

Total Phenolic, Total Flavonoid Contents and Biological Activities of *Mentha Suaveolens* subsp. *timija* Crude Extracts

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ABSTRACT

Mentha suaveolens subsp. *timija* is an aromatic herb with a spearmint flavor that plays an important role in the treatment of a wide range of diseases. It has various biological activities, including serving as a natural source of antioxidants and antibacterial agents. The main objective of the present study was to identify biologically active polyphenols and assess the antioxidant capacity of *Mentha suaveolens* subsp. *timija* leaves and stems using various organic solvents (hexane, ethanol, methanol) and distilled water for extraction. The antioxidant activity was determined using the ABTS, DPPH, and Total Antioxidant Capacity (TAC) free radical scavenging methods. Results revealed that the total flavonoid and phenolic content ranged from 0.30 to 14.47 mg QE/g and 0.30 to 10.86 mg QE/g, respectively, and from 1.47 to 35.34 mg GAE/g and 1.43 to 37.1 mg GAE/g for leaves and stems, respectively. The extracts exhibited significant antioxidant activity in ABTS, DPPH, and TAC assays, which increased with higher concentrations of polyphenol extracts ($P \leq 0.05$). Furthermore, the plant extracts examined in this study displayed remarkable antibacterial activity, particularly against Gram-positive bacteria, with leaf extracts showing better antibacterial activity than stem extracts.

Keywords: Antimicrobial activity; antioxidant activity; Food safety; Medicinal plants, Polyphenolic contents, Radical scavenging activity

INTRODUCTION

Mentha suaveolens subsp. *timija* (Coss. ex Briq.) Harley is a medicinal plant that has long been recognized as a valuable source of bioactive compounds with potential therapeutic properties. It stands out for its historical relevance and numerous applications in traditional medicine. This plant is a strict Moroccan endemic species, specifically found in the High Atlas, Anti-Atlas, Middle Atlas, and Saharan regions of the country (Rankou et al., 2013; Soulaïmani et al., 2021). *Mentha suaveolens* subsp. *timija* is a familiar medicinal plant in Moroccan folk medicine, used to treat various ailments such as diabetes, digestive and respiratory disorders, rheumatism, musculoskeletal illnesses, urogenital issues, as well as otolaryngological and pediatric illnesses (El Hassani, 2020; Nassiri et al., 2016). The plant is used in different forms including infusions for colds and vein palpitations, dried leaf powder for hemorrhoids (Afrokh et al., 2023), and crushed fresh plant for healing, toning, and as a laxative (Aldogman et al., 2022). Additionally, it is commonly used for its essential oils, aromatic flavoring in food, and for preparing traditional dishes (Kasrati et al., 2014).

Unfortunately, extensive harvesting for medicinal and culinary purposes has led to a significant decline in the population of this species. Consequently, the species is currently scarce and vulnerable to extinction in its native environment.

Phenolic compounds, classified as secondary metabolites, are integral to human health and nutrition. Derived from phenylalanine and tyrosine, these phytochemicals are abundant in plants and exhibit remarkable diversity (Lin et al., 2016). Notably, certain phenolics demonstrate superior antioxidant activity compared to synthetic counterparts (Shahidi & Ambigaipalan, 2015). The medicinal properties of phenols primarily stem from their antioxidant capacity, ability to scavenge free radicals, chelation of redox-active metal ions, modulation of gene expression, and interaction with cell signaling pathways. Studies indicate that the radical scavenging and antioxidant activities of phenols are influenced by the arrangement of functional groups within their core structure. The quantity and configuration of H-donating hydroxyl groups are key structural factors determining the antioxidant potential of phenols (Platzer et al., 2022).

The relationship between phenolic compounds and antioxidant capacity is well-documented in the literature. Studies have shown that plants rich in phenolic compounds often

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exhibit high antioxidant activity. For instance, Čavar Zeljković et al. (2021) reported a significant positive correlation between total phenolic content (TPC) and total flavonoid content (TFC) with antioxidant activity in *Mentha suaveolens* (Čavar Zeljković et al., 2021). This suggests that the antioxidant potential of plants like *Mentha suaveolens* is largely due to their phenolic composition. Such findings underscore the importance of phenolic compounds in mitigating oxidative stress, further supporting their application in food preservation and nutraceutical development (Arfaoui, 2021). Despite the recognized medicinal importance of *Mentha suaveolens* subsp. timija, there remains a need for a comprehensive study to investigate the various bioactivities of its extracts. The aim of this study was to address this gap by analyzing its extracts obtained using different solvents (ethanol, methanol, hexane, and water). To address this issue, the research focuses on quantifying the Total Phenolic Content (TPC) and Total Flavonoid Compounds (TFC) in these extracts, providing insights into the different bioactive components. Additionally, their antioxidant potential of each extract was evaluated through several tests, including Total Antioxidant Capacity (TAC), Ferric Reducing-Antioxidant Power (FRAP), Free radical scavenging activity using DPPH and ABTS radicals, and Ferrous chelating ability. These assays aim to assess the effectiveness of its extracts in neutralizing free radicals and preventing oxidative damage, which is often associated with various health conditions. Furthermore, the antibacterial activity of the extracts also was evaluated against a variety of pathogenic microorganisms to offer insight on its possible involvement in the fight against microbial diseases.

MATERIALS AND METHODS

Plant material

Specimens of *Mentha suaveolens* subsp. timija were collected in May 2022 in the Sefrou region of the Moroccan Middle Atlas. Before extraction, species identification was carried out at the Laboratory of Functional Ecology and Environmental Engineering, Faculty of Sciences and Techniques, USMBA-Fez. The leaves and stems were dried at room temperature for two weeks, then ground into powder and stored in opaque containers to maintain their integrity for analysis

Extraction

Five grams (5 g) of powdered samples were weighed and macerated with 100 mL of each solvent (ethanol, methanol, hexane, and water) at

room temperature for a period of 24 hours. Following maceration, the extracts were filtered using Whatman filter paper to remove any particulate matter. Subsequently, all extracts were stored at 4°C until further use.

