical and apoptotic data to elucidate the mechanism of irisin-mediated cytoprotection and to bolster future endeavors in formulating viable treatment solutions against environmental toxicants.

2. MATERIALS AND METHODS

2.1. Chemicals and Reagents

Bisphenol A (BPA; purity ≥99%, Cat. No. 239658) and recombinant irisin (purity ≥95%, Cat. No. SRP8039) were procured from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12; Cat. No. 11320033), fetal bovine serum (FBS; Cat. No. 16000044), penicillin–streptomycin (Cat. No. 15140122), and trypsin–EDTA (Cat. No. 25200056) were sourced from Gibco (Thermo Fisher Scientific, Waltham, MA, USA). The residual compounds were of analytical grade. The research employed ultrapure water (Milli-Q System, Millipore, USA) consistently during the investigation.

2.2. Cell Culture

AML12 cells were cultured in DMEM/F12 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin (ATCC, CRL-2254™) at 37°C in a humidified atmosphere of 5% CO2. The medium was changed every 2 to 3 days and the cells were subcultured to 80 to 90% confluence by trypsin–EDTA. For MTT and LDH assays, cells were plated in 96-well plates at a

density of 1×10^4 cells per well. For biochemical and enzymatic assays, cells were plated in 6-well plates at a density of 2×10^5 cells per well. A method for irisin pretreatment was established to evaluate its protective effectiveness against BPA-induced cellular injury in AML12 hepatocytes. A previous study indicates that 2 hours of irisin pre-incubation enhances antioxidant defense mechanisms and reduces oxidative stress and apoptosis [17]. Furthermore, the equivalent pre-incubation duration was seen to reduce ROS production and apoptotic changes, indicating that a relatively short pretreatment period was appropriate for this study. AML12 cells were seeded onto the plates and allowed to adhere overnight. Subsequently, the cells were pretreated with irisin for 2 h prior to exposure to BPA.

Upon removal of the culture medium, AML12 cells were randomly divided into five experimental groups according to the study design: a control group that received only the culture medium; a BPA group that was administered 100 μM BPA; an irisin group that received solely irisin (100 nM); an IRI 50 + BPA 100 group that was treated with irisin (50 nM) for two hours before exposure to 100 μM BPA; and an IRI 100 + BPA 100 group that received irisin (100 nM) for two hours prior to exposure to 100 μM BPA.

2.3. Cell Viability and Cytotoxicity Assays

An MTT-based colorimetric technique was used to assess the effects of BPA and irisin on AML12 cell survival (Sigma-Aldrich, Cat. No. M5655). Upon completion of the experimental treatments, cells were exposed to MTT solution (0.5 mg/ml) and maintained at 37°C for an additional 3–4 h to allow formazan formation. A BioTek Synergy HTX microplate reader (USA) was used to measure absorbance at 570 nm after the purple formazan products were dissolved with DMSO (Sigma-Aldrich, Cat. No. D2650). The control group was used to standardize the viability values.

Membrane damage was assessed by quantifying lactate dehydrogenase release into the culture medium with a commercial assay kit (Abcam, Cat. No. ab102526, Cambridge, UK). LDH activity was determined based on absorbance measurements performed at 450 nm with a BioTek Synergy HTX microplate reader (USA), and the obtained values were expressed in U/L.

2.4. Preparation of Cell Lysates and Protein Determination

After treatment, AML12 cells were washed with cold PBS and lysed in RIPA buffer (Thermo Fisher Scientific, Cat. No. 89900) supplemented with protease inhibitors (Cat. No. 78430). The lysates underwent clarification by centrifugation (12,000 × g for 10 minutes at 4°C), and the obtained supernatants were utilized for biochemical assessments. Protein levels were subsequently evaluated using the Bradford assay with the Bio-Rad Protein Assay Kit (Cat. No. 5000002), employing bovine serum albumin to generate the calibration curve.

