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Original article

Antibacterial and antioxidant activities of *Mentha piperita* L.

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Abstract

The antibacterial activity of peppermint oil and different extracts of *Mentha piperita* against some Gram-positive and Gram-negative bacterial strains was evaluated in the present research work by agar well diffusion method. It was found that the distilled concentrations of essential oil inhibited the growth of microorganisms and the results were comparable with those of antibiotic gentamycin. Essential oils showed a wider spectrum of activity but less strong inhibition as compared to the investigated commercial antibiotic. Minimum inhibitory concentrations (MICs) for the bacterial species ranged from 0.4% to 0.7% v/v. The oil and extracts also exhibited significant antioxidant activity and the oil showed about half potency when compared to the standard BHT. These results indicated the strong antibacterial and antioxidant activities of peppermint oil but additional investigations need to be performed in order to confirm the safety of these concentrations (MIC) for human consumption. Peppermint oil could be used as a good conservation agent by inhibiting some food borne pathogens.

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Antibacterial; Antioxidant; *Mentha piperita* L; Peppermint oil; MICs

1. Introduction

Mentha piperita L., a medicinally important plant belongs to the Family Lamiaceae (African pharmacopoeia, 1985; The Wealth of India, 1962) and commonly known as peppermint is a hybrid of *M. spicata* L. (spearmint) and *Mentha aquatic*. It was cultivated by the ancient Egyptians and documented in the Icelandic pharmacopoeia of the thirteenth century. It is widely grown in temperate areas of the world, particularly in Europe, North America and North Africa but nowadays cultivated throughout all regions of the world. The medicinal parts are the essential oil extracted from the aerial parts of the flowering plant, the dried leaves, the fresh flowering plant and the whole plant. *M. piperita* is a perennial 50–90 cm high, normally quadrangular and a prototypical member of the mint family (Briggs, 1993; The Wealth of India, 1962). The usually branched stems are often purplish or tinged violet but sometimes they are gray-tomentose. The dark or light green leaves are short-petioled, oblong-ovate and serrate with their margins finely toothed. The flowers are purple or pinkish having false spikes with numerous inconspicuous bracts and rarely bear seeds (Clark and Menory, 1980). The plant is generally sterile and spreads by means of runners. The plant grows in a sunny side and prefers acid, neutral and basic, light, medium soils but can also grow in heavy clay soil (Bradley, 1992).

Peppermint yields 0.1–1% of volatile oil (Leung, 1980) composed primarily of menthol (29–48%), menthone (20–31%), menthofuran (6.8%) and menthyl acetate (3–10%). Other pharmacologically active ingredients include bitter substances, caffeic acid, flavonoids (12%), polymerized polyphenols (19%), carotenes, tocopherols, betaine, choline and tannins (Karuza et al., 1996; Shimada et al., 1992; Sokovic et al., 2009). Measured low to moderate levels of phenolics with antioxidant activity were reported from peppermint (Zheng and Wang, 2001). The chemistry of peppermint oil is very complex and highly variable. The relative concentrations vary depending on climate, cultivar, and geographic location (Hoffmann and Lunder, 1984; Lis-Balchin et al, 1997; Maffei and Sacco, 1987). Peppermint oil and its constituents are commercially used in food, pharmaceutical and cosmetics industries. Menthol is used as a raw material in toothpaste, toothpowder, chewing tobacco, confectionary, mouth fresheners, analgesic balms, cough drops, perfumes, chewing gums, candies and tobacco industry. Tobacco industry constitutes about 40% of the total oil consumption followed by pharmaceutical and confectionary industries. The fresh or dried leaves are the

 nt and are used in breath fresheners, drinks, antiseptic m

and Arab countries. The substances that give the mints their characteristic aromas and flavors are menthol (African pharmacopoeia, 1985; Briggs, 1993; Hoffmann and Lunder, 1984).

In Eastern and Western traditional medicine peppermint and its oil have been used as an antispasmodic, aromatic, antiseptic and also in the treatment of cancers, colds, cramps, indigestion, nausea, sore throat and toothaches (Briggs, 1993). Peppermint oil possesses antibacterial activity *in vitro*. Different commercial preparations exhibit various activities (Lis-Balchin et al., 1997). Peppermint oil and menthol have moderate antibacterial effects against both Gram-positive and Gram-negative bacteria (Diaz et al., 1988). Peppermint is also found to possess antiviral and fungicidal activities (Chaumont and Senet, 1978). Aqueous extracts of the leaves demonstrated significant antiviral activity against Influenza A, Newcastle disease, *Herpes simplex*, *Vaccinia*, Semliki Forest and West Nile viruses in egg and cell culture system (Herrmann and Kucera, 1967). It was also found to reduce the incidence and multiplicity of benzo[α]pyrene-induced lung carcinogenicity and mutagenicity (Samarth et al., 2006). In clinical trials peppermint oil's role in irritable bowel syndrome affirms its effectiveness compared with a placebo with no serious constipation or diarrhea (Kline et al., 2001; Liu et al., 1997; Pittler and Ernst, 1998). In this paper, the antibacterial effects of leaves extracts and essential oil against different bacterial strains, antioxidant activities and phytochemical screening of *M. piperita* are presented.

2. Materials and methods

2.1. Materials

The leaves of *M. piperita* were procured from a local farm house at Gamineus in Benghazi, Libya, identified and confirmed by a taxonomist, a voucher specimen was deposited at the herbarium in the institute (Voucher No. 5386). The absorbance of the reaction mixture was measured with Analytic Jena spectrophotometer, Germany. Mueller–Hinton agar (MHA) was used as base medium for the screening of antibacterial activity, Mueller–Hinton broth (MHB) for preparation of inoculums and both were purchased from Merck, Germany and all other chemicals and reagents used were of analytical reagent grade.