Total phenolic content (TPC)

The total phenolic content (TPC) was assessed spectrophotometrically, following a modified colorimetric method using the Folin-Ciocalteu reagent (Siddiqui et al., 2017). In this method, 50 µL of the sample was combined with 450 µL of a 10-fold diluted solution of the Folin-Ciocalteu reagent. After a 5-minute incubation period at room temperature, 450 µL of a Na₂CO₃ solution (75 g L⁻¹) was added. Subsequently, all samples were kept in the dark for 2 hours at room temperature, and their absorbance was measured at 760 nm using a spectrophotometer. The calibration curve, generated using an ethanolic solution of gallic acid, ranged from 0.062 to 1 mg mL⁻¹ ($y = 1.2116x + 0.1944$, $R^2 = 0.9995$). The experiment was conducted in triplicate, and the results are expressed in mg of gallic acid equivalent (GAE) per gram of dry plant material.

Total Flavonoid Compounds (TFC)

The determination of flavonoid content was conducted using the aluminum chloride colorimetric method described by Abifarín et al., (2019), with certain modifications. Initially, 0.5 mL of the different solvent extracts, prepared at varying concentrations ranging from 0.2 to 1 mg/mL, were added to respective test tubes. Subsequently, 2 mL of distilled water and 0.15 mL of 5% sodium nitrite were added to each test tube, followed by incubation for 6 minutes. Quercetin standard reaction mixtures were prepared in a similar manner. Next, 0.15 mL of 10% AlCl₃ was added to the reaction mixtures, followed by another 5-minute incubation period. Then, 1 mL of 1 M sodium hydroxide was added, and distilled water was added to make up a 5 mL solution. Absorbance was measured at a wavelength of 420 nm. The flavonoid content was determined using the quercetin calibration curve equation: $y = 3.8733x - 0.0455$, with an R^2 value of 0.9994, and expressed as milligrams of quercetin equivalent (mg QE/g).

Antioxidant activity

Total Antioxidant Capacity (TAC)

The total antioxidant activity of the samples was evaluated by measuring the formation of the phosphomolybdenum complex, following the method described by Chater et al., (2024). In this procedure, 50 µL of the sample solution was mixed

with 1 mL of the reagent solution, containing 0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate. The mixture was then incubated in a water bath at 95°C for 90 minutes. Subsequently, the absorbance of the mixture was recorded at 695 nm against a blank using a spectrophotometer. Aqueous solutions of ascorbic acid were used to generate the calibration curve, with a concentration range of 1.0 to 0.0625 mg mL⁻¹ ($y = 1.7355x + 0.235$, $R^2 = 0.9999$). The experiment was conducted in triplicate, and the results, expressed as antioxidant activity in ascorbic acid equivalents, represent average values presented in grams of ascorbic acid equivalents per gram of dry plant material.

Ferric Reducing-Antioxidant Power (FRAP)

The reducing power assay was conducted following the procedure outlined by Türkoğlu & Parlak, (2015). Each sample or standard was combined with 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of potassium ferricyanide [$K_3Fe(CN)_6$] 1%. The mixture was then incubated at 50°C for 20 minutes. Subsequently, 2.5 mL of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3000 rpm for 10 minutes. Next, 2.5 mL of the upper layer solution was mixed with 2.5 mL of distilled water and 0.5 mL of $FeCl_3$ (0.1%), and the absorbance was measured at 700 nm using a spectrophotometer.

Free radical scavenging activity: DPPH

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay was conducted following the protocol outlined by Chater et al., (2023). Specifically, 25 μ L of different concentrations of samples or standards were added to a 1 mL ethanolic solution of DPPH (60 μ M). After an incubation period of 60 minutes at room temperature, absorbance measurements were taken at 517 nm. A negative control containing the same amount of methanol and DPPH solution was used to measure baseline absorption. The experiment was performed in triplicate, and the percentage inhibition of free radical scavenging activity for each extract was calculated using the following formula:

$$\% \text{ inhibition} = \left(\frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}} \right) \times 100 \quad \text{Eq. 1}$$

ABTS radical scanning activity

The ABTS radical cation (ABTS•+) decolorization assay was conducted following the method reported by Tsvetkova et al. (2023), with slight modifications. Stock solutions comprised a 7 mM ABTS•+ solution and a 2.45 mM potassium persulfate solution. A working solution was

prepared by mixing equal volumes of the two stock solutions and allowing them to react for 16 hours at room temperature in the absence of light. This solution was then diluted by combining 1 mL of the ABTS solution with 60 mL of ethanol to achieve an absorbance of 0.832 ± 0.01 units at 734 nm using a spectrophotometer. Fresh ABTS•+ solutions were prepared for each test. The plant extracts (50 μ L) were allowed to react with 950 μ L of the ABTS•+ solution, and absorbance was measured at 734 nm after 6 minutes using a spectrophotometer. Lower absorbance of the reaction mixture indicates higher ABTS•+ scavenging activity. The ability to scavenge ABTS•+ was calculated using the formula provided (Eq. 1).

Ferrous chelating ability

Regarding the ferrous chelating ability, it was evaluated according to the method described by Aazza et al., (2024). Briefly, the samples were incubated with 0.05 mL of $FeCl_2 \cdot 4H_2O$ (2 mM). The addition of 0.2 mL of 5 mM ferrozine initiated the reaction, and after 10 minutes, the absorbance at 562 nm was measured. An untreated sample served as a control. The percentage of chelating power was calculated according to Eq. 1.

Antimicrobial activity

The in vitro antimicrobial activity of hexane extract (HE), methanol extract (ME), ethanol extract (EE), and water extract (WE) was evaluated against four bacterial strains and one pathogenic yeast strain. The bacterial strains tested included two Gram-positive bacteria (*Staphylococcus aureus* and *Bacillus brevis*) and two Gram-negative bacteria (*Pseudomonas aeruginosa* and *Escherichia coli*), while the yeast strain used was *Candida albicans*. These microbial strains were sourced from the Laboratory of Functional Ecology and Environmental Engineering, FST-USMBA, Fez, and were maintained at 4°C on nutrient agar slopes. Before testing, the microbial strains were re-identified based on their morphological, cultural, and biochemical characteristics (Lous et al., 2010) to ensure accuracy.