2.5. Determination of Oxidative Stress Parameters

The following oxidative stress markers were measured: MDA, SOD, TAS, TOS, and OSI. MDA levels were determined using a thiobarbituric acid reactive substances (TBARS) assay kit (Cayman Chemical, Cat. No. 10009055, Ann Arbor, MI, USA). Optical density was measured at 532 nm, and the results were expressed as nmol/mg protein. SOD levels were determined using a colorimetric assay kit (Cayman Chemical, Cat. No. 706002, Ann Arbor, MI, USA). Absorbance was measured at 450 nm, and the results were expressed as U/mg protein. Total antioxidant status (TAS) and total oxidant status (TOS) concentrations were determined using commercial kits (TAS: Cat. No. RL0017; TOS: Cat. No. RL0024) from Rel Assay Diagnostics, Gaziantep, Türkiye, in accordance with the manufacturer's instructions. TAS concentrations were expressed as mmol Trolox equivalent per liter, and TOS concentrations as μmol H2O2 equivalent per liter. The oxidative stress index (OSI) was

expressed in arbitrary units and was calculated by dividing total oxidant status by total antioxidant status and multiplying the result by 10 (OSI = TOS/TAS × 10).

2.6. Caspase-3 Activity Assay

Assessment of caspase-3 activity was performed with a commercial colorimetric kit (Abcam, Cat. No. ab39401). Equal quantities of protein from each sample were reacted with DEVD-pNA, and the liberated p-nitroaniline product was detected by measuring absorbance at 405 nm. The data represent U per milligram of protein.

2.7. Statistical Analysis

Statistical computations were conducted in the GraphPad Prism environment (version 11.0.0; GraphPad Software, San Diego, CA, USA), and numerical outcomes are presented as mean ± SD. Variance homogeneity was examined by Brown–Forsythe and Bartlett tests, whereas data distribution was evaluated using a normality test. Comparisons among experimental groups were performed using one-way analysis of variance (ANOVA), and Tukey's multiple comparison test was subsequently applied to identify differences between individual groups. A probability value below 0.05 was accepted as statistically significant, whereas p values lower than 0.01, 0.001, and 0.0001 were interpreted as reflecting increasing levels of statistical confidence. In addition, the coefficient of determination (R^2) was calculated to estimate the proportion of variability accounted for by the fitted model.

3. RESULTS

3.1. Effects of Irisin on Cell Viability and Cytotoxicity in BPA-treated AML12 Cells

The MTT and LDH assays were used to evaluate BPA-induced cytotoxicity and the protective effects of irisin pretreatment in AML12 cells. To evaluate treatment-dependent effects, one-way ANOVA was performed, followed by Tukey's post hoc analysis for comparisons between individual groups (Figure 1). Cell viability varied significantly between the experimental groups, according to a one-way ANOVA ($F(4, 10) = 60.55$; $p < 0.0001$; $R^2 = 0.9603$).Tukey's post-hoc test showed that treatment with 100 μM BPA significantly reduced cell viability to 59.30 ± 1.51% compared with the control group (97.36 ± 3.82%), indicating marked toxicity ($p < 0.0001$, Figure 1A). Pretreatment with irisin (50 and 100 nM) for 24 hours significantly attenuated this toxicity in a dose-dependent manner, with the greatest protective effect observed at 100 nM irisin (85.59 ± 3.53%; $p < 0.0001$ vs. BPA). The effect of 100 nM irisin was also significantly greater than that of 50 nM irisin (74.95 ± 4.22%; $p = 0.0213$). Treatment with irisin alone (100 nM) did not result in a significant difference relative to the control group (92.73 ± 3.19%; $p = 0.4893$), indicating that the dose used was not cytotoxic to AML12 cells.

LDH leakage measured cell membrane damage was significantly different between the experimental groups ($F(4, 10) = 262.1$; $p < 0.0001$; $R^2 = 0.9906$). The Brown-Forsythe test for homogeneity of variance was found to be homogeneous ($p = 0.9588$), indicating a lack of statistical artefact of the data (Figure 1B). Exposure to 100 μM BPA significantly increased LDH leakage to 454.2 ± 9.18 U/L compared with the control group (136.7 ± 11.24 U/L), indicating severe membrane disruption ($p < 0.0001$). Pretreatment with irisin significantly reduced BPA-induced leakage in a dose-dependent manner. Cells pretreated with 100 nM irisin showed a more pronounced protective effect than those pretreated with 50 nM irisin, with LDH levels of 186.1 ± 13.78 U/L and 287.3 ± 17.31 U/L, respectively ($p < 0.0001$ vs. BPA). Treatment with 100 nM irisin alone (129.4 ± 18.88 U/L) did not significantly alter LDH leakage compared with the control group ($p = 0.9694$).

