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Original Article

ANTIBACTERIAL, ANTIOXIDANT AND PHYTOCHEMICAL SCREENING OF PALESTINIAN MALLOW, *MALVA SYLVESTRIS* L.

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ABSTRACT

Objective: To evaluate antibacterial, antioxidant activities, the existence of the major secondary metabolites, and volatile compounds in methanolic extracts from *M. sylvestris* leaves.

Methods: Antibacterial activity was assessed using a well diffusion method. Antioxidant activity was assessed using ABTS$^{\bullet+}$and DPPH$^{\bullet}$ free radical scavenging assays. Phytochemical screening for secondary metabolites and volatile compounds were done following standard techniques and gas chromatography-mass spectrometry (GC–MS).

Results: Methanolic extracts exhibited moderate antibacterial activity compared with the positive control against the gram-negative *Klebsiella pneumoniae* and the gram-positive bacteria's *Staphylococcus aureus* by 47.2 and with 47.1% respectively. The average percentage of scavenging was 97.82±0.05 and 79.49±0.4 for ABTS$^{\bullet+}$and DPPH$^{\bullet}$, correspondingly. Total phenols were quantitatively estimated and found to be 78.9±9.55 mg GAE/g. Phytochemical screening assays revealed the presence of a wide range of phytochemical groups such as alkaloids flavonoids, phenols, tannins, quinones, saponins, steroids, tannins, terpenoides with at least sixteen volatile compounds detected in the plant.

Conclusion: The present study confirmed the antioxidant activity of the methanolic extract of *M. sylvestris* and the existence of is the volatile compounds (phytol), which mediate, even partially, the antioxidant and the claimed analgesic activity of the plant.

Keywords: Mallow Mallow, Antioxidant, DPPH, Phytol

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INTRODUCTION

Medicinal plants also called medicinal herbs have been frequently used over the years for anticipation and treatment of many diseases as well as for healthiness. Statistically, it is estimated that there are 750,000 plant species on Earth, of which 1-10% are used as food and medicine by both humans and animals [1, 2]. Medicinal plants display several pharmacological properties such as antioxidants [3], anti-diabetes [4], antibacterial [5], antiviral [6], anticancer [7], and anti-ulcer activities [8]. The medicinal value of these plants lies in a group of bioactive organic compounds (metabolites, secondary compounds) generally classified into alkaloids, saponins, flavonoids, tannins, glycosides, anthraquinones, steroids, and terpenoids and present at different quantities and qualities within the plants [9].

Mallow (*Malva sylvestris* L., Malvaceae) is a biennial–perennial ethnobotanical herb native to Northern Africa, Europe, and Asia. The plant generally grows naturally in moist areas such as near marshes, ditches, riverbanks, and meadows [10]. For many decades, this plant is widely used in the traditional Palestinian culture as food and in curing a large number of diseases [11]. Indeed, leaf extracts have been used traditionally as medicine for their anti-inflammatory, analgesic, antioxidant, neuroprotective, antibacterial, and antifungal activities [12]. For example, decoction from aerial parts of *M sylvestris* has demonstrated remarkable anti-ulcerogenic activity against an ethanol-induced gastric ulcer in rat models [13]. However, hexane extract of *M.* leaves is reported to play a critical role in diabetes management by inhibiting insulin resistance, lipid abnormalities, and oxidative stress [14]. Successive petroleum ether extraction of *Malva* leaves has been reported to exhibit a counter-irritant effect on the rabbit's ear [15]. Due to these pharmacological activities of *Malva* species and others reported by Gasparetto and colleagues (2012), the interest in finding other pharmacological activities or confirming the traditional use of *Malva* species continues to grow given the large scale at which *Malva* species grow and the recent existing literature highlighting its effectiveness in treating diseases in Palestine and other parts of the world [5, 8, 11]. The present study was designed to assess the antimicrobial and antioxidant activities of the methanolic extracts from *M. sylvestris* leaves, and to determine the profile of the secondary and volatile compounds.

MATERIALS AND METHODS

Collection of plant materials

Leaves of mature local mallow plants were collected in April/2018 from Hebron city (Lat: 31.538629, Lon: 35.085769). Botanically, the plant was identified as *M. sylvestris.* Morphological identification of the plant was carried out by referring to the Traditional Arabic Palestinian Herbal Medicine, TAPHM by Ali-Shtayeh, and Flora of Israel Online by Avinoam Danin (http://flora.org.il/en/plants/ephfoe/). A voucher specimen (Pharm-PCT-2743) was preserved for identification in the phytochemical analysis laboratories at Hebron University. Leaves were isolated from plant stems, then cleaned, shade–dried at room temperature, grounded to a coarse powder, and stored in airtight containers.