For the antimicrobial assay, 1 to 2 colonies from a 24-hour pure agar culture of each strain were transferred to sterile saline solution and vortexed thoroughly. The turbidity of the solutions was adjusted to 0.5 McFarland standard.

The minimum inhibitory concentration (MIC), defined as the lowest concentration of the extract inhibiting microbial growth, was determined using broth microdilution according to (Mekky et al., 2021). The microplates were then incubated at 37°C for 24 hours for bacteria and at 30°C for 48 hours for yeast. After incubation, 10 μ L

of resazurin was added to visualize microbial growth.

To determine the minimum bactericidal concentration (MBC) or minimum fungicidal concentration (MFC), 5 μ L of the negative wells were plated onto LB (for bacteria) or YPG (for yeast) agar plates and incubated at 37°C for 24 hours or at 30°C for 48 hours, respectively. MBC or MFC was recorded as the lowest concentration showing no visible growth. Additionally, the nature of the antimicrobial effect was assessed by calculating the MBC/MIC ratio (Levison, 2004).

Statistical analysis

All analyses were conducted in triplicate, and the results are presented as the mean $\pm$ standard deviation (SD). Statistical analysis was performed using the free version of STATISTICA version 10 software (STATSOFT, INC., 2011) and GraphPad Prism 8.4.3 (GraphPad Software, LLC). One-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference post hoc test was employed to compare the data. Differences with a significance level of $P \leq 0.05$ were considered statistically significant.

RESULTS

Total phenolic content (TPC)

The quantification of total phenolic compounds (TPC) and total flavonoid content (TFC) in *M. suaveolens* extracts was conducted using four different solvents to enhance result consistency, gain a more comprehensive understanding of the phenolic content in the plant, and to study the influence of solvent type on the extraction of bioactive compounds. The assessment of phenolic content across various extracts (depicted in Figure 1) revealed distinct outcomes. Notably, water extracts consistently exhibited the highest TPC values in both stems (37.10 ± 0.03 mg GAE/g) and leaves (35.34 ± 0.02 mg GAE/g), closely followed by methanol and ethanol extracts recording 33.19 ± 0.02 and 29.77 ± 0.02 mg GAE/g for leaves, and 33.03 ± 0.02 and 32.05 ± 0.02 mg GAE/g for stems, respectively. In contrast, hexane extracts displayed substantially lower phenolic content, recording 1.47 ± 0.02 mg GAE/g for leaves and 1.44 ± 0.03 mg GAE/g for stems.

Total flavonoid content (TFC)

The quantification of total flavonoid content (TFC) in the extracts highlights the influence of the nature of extracting solvents on the TFC. Figure 2, illustrates the results of the quantitative analysis of flavonoids in the different extracts, revealing significant variation across the extracts, ranging

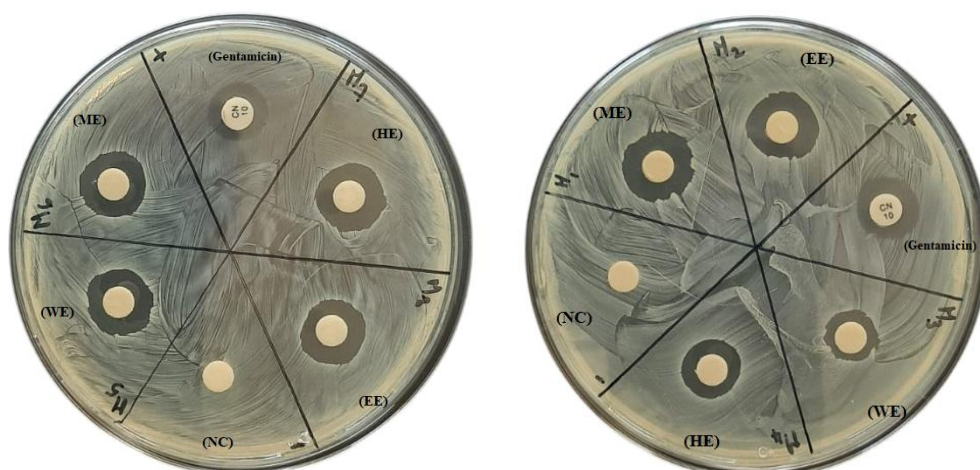
from 0.30 ± 0.00 to 14.47 ± 0.01 mg QE / g for leaves, and 0.30 ± 0.01 to 10.86 ± 0.01 mg QE / g for stems. The leaf water extract exhibited the highest flavonoid content (14.47 ± 0.01 mg QE / g), followed by the methanol leaf extract (13.31 ± 0.01 mg QE / g) and the ethanol leaf extract (11.91 ± 0.01 mg QE / g). In both leaves and stems, water and methanol extracts consistently exhibited the highest TPC values, followed by ethanol. Whereas the hexane extract showed considerably lower flavonoid amounts (0.30 mg QE / g). Across different plant parts, leaves exhibited higher TPC and TFC compared to stems. In summary, polar solvents, particularly water, and methanol, proved more effective in extracting bioactive compounds from *M. suaveolens*.

Antioxidant activity

Total Antioxidant Capacity (TAC)

The phosphomolybdate method has been routinely used to evaluate the total antioxidant capacity of plant extracts (Pisoschi et al., 2016). In the presence of the extracts, the Mo (VI) is reduced to Mo(V) and forms a green-colored phosphomolybdenum-V complex, which has an absorption maximum of 695 nm. The evaluation of total antioxidant capacity (TAC) across different extracts, employing solvents such as methanol, ethanol, hexane, and water in leaves and stems of *M. suaveolens*, expressed as equivalents of ascorbic acid, reveals significant trends and distinctions. Figure 3, provides a visual representation of these findings.