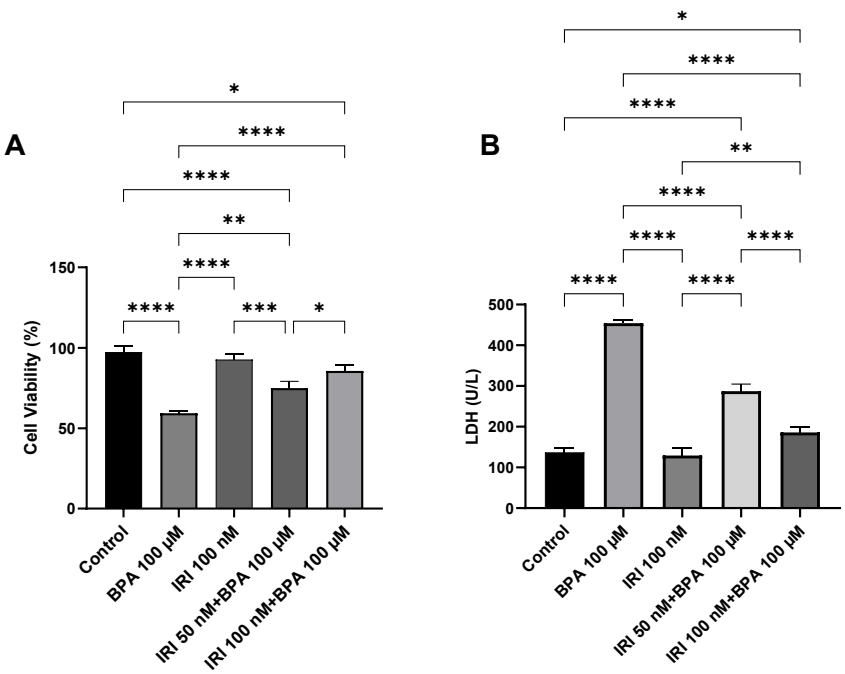

Figure 1. Effects of irisin on cell viability and cytotoxicity in BPA-treated AML12 hepatocytes. (A) Cell viability (%) assessed by the MTT assay. BPA exposure (100 µM) significantly reduced cell viability compared to the control group, whereas irisin pretreatment (50 and 100 nM) restored viability in a dose-dependent manner. (B) LDH release (U/L) as an indicator of cytotoxicity. BPA markedly increased LDH levels, while irisin pretreatment significantly attenuated this increase. Data are presented as mean ± standard deviation (SD) (n = 3). Statistical analysis was performed using one-way ANOVA followed by Tukey's post-hoc test. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

3.2. Effects of Irisin on BPA-induced Oxidative Stress and Apoptosis

The level of MDA, a terminal product of lipid peroxidation and one of the most common biomarkers of oxidative stress, was assessed following BPA exposure and irisin treatment ($F(4, 10) = 96.37$; $p < 0.0001$; $R^2 = 0.9747$). One-way ANOVA followed by Tukey's multiple comparison test revealed that exposure to 100 µM BPA significantly increased MDA levels compared with the control group (4.87 ± 0.24 vs. 1.62 ± 0.38; $p < 0.0001$). This observation suggests a considerable membrane lipid damage in this experimental group (Figure 2A). However, such oxidative burden was markedly ameliorated by irisin pretreatment with increasing efficacy at higher concentrations. The 100 nM dose of irisin produced the most effective suppression of MDA formation (2.26 ± 0.26; $p < 0.0001$ vs. BPA). In addition, the 100 nM dose of irisin exerted significantly ($p = 0.0025$) a stronger inhibitory activity on lipid peroxidation (3.47 ± 0.28) than the 50 nM dose. Finally, the level of MDA in the 100 nM irisin group (0.82 ± 0.19) was lower than that in control group ($p = 0.0379$).