Antibacterial activity

Extract preparation

The methanolic extract of *M sylvestris* leaves was performed based on the method described by [16] with minor modifications as described below. A 10 g of *M. sylvestris* grounded leaves were extracted in absolute methanol (100 ml) for 24 h at room temperature. The extracts were filtered and concentrated to a final volume of 20 ml and subjected to antibacterial analysis.

Bacterial samples

Four pathogenic bacterial strains were obtained from the microbiology department of Hebron Governmental Hospital. A gram-positive bacteria (*Staphylococcus aureus*), and three strains of

gram-negative bacteria including *Escherichia coli, Pseudomonas aeruginosa,* and *Klebsiella pneumoniae*, were cultured on nutrient agar and incubated at 37 °C for 24 h (Heratherm incubator, Thermo Scientific, Germany). Cultured plates from all bacterial strains were preserved in a refrigerator at 2–4 °C until further use.

Media preparation

Differential media Muller Hinton agar (MHA), Eosin methylene blue (EMB), Mannitol Salt agar (MSA) (all from HiMedia Laboratories, India), and nutrient agar (NA, BioMaxima, Poland) were prepared based on manufacturers recommendations. All prepared media samples were autoclaved at 121 °C for 15 min (Labtech, Korea). The sterile media were poured in sterile Petri dishes (90 × 16 mm) and stored in the refrigerator at 2–4 °C for later use.

Bacterial culture and subculture

All bacterial strains were subcultured on nutrient agar plates and incubated at 37 °C for 24 h. The grown bacteria were further subcultured in differential media as the following: *S. aureus* on MSA, *E. coli,* and *K. pneumoniae* on EMB and *P. aeruginosa* on nutrient agar. Accordingly, all plates were incubated at 37 °C for 24 h.

Sensitivity testing

Sensitivity testing was performed for all bacterial strains using well diffusion method on Muller Hinton agar plates as described in [17]. Bacterial suspensions were prepared to a density of 0.5 McFarland units which is equivalent to $15*10^8$ CFUs/ml from 18-24 h old bacteria colonies in saline solution and spread on Muller Hinton agar plates by a sterile cotton swab. Then, four holes in each plate were made, in which 10 µl extract were added onto each of the first three holes, whereas the fourth hole was used for negative control (methanol). Positive control disks used in this study include vancomycin (30 µg, Biomaxima, Poland) for *S. aureus* bacteria and meropenem (10 µg, Biomaxima, Poland) for all other bacterial strains. The zone of inhibition of the positive controls and *M. sylvestris* extracts were measured (mm) after 24 h incubation at 37 °C, and expressed as a percentage (%) of positive control.

Antioxidant activity

Extract preparation

The leaf extract was prepared following the protocol by [16]. A 200 mg of grounded leaves of *M. sylvestris* were extracted using 4 ml methanol 80% on a shaker (Labtech, Korea) for 24 h at 80 rpm, at 25 °C. The extract (1.5 ml) was transferred into Eppendorf tubes and spun down for 5 min at 4000 rpm) using (MicroCl 17, Thermo Scientific, Germany). The supernatants were transferred to another clean Eppendorf tube for ABTS•+and DPPH•assays.

ABTS•+assay

The ABTS•+solution was prepared by mixing a stock solution of ABTS•+(7 mmol; prepared by dissolving 18 mg of ABTS•+reagent in 5 ml distilled water) with 88 µl potassium persulfate solution (2.45 mmol, prepared by dissolving 75 mg in 2 ml distilled water). The ABTS•+solution was incubated overnight in a dark place. The working solution of ABTS•+was prepared by diluting a stock solution of ABTS•+with 80% methanol to final absorbance 0.7000±0.02 at 734 nm. A 30 µl of diluted plant extracts (1:4) solutions were mixed with 3 ml ABTS•+working solution in micro cuvettes. For control, 30 µl methanol (80%) was mixed. All cuvettes were mixed and incubated in a dark place for 30 min at room temperature. The absorbances of plant extracts (A_{sample}) and the methanol ($A_{control}$) were measured at 734 nm using the Genway UV/Visible spectrophotometer (Cole-Parmer Ltd, UK). The percentage scavenging of ABTS•+was calculated according to the equation:

$$\text{ABTS Scavenging (\%)} = [(A_{control} - A_{sample})/A_{control}] \times 100\%$$

Data Analysis: Data were expressed as means of triplicates±standard deviation.