The water extract of leaves exhibited the highest activity at 28.66 ± 0.02 mg AAE/g dry plant, followed by the methanol extract of leaves at 27.18 ± 0.01 mg AAE/g dry plant, and the water extract of stems at 26.81 ± 0.02 mg AAE/g dry plant. In contrast, hexane extracts from both the leaf and stem displayed the lowest activity, recording 5.92 ± 0.03 and 3.48 ± 0.01 mg AAE/g dry plant, respectively. Methanol consistently yields high TAC values, averaging 27.18 ± 0.01 mg AAE/g dry plant in leaves and 24.86 ± 0.03 mg AAE/g dry plant in stems. Ethanol, even slightly lower than methanol, exhibits respectable TAC values with averages of 24.88 ± 0.01 mg AAE/g dry plant in leaves and 22.43 ± 0.02 mg AAE/g dry plant in stems. Hexane, in contrast, demonstrates significantly lower TAC values in both plant parts, averaging 5.92 ± 0.03 mg AAE/g dry plant in leaves and 3.48 ± 0.01 mg AAE/g dry plant in stems, indicating its limited efficiency in extracting antioxidant compounds. In summary, the TAC results reveal distinct solvent-dependent and plant part-specific patterns. Water emerges as a promising solvent for extracting antioxidants from



NC: Negative control; Gentamicin: Positive control; EE: Ethanol extract; HE: Hexane extract; ME: Methanol extract; WE: Water extract.

Figure 1. a: Inhibition zones for antibacterial activity of *M. suaveolens* subsp. *timija* leaf extracts against *E. coli*, using the disc diffusion method; b: Inhibition zones for antibacterial activity of *M. suaveolens* subsp. *timija* root extracts against *E. coli*, using the disc diffusion method.

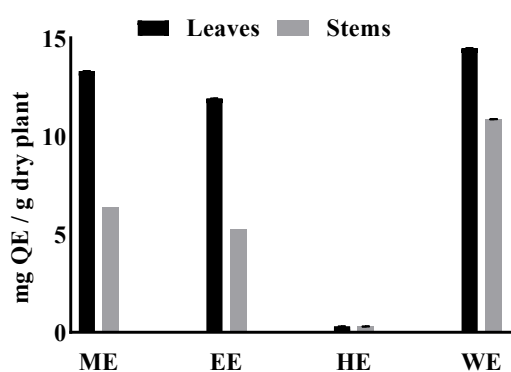


Figure 2. Total Flavonoid compounds of different extracts

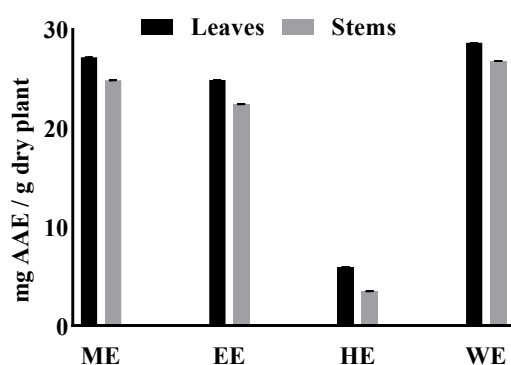


Figure 3. Total antioxidant capacity of different extracts

M. suaveolens, particularly in leaves, and could be further explored for its potential in extracting specific bioactive compounds. The study

underscores the importance of considering both solvent and plant parts in designing efficient extraction protocols for antioxidant-rich plant materials.

Ferric Reducing-Antioxidant Power (FRAP)

The conversion of Fe^{3+} to Fe^{2+} in the presence of various fractions was measured to determine the reducing power. The reducing power of a compound generally depends on the presence of reducing agents (antioxidants), which exert the antioxidant effect by breaking the free radical chain by releasing a hydrogen atom (Armstrong, 2015).

The analysis of ferric reducing antioxidant power (FRAP) of various solvent extracts (methanol, ethanol, hexane, and water) from leaves and stems of the investigated plant reveals distinct trends. The results are shown in Figure 4. All extracts were able to catalyze the reduction of $\text{Fe}(\text{III})$ to $\text{Fe}(\text{II})$. Water extracts consistently stand out with the highest FRAP values, averaging 2.06 ± 0.00 in leaves and 2.04 ± 0.00 in stems, underscoring the remarkable ferric-reducing antioxidant power. Methanol extracts exhibited moderate FRAP values in both leaves and stems, with averages of 1.96 ± 0.00 and 1.89 ± 0.00 . while, ethanol had lower FRAP values, with an average of 1.92 ± 0.00 in leaves and 1.60 ± 0.00 in stems, showing significant antioxidant potential. Because of its nonpolar nature, hexane consistently produces the lowest FRAP values among all solvents, with averages of 0.76 ± 0.00 in leaves and 0.58 ± 0.00 in stems. The plant part comparison reveals that leaves generally possess higher FRAP

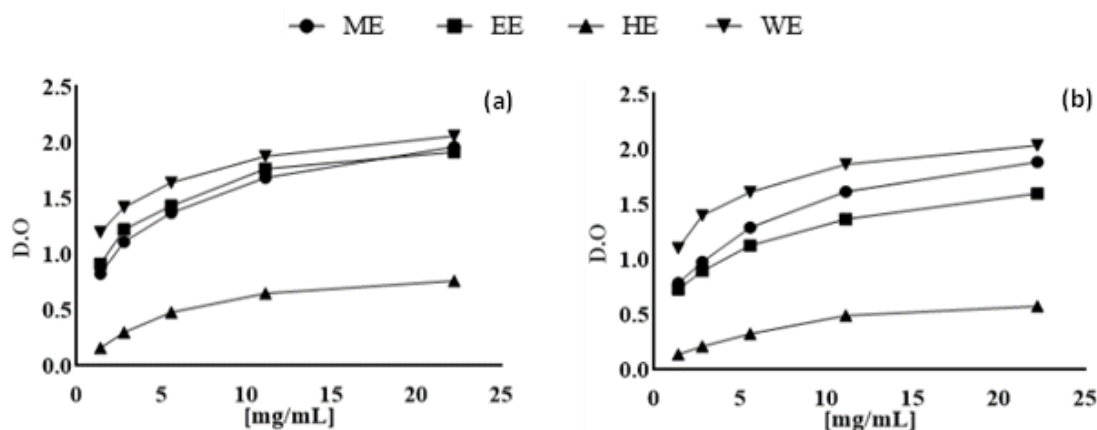


Figure 4. Ferric Reducing-Antioxidant Power of different extracts of leaves (a) and stems (b)