One-way ANOVA showed that there was a highly significant difference in SOD activity between the experimental groups ($F(4,10) = 15.73$; $p = 0.0003$, $R^2 = 0.8629$) (Figure 2B). Tukey's post-hoc test showed that BPA (100 µM) significantly inhibited SOD activity (3.44 ± 0.21) compared with the respective control group (9.03 ± 0.08) ($p < 0.001$) implying a long-term suppressed antioxidant defence system. Likewise, the BPA-induced oxidative challenge was significantly recovered by irisin. Irisin at 50 and 100 nanomoles significantly improved superoxide dismutase (SOD) activity over and above the BPA-only level ($p = 0.0072$ and $p = 0.0030$, respectively). Furthermore, SOD activity in both irisin-treated groups (50 nM 7.16 ± 1.16; 100 nM 7.66 ± 0.43) was not significantly different from the respective control group ($p > 0.05$); i.e., irisin exerted a protective effect that restored SOD activity to almost baseline levels. The standard errors for the 50 nM and 100 nM group in the presence

of BPA were 2.38 and 1.56 respectively. A similar trend was observed for the apoptotic profile but in the opposite direction. Again, there was a highly significant difference in caspase-3 activity between the experimental groups ($F(4, 10) = 225.3$; $p < 0.0001$). BPA significantly increased caspase-3 activity (3.00 ± 0.07) to more than 6-fold that of the control group (0.47 ± 0.09; $p < 0.001$) (Figure 2C), confounding the apoptotic system. The anti-apoptotic effect of irisin was dose-dependent. Namely, although 50 nM irisin significantly suppressed caspase-3 activity down to 1.84 ± 0.02 ($p < 0.0001$), irisin at 100 nM produced a more marked suppression bringing it down to 0.86 ± 0.07 ($p < 0.0001$). In other words, the substantial reduction in BPA-induced caspase-3 activity was directed to a much more favorable level.

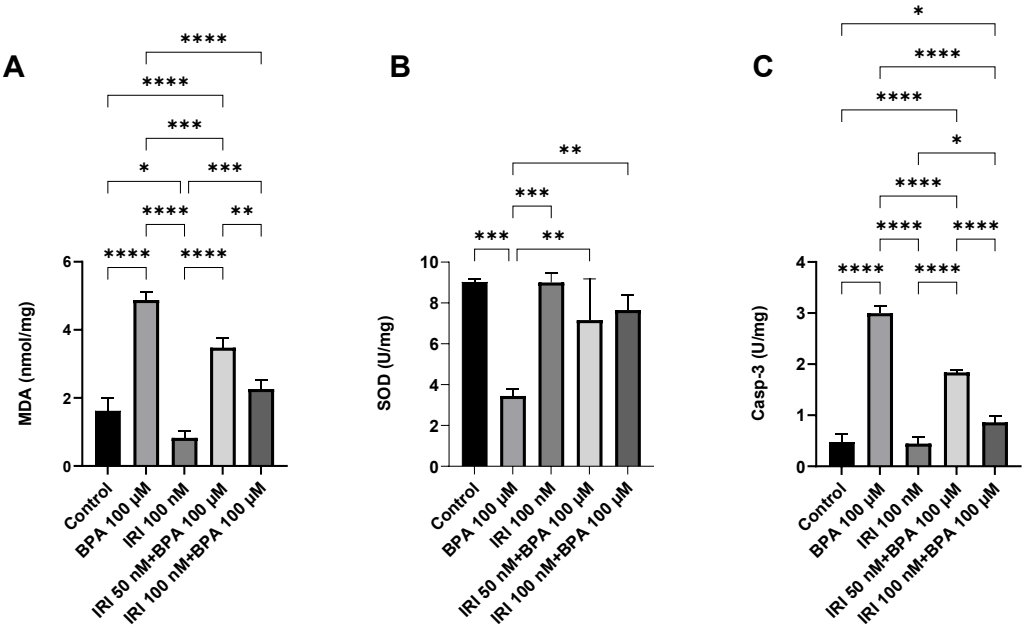

Figure 2. Effects of irisin on oxidative stress markers and apoptosis in BPA-treated AML12 hepatocytes. (A) Malondialdehyde (MDA) levels (nmol/mg protein) as an indicator of lipid peroxidation. BPA exposure significantly increased MDA levels, while irisin pretreatment reduced lipid peroxidation in a dose-dependent manner. (B) Superoxide dismutase (SOD) activity (U/mg protein). BPA markedly decreased SOD activity, whereas irisin pretreatment restored antioxidant enzyme activity. (C) Caspase-3 activity (U/mg protein) as a marker of apoptosis. BPA exposure significantly elevated caspase-3 activity, while irisin treatment attenuated apoptotic activation in a dose-dependent manner. Data are presented as mean ± standard deviation (SD) (n = 3). Statistical analysis was performed using one-way ANOVA followed by Tukey's post-hoc test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