DPPH• assay

A stock solution of DPPH• was prepared by dissolving 2.3 mg of DPPH• with 5.57 ml of 80% methanol. A 200 µl of DPPH• stock solution was mixed with 2 ml 80% methanol and 20 µl of diluted plant extract (1:4, Sample) or 20 µl of methanol (80%, control) in plastic cuvettes. All cuvettes were mixed and incubated in a dark place at room temperature for 1 h. The absorbances of plant extracts (A_{sample}) and the methanol ($A_{control}$) were measured at 734 nm using Genway UV/Visible spectrophotometer (Cole-Parmer Ltd, UK) at 517 nm. The radical scavenging activity was calculated as a percentage of DPPH• discoloration using the following equation:

$$\text{DPPH Scavenging (\%)} = [(A_{control} - A_{sample})/A_{control}] \times 100\%$$

Phytochemical screening

Malva sylvestris powder (3 g) was extracted in 60 ml methanol 80% for 24 h at room temperature with continuous stirring in a shaking incubator. The extracts then filtered through the vacuum and used to screen for the presence of alkaloids, anthraquinones, anthocyanins, cardiac glycosides, coumarins, flavonoids, glycosides, phenolics, phlobatannins, quinones, saponins, steroids, tannins and terpenoids based on the methods described in [18].

Determination of total phenols using folin–ciocalteu

Leaves samples were extracted following the protocol described in [16]. Dried leaves (2 g) were extracted with 20 ml methanol (70%) at room temperature for 30 min with continuous stirring. After filtration, the resulting raffinate was re-extracted with 10 ml methanol (70%) for 15 min with continuous stirring and filtrated. The combined filtrate was defatted twice with 20 ml of *n*-hexane. Consequently, the defatted extract was filtered twice by GF⁄F filter paper and 0.45 µm nylon syringe filter. Obtained extracts were stored at–20 °C until analysis.

Total phenols were assessed based on the method described in [16]. Plant extracts (20 µl) were mixed with 1.58 ml distilled water, 100 µl Folin–Ciocalteu reagent (Sigma, Israel), and 30 µl of aqueous Na_2CO_3 (20 %, w⁄v) in plastic macro-cuvettes. All cuvettes were mixed and incubated in the dark for 1 h. The absorbance of resulting solutions was measured at 760 nm, data were expressed as milligrams of gallic acid per gram of dried plant leaves (mg GAE⁄g). The assay was done in triplicate.

GC–MS analysis

Volatile compounds in *M. sylvestris* leaves (3 g) were extracted in absolute methanol (10 ml) overnight and analyzed using a GC–MS (Clarus SQ 8S, Perkin Elmer, USA) fitted with a BD-5 ms capillary column (30 m, 0.25 µm film thickness, 0.25 µm bore diameter) based on the method described by Qawasmeh and others [19] with minor modifications as described below. The injection volume was 1 µl. The oven temperature was maintained at 80 °C for 2 min and was programmed to rise to 280 °C at the rate of 30 °C/min. The temperatures of the injector and the detector were maintained at 250 °C and 260 °C, respectively. Helium was used as the carrier gas; the total-gas flow and velocity were maintained at 134.3 ml/min and 43.1 cm/s, respectively. MS scan speed was 1000 amu/s and the molecular masses (*M/Z*) of the compounds between 50 and 500 *M/Z* were acquired. The analysis for each sample was repeated 3 times. Compounds were tentatively identified using the NIST05 mass spectral library, and when applicable, their mass spectra were compared with those published in the literature.

Table 1: Mean antibacterial activity (% of positive control) of *Malva sylvestris* leaves methanolic extracts against several bacterial species

Bacterial species		Positive control (mm)	Antibacterial activity (%)*
Escherichia Coli	G^-	25	24.2±4.6
Klebsiella pneumonia	G^-	10	47.1±5.9
Pseudomonas aeruginosa	G^-	25	29.9±5.0
Staphylococcus aureus	G^+	30	47.2±4.6

*Values are mean of replicate determination (n=3)±standard deviation. G^-, gram-negative; G+, gram-positive

RESULTS

Antibacterial activity

Methanolic extract of *M. sylvestris* leaves displayed antibacterial activity against the gram-negative *K. pneumoniae* (47.1%) and the gram-positive *S. aureus* (47.2%) bacteria compared with the positive control (table 1). Other bacterial species were little affected by the methanolic extract table 1.

Antioxidant activity

The diluted methanolic extract of *M. sylvestris* leaves displayed antioxidant capacity using the two stable free radical scavenging assays, namely, $ABTS^{\bullet+}$and $DPPH^{\bullet}$. The average percentage of scavenging was 97.82±0.05 and 79.49±0.4 for $ABTS^{\bullet+}$and $DPPH^{\bullet}$, respectively.