Table I. IC₅₀ Antioxidant activities of *M. suaveolens* subsp. *timija* leaves and stem extracts

Extracts		DPPH	ABTS	CP
Leaves	Methanol	0.09 ± 0.00 ^d	0.16 ± 0.00 ^a	0.16 ± 0.00 ^a
	Ethanol	0.07 ± 0.00 ^b	0.24 ± 0.00 ^f	0.18 ± 0.00 ^c
	Hexane	-	0.23 ± 0.00 ^e	-
	Water	0.08 ± 0.00 ^c	0.18 ± 0.00 ^b	0.17 ± 0.00 ^b
Stems	Methanol	0.11 ± 0.00 ^e	0.22 ± 0.00 ^d	0.18 ± 0.00 ^c
	Ethanol	0.08 ± 0.00 ^c	0.24 ± 0.00 ^f	0.22 ± 0.00 ^d
	Hexane	-	0.18 ± 0.00 ^b	-
	Water	0.06 ± 0.00 ^a	0.21 ± 0.00 ^c	0.23 ± 0.00 ^e

values than stems across all solvents, emphasizing potential variations in antioxidant concentrations between these tissues.

Free radical scavenging activity: DPPH

The DPPH assay stands as a frequently employed technique to evaluate the antioxidant potential of plant extracts (Gulcin & Alwasel, 2023). This technique relies on the shift in color from the initial purple hue of the stable nitrogen-based radical (DPPH) to the yellow compound referred to as diphenyl picrylhydrazine, following the addition of the extracts. The reduction in the number of DPPH molecules can be correlated with the number of available hydroxyl groups (Baliyan et al., 2022).

The extracts from *M. suaveolens* were tested for their ability to scavenge DPPH free radicals, and the findings are displayed in Figure 5, with the IC₅₀ values reported in Table I. The DPPH assay highlighted notable variations in antioxidant activity among the different solvents employed for the extraction, particularly within the same plant parts. The highest concentration of 1.22 mg/mL, water extracts demonstrated the most pronounced DPPH radical scavenging activity for both leaves (87.69±0.18 %) and stems (85.03±0.14 %),

surpassing the activities of methanol (83.17±0.12 % and 79.66±0.12 %), ethanol (82.65 ± 0.18 % and 77.68±0.28 %), and hexane extracts (48.22±0.07 % and 49.59±0.18 %) in descending order ($P \leq 0.05$). The IC₅₀ values, representing the concentration required to scavenge 50% of the radicals, revealed that the ethanol extract exhibited the lowest IC₅₀, indicating its potent DPPH radical scavenging activity (Table I). Notably, all extracts exhibited significantly higher inhibition percentages, reflecting a stronger ability to donate hydrogen, and these values positively correlated with the total phenolic content. Methanol extracts from both leaves and stems consistently displayed the lowest IC₅₀ values, suggesting superior free radical scavenging capacity attributed to the efficiency of methanol in extracting bioactive compounds with strong DPPH radical scavenging potential. Furthermore, in comparing plant parts for each solvent, leaves consistently exhibited lower IC₅₀ values than stems, underscoring the superior antioxidant properties of the leaves.

ABTS radical scanning activity

The ABTS radical scavenging assay is a method used to measure the ability of substances to neutralize harmful free radicals. It involves

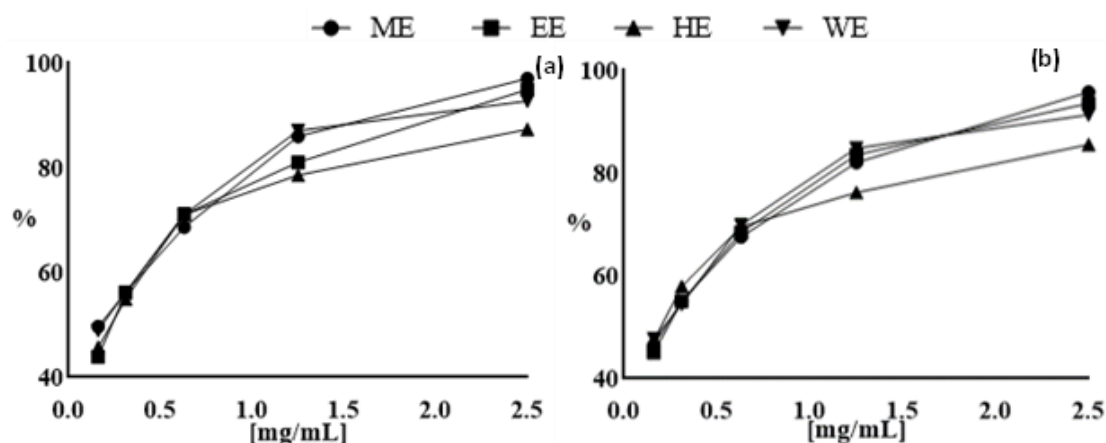


Figure 6. ABTS radical scanning activity of different extracts of leaves (a) and stems (b)

combining ABTS and potassium persulfate to create a blue/green compound called ABTS⁺. The process involves oxidizing ABTS with potassium persulfate, which results in the formation of the ABTS radical cation. The presence of antioxidants that can donate hydrogen atoms causes a reduction in the ABTS radical cation, and this reduction can be measured using a spectrophotometer at a wavelength of 745nm (Ilyasov et al., 2020). As shown in Figure 6, the extracts' ABTS scavenging activity increases with concentration, achieving statistical significance ($P \leq 0.05$). Methanol, ethanol, and water extracts consistently displayed the greatest mean% inhibition at the highest dose (2.5 mg/mL). The concentration required to scavenge 50% of the radicals (IC_{50}) indicated the following order: Hexane Extract (HE) > Ethanol Extract (EE) > Methanol Extract (ME) > Water Extract (WE) (Table I). Notably, methanol extracts regularly had lower IC_{50} values, suggesting improved extraction of chemicals with ABTS radical scavenging properties. The contrast between leaves and stems was more noticeable in the ABTS assay than in the DPPH assay, emphasizing the need to use many assays to gain a thorough picture of antioxidant activity.