3.3. Effects of Irisin on TAS, TOS, and OSI Levels Against BPA-Induced Oxidative Stress

Descriptive statistics and overall TAS level analysis supported the recovery pattern observed in individual antioxidant enzymes (Figure 3A). The BPA 100 µM group exhibited a marked depletion of the total antioxidant pool, with a mean TAS level of 1.01 ± 0.06, representing nearly a 45% reduction compared with the Control group (1.84 ± 0.07). Irisin alone dose-dependently reversed this effect, with the 50 nM irisin + BPA group reaching 1.36 ± 0.06 and, more notably, the 100 nM irisin + BPA group reaching 1.61 ± 0.04. Considered together with the previous SOD data, these TAS findings indicate that irisin not only elevated a single enzyme but also restored the overall redox buffering capacity, with 100 nM irisin providing consistent protection against BPA-induced redox imbalance.

Descriptive statistics for TOS levels revealed a substantial induction of oxidant levels by BPA and a pronounced reduction induced by irisin in a dose-dependent manner (Figure 3B). In the BPA 100 µM group, TOS reached a mean of 18.72 (± 0.15), more than fourfold higher than in the Control group

(4.27 ± 0.21). This induction was further reflected by the narrow range (0.54) and low SD (SD: 0.27) observed in the BPA-exposed group. Irisin effectively counteracted this BPA-induced oxidative shift. In the 50 nM irisin + BPA group, the mean oxidant level decreased to 10.59 (± 0.15), whereas in the 100 nM irisin + BPA group, it declined further to 7.41 (± 0.56). The difference between the 50 nM irisin + BPA group and the 100 nM irisin + BPA group was statistically significant ($p = 0.0002$), indicating a clear dose-dependent effect of irisin in reducing the overall oxidant load. Together with the TAS analysis, these data suggest that irisin concentration-dependently increased antioxidant capacity while decreasing the total oxidant load induced by BPA. In this study, the OSI, a comprehensive index summarizing redox status by incorporating TAS and TOS measurements, showed a highly significant difference among the groups ($F(4, 10) = 137.0$; $p < 0.0001$, $R^2 = 0.9821$) (Fig. 3C). Exposure to 100 μM BPA resulted in a substantial shift toward a pro-oxidative state, with the OSI reaching 1.868 ± 0.11, representing an almost eightfold increase relative to the control group (0.234 ± 0.02; $p < 0.0001$). Administration of irisin counteracted this oxidative alteration in a dose-dependent manner. The 50 nM irisin + BPA group showed a significant reduction in OSI compared with the irisin alone group (i.e., 50 nM irisin group), reaching 0.779 ± 0.04 ($p < 0.0001$). Notably, treatment with 100 nM irisin + BPA yielded an even stronger effect ($p = 0.0197$ when compared with 50 nM irisin + BPA), reducing the OSI to 0.459 ± 0.02, which was statistically indistinguishable from the control group ($p = 0.1160$) as well as from the irisin alone group (100 nM irisin group; $p = 0.1523$). These data indicate that administration of irisin, even at the lower dose, was able to reverse the oxidative effects of BPA, whereas administration of irisin at 100 nM was able to normalize the OSI and thereby mitigate BPA toxicity with respect to redox homeostasis.