Phytochemical screening

The Phytochemical screening assays for the methanolic extracts of *M.* sylvestris leaves revealed the presence of a wide range of phytochemical groups such as alkaloids flavonoids, phenols, tannins, quinones, saponins, steroids, tannins, terpenoides. Other groups were not detected as summarized in table 2.

Determination of total phenols using folin–ciocalteu

The total phenols in the methanolic extracts of *M. sylvestris* leaves were quantitatively estimated and found to be 78.9±9.55 mg GAE/g (n=3).

GC–MS analysis

The GC–MS analysis revealed the presence of at least 16 volatile compounds fig. 1. Major volatile compounds detected in the methanolic extract of *M. sylvestris* leaves were tetradecenol (Rt = 7.8), oxirane (Rt = 7.958.2), Octadecatrienoic acid (Rt = 8.71) and Phytol (Rt = 8.75). Other volatile compounds identified include but not limited to vitamin A aldehyde (retinal), hexadecenol, nonadecanoic acid, and trans benzyl cyclohexanol table 3.

Table 2: Phytochemical screening for the methanolic extracts from *M. sylvestris* leaves

Phytochemical groups	Extract
Alkaloids	+
Anthraquinone	-
Anthocyanin	-
Cardiac Glycoside	-
Coumarins	-
Flavonoid	+
Glycosides	-
Phenols	+
Phlobatannins	-
Quinones	+
Saponins	+
Steroids	+
Tannins	++
Terpenoids	+

+, present,-, absent

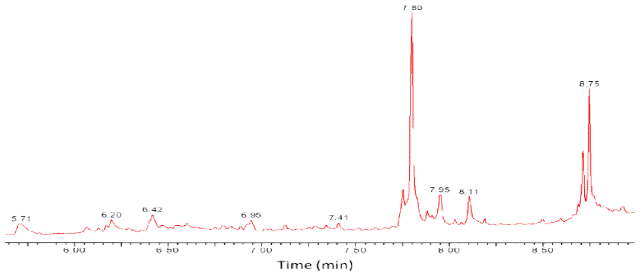

Fig. 1: Representative GC–MS total-ion mass chromatograms of the volatile compounds detected in the methanolic extracts of Malva sylvestris leaves. Numbers on peaks represent the retention time (*Rt*) in minutes for each peak

Table 3: Major compounds detected in *M. sylvestris* extracts with their retention time (*Rt*) and molecular weight (MW), Molecular masses (M/Z) and the molecular formula (MF)

t	M/Z	Compound identification	MW	MF
5.71	55,84,91	1-Pentene,2,3,3-trimethyl-5-phenyl	188	$C_{14}H_{20}$
6.04	58,84,105	1-Oxaspiro(2.2)pentane,5-isopropylidene-tetramethyl	166	$C_{11}H_{18}O$
6.07	93,105,132	5,9-Tetradecadiyne	190	$C_{14}H_{22}$
6.13	55,73,127	Caprylic anhydride	270	$C_{16}H_{30}O_3$
6.20	73/98/147	N-phenethyl-2-methylbutylidenimine	189	$C_{13}H_{19}N$
6.42	55,91,107	Vitamine A aldehyde	204	$C_{15}H_{24}$
6.47	55,91,147	Hexanoic acid	280	$C_{16}H_{24}O_4$
6.95	73,117,156	Pentanedioic acid 3,3-dimethyl-dimethyl ester	188	C9H16O4
7.41	55,117,173	Transbenzylcyclohexanol	242	$C_{16}H_{22}N_2$
7.76	57,68,95	Hexadecen-1-ol, tetramethyl	296	$C_{20}H_{40}O$
7.80	57,68,95	Tetradecen-1-ol, methylpropionate	282	$C_{18}H_{34}O_2$
7.95	57,81,95	Oxirane, heptadecyl	282	$C_{19}H_{38}O$
8.03	57,73,147	1,2-15,16-Diepoxyhexadecane	254	$C_{16}H_{30}O_2$
8.11	74,87,143	Nonadecanoic acid, methyl ester	312	$C_{20}H_{40}O_2$
8.71	57,67,79	Octadecatrienoic acide	320	$C_{18}H_{30}O$
8.75	55,71,123	Phytol	296	$C_{20}H_{40}O$