Figure 6 shows that the ABTS scavenging activity of the extracts increased with increasing concentration ($P < 0.05$). At the maximum concentration (2.5 mg / mL), the highest mean % inhibition was produced by methanol (97.12 ± 0.12 %) and (95.87 ± 0.14 %), surpassing the activities of ethanol (95.03 ± 0.18 % and 93.71 ± 0.18 %), water (92.87 ± 0.07 % and 91.47 ± 0.12 %), and hexane extracts (87.50 ± 0.12 % and 85.70 ± 0.12 %) in descending order ($P \leq 0.05$) for both leaves and stems respectively. The IC_{50} values for methanol extracts were consistently lower, indicating that

methanol is better at extracting compounds with ABTS radical scavenging abilities. The differences between leaves and stems were more pronounced in the ABTS assay than in the DPPH assay, highlighting the importance of studying antioxidant activity using various tests.

Chelation capacity

The capacity of *M. suaveolens* subsp. timija extracts to chelate metals was assessed by monitoring the formation of the ferrozine complex with iron ions. This complex, distinguished by its red color, absorbs at 562 nm (Yamaguchi et al., 2000). Chelating agents that establish a σ -bond with metals have been recognized for their effectiveness as secondary antioxidants. This efficacy arises from their capacity to reduce the redox potential, consequently stabilizing the oxidized state of the metal ion (Duh, 1999). The metal-chelating capacity of *M. suaveolens* extracts was evaluated, and the outcomes are illustrated in Table I. The introduction of the extracts influenced the iron-ferrozine complex, leading to a discernible reduction in the red color of the complex as the extract concentration increased. All the extracts trapped ferrous ions before ferrozine and thus have the ability to chelate ferrous ions, suggesting their antioxidant potential (Figure 7). The evaluation of chelating power exhibited a distinct pattern, with methanol and water extracts consistently demonstrating superior metal ion-binding capacities. The chelating power of the extracts, as evidenced by their IC_{50} values, revealed notable distinctions between leaves and stems. Methanol extracts from leaves exhibited the lowest IC_{50} value (0.16 ± 0.00), closely followed by water extracts (0.17 ± 0.00), demonstrating a strong metal ion-binding capacity. Similarly, in stems, methanol extracts exhibited a potent chelating power, as

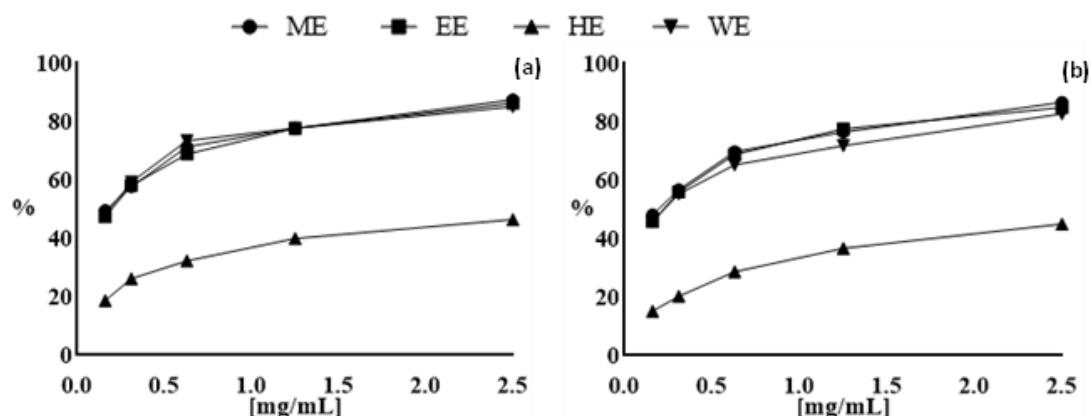


Figure 7. chelating power of different extracts of leaves (a) and stems (b)

indicated by the lowest IC_{50} value (0.18 ± 0.00), with water extracts also showing important chelating ability (0.23 ± 0.00). These findings underscore the impact of both solvent choice and plant part on the chelating properties of the extracts.

Antimicrobial activity

Plant extracts were evaluated for their antibacterial properties against both Gram-positive and Gram-negative bacteria. This assessment involves determining the inhibition zones for each extract (Figures 1a and 1b) and further confirming the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). Table II shows the antimicrobial efficacy of hexane (HE), ethanol (EE), methanol (ME), and water (WE) extracts obtained from leaves and stems, against various microorganisms, including bacteria (*S. aureus*, *B. subtilis*, *E. coli*, *P. aeruginosa*) and fungi (*C. tropicalis*). The results of this study showed that both gram-positive and gram-negative bacteria were susceptible to all the examined extracts. Overall, leaf extracts appear to have stronger antibacterial activity than stem extracts. This is demonstrated by the reduced MIC and MBC values found for the majority of bacteria examined. For instance, in the case of the ME extract, the MIC and MBC values are generally lower for leaf extracts compared to stem extracts against most microorganisms. Conversely, stem extracts generally require higher concentrations (higher MIC and MBC values) to inhibit the growth of microorganisms compared to leaf extracts. However, certain stem extracts, such as the EE extract, have comparable effectiveness to leaf extracts against particular bacteria, such as *B. subtilis* and *E. coli*.