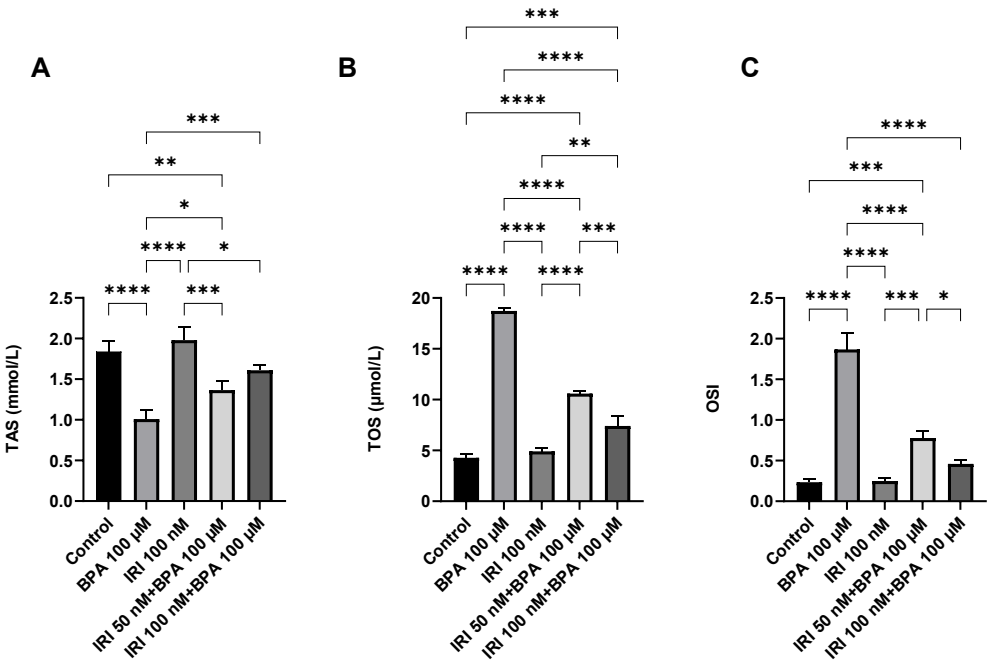

Figure 3. Effects of irisin on total antioxidant status (TAS), total oxidant status (TOS), and oxidative stress index (OSI) in BPA-treated AML12 hepatocytes. (A) Total antioxidant status (TAS) levels (mmol/L). BPA exposure significantly reduced TAS levels, whereas irisin pretreatment restored antioxidant capacity in a dose-dependent manner. (B) Total oxidant status (TOS) levels (μmol/L). BPA markedly increased TOS levels, while irisin pretreatment significantly decreased oxidant load. (C) Oxidative stress index (OSI), calculated as the ratio of TOS to TAS (OSI = TOS/TAS × 10), reflecting the overall redox balance. BPA exposure caused a significant increase in OSI, while irisin treatment effectively normalized oxidative stress in a dose-dependent manner. Data are presented as mean ± standard deviation (SD) (n = 3). Statistical analysis was performed using one-way ANOVA followed by Tukey's post-hoc test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

4. DISCUSSION

This study demonstrates that irisin mitigates BPA-induced hepatotoxicity via modulating oxidative stress and apoptotic pathways in AML12 hepatocytes. The findings of this study suggest that exposure to BPA (bisphenol A) leads to a decline in cell viability and a substantial increase in LDH (lactate dehydrogenase) release. These findings provide further evidence for the hepatotoxic effects of this environmental toxicant, which is in line with recent investigations that have established its hepatocellular damaging effects via oxidative and inflammatory pathways [15, 18]. The primary conclusion of this study is that exposure to BPA leads to a substantial increase in oxidative stress, as indicated by increased levels of MDA, TOS, and OSI, alongside diminished TAS and SOD activity. Rather than characterizing the up- or downregulation of specific oxidative markers, it may be more informative to evaluate combined markers that provide a more comprehensive representation of the redox homeostasis status of a biological system [19]. Indeed, recent literature suggests that integrated oxidative stress indices are superior to single biomarkers for assessing in vivo and ex vivo oxidative burden, as demonstrated by OSI in this study [20,21]. Accordingly, the marked elevation of OSI in BPA-treated cells confirms the pronounced pro-oxidant intracellular status induced by BPA.

The present findings indicate that irisin-mediated cytoprotection occurred in a dose-dependent manner, with the 100 nM concentration exerting the greatest protective effect. This response, which is dose-dependent, suggests that irisin may act through threshold-dependent activation of cellular defence mechanisms. Recent studies have demonstrated that irisin enhances cellular resilience by modulating redox-sensitive signalling pathways and improving mitochondrial function [22-24]. Accumulating evidence indicates that irisin supports mitochondrial integrity and suppresses oxidative damage by reducing reactive oxygen species formation [25], which may account for the marked reduction in MDA and TOS levels observed in the present study.