DISCUSSION

Although Palestine is a small country, it has a wide range of agro-ecological concerns and hosts a large variety of plants [20-22]. Such plant richness might relate to its unique geographical and ecological environment preciously to its location between the continents Asia, Europe, and Africa, and between the eastern Mediterranean Sea, Red Sea, Dead Sea, and Jordan River; in addition to its elevation span ranges from-430 meters (Dead Sea) to+1100 meter above the sea level). Such a situation allowed the survival of a wide range of plants, some of which are edible as a food and/or as a medicinal plant. *Malva sylvestris* is among the traditional edible and medicinally important plants in Palestine. However, only a few ethnobotanical studies on *M. sylvestris* plant have been accomplished [23, 24] with a slight emphasis on its biological activities and phytochemical constituents [12]. Given the large scale at which the plant is wildly grown, from sea level up to the high mountains and its multiple usages among Palestinians, it is crucial to evaluate its antimicrobial and antioxidant activities, profile the major phytochemical groups and appraise the existence of the volatile compounds.

Our results showed that *M. sylvestris* leaves showed potential antimicrobial activities against *K. pneumoniae* and *S aureus*. The zone of inhibition recorded for both strains was almost half of that recorded for the positive control antibiotics (meropenem and vancomycin, respectively). These findings are consistent with the study of Dulger and Gonus (2004) reporting moderate activity of *M. sylvestris* aerial parts (methanolic extract) against *K. pneumoniae* and *S aureus* using disc diffusion methods [25]. In this study, *E. coli* and *P. aeruginosa* were little affected by the methanolic extracts of *M. sylvestris* leaves (zone of inhibition<30% of the positive control). These findings are in accordance with other studies reporting *E. coli* particularly is either resistant [25, 26] or moderately sensitive [10] to the methanolic extract of *M. sylvestris* aerial parts. Although some studies have reported antimicrobial activities against some bacterial strains such as *Streptococcus mutans* [27], based on our research findings, the antibacterial activities of *M. sylvestris* leaves *in–vitro* remain inconclusive and warrant further investigations.

The antioxidant activity of *M. sylvestris* was evident using ABTS$^{\bullet+}$and DPPH$^{\bullet}$ assays. The percentage scavenging activity was 97.82±0.05 and 79.49±0.4 for ABTS$^{\bullet+}$and DPPH$^{\bullet}$, respectively. This antioxidant activity has been consistently attributed to the presence of phenolic compounds (flavonoids, phenols, and tannins) containing a hydroxyl group capable of scavenging the free radical [16, 28, 29]. Phytochemical screening revealed the presence of phenols, flavonoids, and tannins in the methanolic extract of *M. sylvestris,* confirming the existence of the antioxidant activity. Detection of alkaloids in *M. sylvestris* is of particular interest due to the potential pharmacological activities of these compounds. In this study and others [30, 31] alkaloids have been detected qualitatively in *M. sylvestris* and the related species such as *M. parviflora* [32], whereas only two alkaloids, sanguinarine, and berberine, were reported to occur in the flowers of *M. sylvestris* plant at concentrations (w/w %) 0.10126% and 0.00059%, respectively [31]. Whether these alkaloids or others exist in the leaves of Palestinian *M. sylvestris* has not been established and further studies are needed.

Here, we confirmed for the first time the presence of 16 volatile compounds in the methanolic extract of Palestinian *M. sylvestris* leaves using GC–MS. These compounds were tentatively identified as described in table 3. Volatile compounds in *M. sylvestris* have been studied by Tabaraki and others from Iran involving plants' flowers [33] and by Al-Rubaye and others from Iraq involving plants' leaves [34]. Notably, some similarities in the identity of the volatile compounds have been observed between this study and previous studies [33, 34]. Phytol, hexadecenol (ipalmitic acid analog), and octadecatrienoic acid (linolenic acid analog) derivatives were all reported in the leaves of *M. sylvestris* plants [34]. Although it is difficult to associate the pharmacological activities of *M. sylvestris* with a single volatile compound, phytol has been reported to exhibit antioxidant, anticancer, diuretic, and antinociceptive (analgesic) activities.[34, 35]

CONCLUSION

Malva sylvestris leaves are a source of chemically diverse compounds, some of which may represent a starting compound in drug development. Identifying phytochemical compounds in *M. sylvestris* leaves–plant commonly used in Palestinian herbal medicine–remains in its early stages. Several experimental models are required to approve its ethnopharmacological uses and identifying the underlying compounds responsible for the plants' activity. To the best of our knowledge and despite the plants' antioxidant activity, there are no pharmaceutical dosage forms that have been designed where *M. sylvestris* is part of their active constituents.

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AUTHORS CONTRIBUTIONS

All authors contributed equally in writing the manuscript.

CONFLICT OF INTERESTS

This statement is to declare that all authors involved in this manuscript have no conflict of interest.

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