The leaf extracts yielded using organic solvents, namely, ethanol, methanol, and hexane

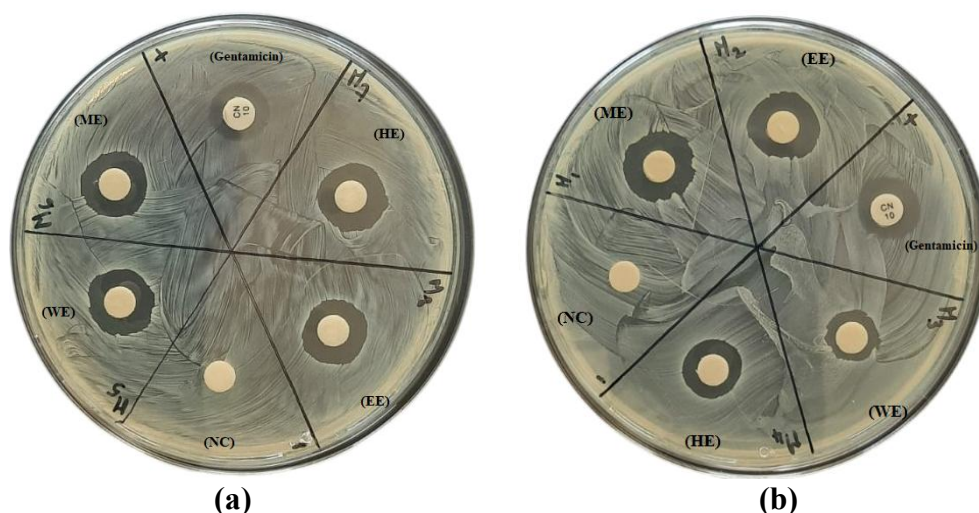
exhibited the most potent inhibitory activity against *B. subtilis* and *E. coli*. Whereas, they showed less uniform efficiency against the rest of the bacterial strains including *P. aeruginosa*, and the fungi *C. tropicalis*, for which, the MIC values were equal to the ones of MBC. However, against *S. aureus*, the extracts showed the same inhibitory activity, but less bactericidal potency.

The EE extract exhibits variable antimicrobial activity, with some instances of potent inhibition (e.g., against *S. aureus*) and others showing moderate activity. This variability may be attributed to the diverse nature of compounds extracted by ethanol.

Methanol extracts (ME) from both leaves and stems showed strong antibacterial activity, with effective Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal/Fungicidal Concentration (MBC/MFC) values against *S. aureus*, *B. subtilis*, and *E. coli*. Ethanol extracts (EE) had comparable activity, but hexane extracts (HE) had decreased but still substantial antibacterial effects. Water extracts (WE) showed modest efficacy, especially against *B. subtilis* and *E. coli*. Leaves had somewhat stronger antibacterial activity than stems, particularly in hexane extracts. The observed differences emphasize the effect of solvent selection on antibacterial component extraction and *Thymus vulgaris*'s potential as a source of natural antibacterial agents. Further research is needed to identify particular bioactive chemicals that contribute to the reported benefits.

DISCUSSIONS

Phenols naturally possess antioxidant properties and have potential as therapeutics against a variety of diseases, including neurodegenerative disorders, cancer, diabetes, cardiovascular dysfunction, inflammatory diseases, and aging (Muscolo et al., 2024). The



NC: Negative control; Gentamicin: Positive control; EE: Ethanol extract; HE: Hexane extract; ME: Methanol extract; WE: Water extract.

Figure 8. (a) Inhibition zones for antibacterial activity of *M. suaveolens* subsp. timija leaf extracts against *E. coli*, using the disc diffusion method; (b) Inhibition zones for antibacterial activity of *M. suaveolens* subsp. timija root extracts against *E. coli*, using the disc diffusion method.

Table II. MIC and MBC/MFC values of the different extracts against all microorganisms

Extracts	MIC MBC/MFC	Gram +		Gram -		Fungi
		<i>S. aureus</i>	<i>B. subtilis</i>	<i>E. Coli</i>	<i>P. aeruginosa</i>	<i>C. tropicalis</i>
Leaves	ME	MIC	6.25	3.12	3.12	6.25
		MBC/MFC	12.5	6.25	12.5	6.25
	EE	MIC	6.25	3.12	3.12	6.25
		MBC/MFC	12.5	6.25	12.5	6.25
	HE	MIC	6.25	3.12	3.12	6.25
		MBC/MFC	12.5	12.5	12.5	6.25
	WE	MIC	-	6.25	12.5	12.5
		MBC/MFC	25	12.5	12.5	12.5
	ME	MIC	6.25	6.25	6.25	6.25
		MBC/MFC	12.5	25	12.5	12.5
Stems	EE	MIC	12.5	6.25	6.25	6.25
		MBC/MFC	12.5	25	12.5	12.5
	HE	MIC	6.25	3.12	6.25	6.25
		MBC/MFC	12.5	12.5	12.5	12.5
	WE	MIC	12.5	6.25	12.5	12.5
		MBC/MFC	25	25	25	12.5

extraction of phenolic compounds showed that water extracts had the highest total phenolic content (TPC) in both stems and leaves, followed by methanol and ethanol extracts. Hexane extracts had significantly lower phenolic content compared to the other solvents. This observation is consistent with the results reported by Chater et al. (2024), where the Total Phenolic Content (TPC) for hexane extract was minimal (0.00 ± 0.00 mg GAE/g) (Chater et al., 2024).

Water is an efficient solvent for extracting polar molecules and is known for effectively extracting a wide range of phenolic compounds,

including flavonoids and other polyphenols. The solubility of highly hydroxylated aglycone forms of phenolic compounds in water, alcohols (ethanol, methanol), and their mixtures is well-established, whereas less polar and highly methoxylated aglycone forms are preferentially extracted into less polar solvents such as ethyl acetate, acetone, and chloroform (Dorta et al., 2012). Additionally, water extracts exhibited relatively high yields for both plants and their organs, contrasting with petrol ether and ethanol, which demonstrated the lowest yields (Kaczorová et al., 2021). These findings align with those reported by Chater et al.

(2024) of the same plant species. Their findings further highlight the importance of solvent composition in the extraction of Total Phenolic Content (TPC), with water extraction giving the highest amount TPC (Chater et al., 2024). The elevated TPC values in methanol extracts align with the solvent's efficiency in extracting diverse phenolic compounds, as reported in previous studies. The findings from the extraction of *Juniperus procera* indicated that methanol is the most effective solvent for recovering TPC from leaf extract compared to other solvents (Salih et al., 2021). Furthermore, in the comparison of various solvents, methanol has generally proven more efficient in extracting lower molecular weight polyphenols, whereas higher molecular weight flavonoids are better extracted with aqueous acetone (Salih et al., 2021). Ethanol extracts also demonstrated substantial TPC values, indicating their effectiveness in extracting phenolic compounds, although slightly lower than the ones obtained with methanol and water. Likely, protic solvents such as ethanol and methanol were found to be effective in extracting phenolic compounds from the roots of *Asparagus officinalis*, resulting in the highest concentration of total phenolics compared to other solvents (Ozdemir et al., 2024). Conversely, hexane, being nonpolar, showed markedly lower effectiveness in extracting TPC, aligning with the solvent's limited ability to extract polar compounds. In summary, the results underscore the importance of solvent selection in optimizing the extraction of phenolic compounds. The polar solvents, particularly water and methanol, proved to be more effective in extracting these bioactive compounds.