Furthermore, the restoration of antioxidant capacity is a pivotal component of the protective impact of irisin. The restoration of SOD activity and TAS levels suggests that, in addition to its role in inhibiting oxidant production, irisin may also enhance endogenous antioxidant mechanisms. Irisin has been demonstrated to modulate the expression of antioxidant genes by means of the activation of transcriptional cascades, such as Nrf2, thus conferring protection against oxidative stress [12, 26]. It should be noted that molecular analyses were not performed in the present investigation. The biochemical data obtained offer significant evidence for the existence of these pathways.

Besides its antioxidant properties, we found that irisin significantly reduced BPA-induced apoptosis, as shown by caspase-3 activity. This observation is consistent with the well-known link between oxidative stress and apoptosis [27]. Excessive ROS production can cause mitochondrial dysfunction and activate caspase-dependent pathways [28]. Irisin-mediated protection against apoptosis has been linked to the modulation of multiple intracellular pathways, with PI3K/Akt signaling and maintenance of mitochondrial homeostasis playing key roles [6]. Consequently, the observed reduction in caspase-3 activity could be attributed to the inhibition of the apoptotic pathways. In addition, our data demonstrated that irisin itself has no cytotoxic effect on AML12 hepatocytes. This observation is of major interest given that irisin has been increasingly proposed as a new therapeutic molecule. Unlike many synthetic antioxidants, irisin is an endogenous peptide involved in cellular homeostasis and energy metabolism and may thus have stronger translational potential [29, 30].

The strength of our study lies in the complete analysis of oxidative stress through the determination of TAS, TOS, and OSI. These parameters allow for the most precise determination of the redox state, strengthening the conclusion that irisin has a protective effect. Another strength of our study is that we could observe the dose-response relationship clearly in several parameters. However, we also have some limitations: First, we used only one dose of BPA, so we were not able to establish the complete dose-response. Future studies should use higher and lower doses of BPA to estimate the IC50 and define more

precisely the threshold of toxicity. Second, we did not perform molecular analyses, which limits our conclusions. Therefore, future studies should aim to determine the signaling pathways possibly responsible for irisin's protective action, such as Nrf2/HO-1, mitochondria, and inflammatory mediators.

Our study shows that irisin protects AML12 hepatocytes from BPA-induced oxidative stress and apoptosis in a dose-dependent manner. Irisin restores antioxidant capacity and attenuates lipid peroxidation and caspase-3 activation, thus helping the maintenance of cellular homeostasis. Irisin may represent a potential therapeutic candidate in liver injury induced by environmental toxins.

5. CONCLUSION

This study demonstrates that irisin significantly protects AML12 hepatocytes from BPA-induced damage. It is evident that exposure of cells to BPA (bisphenol A) has been demonstrated to induce considerable oxidative stress and diminish antioxidant capacity. In addition, this exposure has been shown to promote apoptotic pathways, as evidenced by elevated levels of MDA, TOS, OSI and caspase-3, and decreased activity of SOD and TAS. Additionally, irisin treatment alleviated the effects of BPA in a dose-dependent manner, and at the higher dose (100 nM), showed a stronger impact in counteracting cellular toxicity. These results suggest that irisin reduces cellular damage by restoring redox balance, increasing antioxidant status, and decreasing apoptotic pathways. In addition, the comprehensive evaluation of oxidative stress parameters verified the significant effect of irisin on maintaining cellular homeostasis under toxic conditions.

In summary, the present data indicate that irisin may represent a potential therapeutic candidate for protecting the liver against environmental toxin-induced injury. Subsequent studies employing *in vivo* models will be essential to better understand the mechanistic basis of irisin-mediated protection and to assess its translational potential.

ACKNOWLEDGEMENT

The author would like to thank Nuran Karabulut for her contributions and support throughout this study.

CONFLICT OF INTEREST

The author stated that there are no conflicts of interest regarding the publication of this article.

CRediT AUTHOR STATEMENT

Gözde Karabulut: Conceptualization, Investigation, Formal analysis, Data interpretation, Visualization, Writing – Original Draft, Review & Editing.

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