Flavonoids are a group of polyphenolic compounds that exhibit various biological effects, such as anti-inflammatory, antihepatotoxic, antiallergic, antiviral, and anticancer activities, while also inhibiting enzymes such as aldose reductase and xanthine oxidase (Ullah et al., 2020). Due to their phenolic hydroxyl groups, they are able to effectively scavenge reactive oxygen species and are potent antioxidants (Rudrapal et al., 2022). The quantification of total flavonoid content (TFC) revealed significant variation across extracts, influenced by the solvent type. Water and methanol extract consistently showed the highest TFC in both leaves and stems, followed by ethanol. Hexane extracts had considerably lower flavonoid amounts. Leaves exhibited higher TFC and TPC compared to stems, a result in agreement with findings such as *Juniperus procera* leaves having the highest TFC in ethanol extracts (Salih et al., 2021).

In summary, polar solvents, particularly water, and methanol, proved more effective in extracting bioactive compounds from *M. suaveolens*. Additionally, a general trend observed by Mathipa et al. (2022) indicates that leaves tend to have higher concentrations of phytoconstituents compared to stems (Mathipa et al., 2022).

To comprehensively evaluate the antioxidant potential of *M. suaveolens*, several assays were conducted, including Total Antioxidant Capacity (TAC), Ferric Reducing-Antioxidant Power (FRAP), free radical scavenging activity (DPPH and ABTS), and metal chelation capacity. These assays collectively highlight the ability of the plant to neutralize free radicals, reduce oxidative stress, and chelate metal ions, underscoring its potential as a source of natural antioxidants.

Water and methanol extract consistently demonstrated the highest antioxidant activity across all assays, with water extracts of leaves showing the most pronounced activity. Methanol extracts also exhibited strong performance, particularly in ABTS and metal-chelating assays, while ethanol extracts showed slightly lower but still significant antioxidant potential. Notably, ethanol extract exhibited the highest DPPH scavenging activity (Nguyen et al., 2020), further supporting its efficacy in free radical neutralization. In contrast, hexane extracts, due to their nonpolar nature, consistently displayed the lowest activity, emphasizing the limited efficiency of nonpolar solvents in extracting antioxidant compounds.

The influence of plant parts was also evident, with leaves generally exhibiting superior antioxidant properties compared to stems across all solvents. This was reflected in higher FRAP values, lower IC₅₀ values in DPPH and ABTS assays, and stronger metal-chelating capacities. The pronounced differences between leaves and stems in the ABTS assay further highlight the importance of using multiple assays to gain a comprehensive understanding of antioxidant activity.

Overall, the findings emphasize the critical role of solvent choice and plant part in optimizing extraction protocols for antioxidant-rich materials. Water and methanol emerged as the most effective solvents, particularly for leaves, reinforcing the potential of *M. suaveolens* as a valuable natural resource for developing antioxidant-rich extracts. The positive correlation between antioxidant activity and total phenolic content further supports the presence of bioactive compounds responsible for the observed effects. These insights contribute

to the broader understanding of solvent selection and plant part utilization in maximizing the antioxidant potential of medicinal plants.

The antimicrobial capacity of *M. suaveolens* extracts was evaluated against Gram-positive and Gram-negative bacteria, as well as fungi, by determining inhibition zones, minimum inhibitory concentration (MIC), and minimum bactericidal concentration (MBC). Leaf extracts generally exhibited stronger antibacterial activity than stem extracts, as evidenced by lower MIC and MBC values, consistent with findings for *Carissa bispinosa* (Shekwa et al., 2023). Organic solvent extracts (ethanol, methanol, and hexane) demonstrated potent inhibitory activity against *B. subtilis* and *E. coli*, while water extracts were less effective, aligning with results reported by Mela et al. (2020).

Hexane extracts, though modest in activity, showed antibacterial potential due to non-polar compounds like alkaloids and terpenoids (Joana Gil-Chávez et al., 2013; Asgharpour et al., 2020). Ethanol extracts, capable of extracting a wide range of polar and moderately non-polar compounds (Plaskova & Mlcek, 2023), exhibited variable antimicrobial activity, with notable efficacy against *S. aureus*. Methanol extracts displayed strong antibacterial activity, particularly against *S. aureus*, *B. subtilis*, and *E. coli*, while water extracts showed modest effects.

The superior antimicrobial activity of leaves over stems, especially in hexane extracts, highlights the influence of solvent choice and plant part on bioactive compound extraction. The observed effects are attributed to synergistic interactions among phytochemicals with antimicrobial properties, as seen in studies on *Bidens pilosa* and *Moringa oleifera* (Falowo et al., 2016). However, the resistance of Gram-negative bacteria to plant extracts, as noted by Biswas et al. (2013), underscores the complexity of antimicrobial mechanisms. These findings position *M. suaveolens* as a promising source of natural antimicrobial agents, warranting further research to identify specific bioactive compounds responsible for the observed activities.

CONCLUSION

The research underscores the presence of potent antioxidant and antibacterial compounds in both *Mentha suaveolens* subsp. *timija* leaves and stems. It also demonstrates how the solvent polarity choice significantly influences both the extraction efficiency and the biological efficacy of the resulting extracts. Generally, leaf extracts

usually demonstrate higher levels of total flavonoid compounds, and stronger antioxidant and antibacterial activities than stem extracts. The findings suggest that *M. suaveolens* subsp. *timija* might be a useful natural source of antioxidants and antibacterial agents, with uses in food, pharmaceuticals, and other sectors.

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