2.2. Distillation of oil

Hydrodistillation was conducted by a standard procedure (Clevenger apparatus) with dried peppermint leaves which had previously been chopped in a domestic blender. The isolation experiment was carried out continuously on a heating mantle at the temperature 60–80 °C until no further oil was extracted. The essential oil was dried over anhydrous Na_2SO_4 and after dark bottle at 4 °C until tested and analyzed. The yield of

2.3. Extraction of M. piperita L. leaves

The chopped, dried leaves of *M. piperita* (1 kg) were transferred into a round bottom flask and subjected to hot extraction by refluxing it with petroleum ether (2 × 2 L) for 2 × 3 h. The extract was cooled and filtered. Further, the plant material was refluxed with chloroform, ethyl acetate, and ethanol, finally with distilled water (2 × 2 L each) for 2 × 3 h, cooled and filtered after each extraction. Each filtrate was evaporated under reduced pressure in rotary evaporator to obtain an oily mass (32.4 g after deep freeze cooling) in case of pet. ether, a viscous mass (28.7 g) for chloroform, 14.2 g for ethyl acetate, 11.8 g for ethanol and 16.3 g for water extract.

2.4. Preparation of solutions

The dried mass (50 mg) of each extract was redissolved at room temperature (28 ± 1 °C) in the corresponding solvent (100 mL) by simple dissolution techniques to prepare a stock solution having concentration 500 μg/mL. Further, 1 mL of stock solution of each extract was dissolved in 100 mL to prepare a solution of 5 μg/mL and all the experiments were conducted within this range (5–500 μg/mL). In case of oil distilled concentration was diluted in ethanol (75%).

2.5. Phytochemical analysis

Each extract of the leaves (2–3 mg/mL) of *M. piperita* was subjected to a preliminary phytochemical analysis for the detection of different chemical groups (Harborne, 1998) by using the different tests and the results are presented in Table 1.

Table 1. The results of the preliminary phytochemical screening of leaves extracts of *M. piperita*.

Chemical groups	PEE[a]	CE[b]	EAE[c]	EE[d]	AE[e]
Terpenoids	+	+	–	–	–
Steroids	–	+	+	–	–
Phenols	–	+	+	–	–
Flavonoids	–	+	+	–	–
Alkaloids	–	–	–	–	–
Tannins	–	–	–	+	+

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b
Chloroform extract.

c
Ethyl acetate extract.

d
Ethanol extract.

e
Aqueous extract.

2.6. Antioxidant activity

2.6.1. Antioxidant capacity

The antioxidant capacity of samples was measured by applying the procedure described previously (Arnao et al., 2001) with little modifications. A 4.4 units/mL of peroxidase, 50 μM of H_2O_2, 100 μM of 2, 2-azino-bis (3-ethylbenz-thiazoline-6-sulfonic acid) and 1 mL distilled water were mixed and kept in dark for 1 h for the reaction. After the addition of 1 mL of plant material absorbance was determined at 734 nm. The antioxidant capacity was calculated by the following formula:

$$\mathbf{Antioxidant activity}\ (\%) = 100 \times (1 - A_{\mathbf{sample}}/A_{\mathbf{blank}}).$$

2.6.2. Free radical scavenging activity

The antioxidant activity of the essential oil and extracts was assessed by their ability to scavenging 2,2-diphenyl-1-picrylhydrazyl stable radicals (DPPH) by using the method described previously (Shimada et al., 1992). Briefly, 1 mL of methanolic extract and 5 mL of freshly prepared 0.1 mM DPPH methanolic solution were thoroughly mixed and kept in the dark for 60 min. The absorbance of the reaction mixture at 517 nm was measured with a spectrophotometer. The blank was prepared by replacing the extract with methanol (1 mL). The percentage of free radical scavenging activity was calculated as follows:

$$\mathbf{Radical\ scavenging}\ (\%) = 100 \times (1 - A_{\mathbf{sample}}/A_{\mathbf{blank}}).$$

Where A_{blank} is the absorbance of the control and A_{sample} is the absorbance of the test essential oil/extracts or BHT. IC_{50} is defined as the concentration sufficient to obtain 50% of a

2.6.3. Reducing power

Reducing power was measured according to the method described by Duh and Yen (1997). One mL of oil or plant extract of *M. piperita* was mixed with phosphate buffer (0.2 M, pH 6.6, 0.5 mL) and potassium hexacyanoferrate solution (1% v/w) in a test tube and heated at 50 °C for 20 min. After cooling the tube on ice, 0.5 mL 10% trichloroacetic acid was added. After centrifugation for 10 min, 1 mL distilled water and 0.1 mL ferric chloride (0.1%) were mixed with 1 mL of aliquot supernatant. Finally, the absorbance at 700 nm was measured, increased absorbance of the reaction mixture indicated an increased reducing power.

2.7. Determination of antibacterial activity

2.7.1. Bacterial stains

Two Gram-positive (*Staphylococcus aureus* ATCC 25923 and *Streptococcus pyogenes* ATCC19615), and two Gram-negative (*Escherchia coli* ATCC 25922 and *Klebsiella pneumonia* ATCC 13883) bacteria were selected for antibacterial activity assay. The cultures of bacteria were maintained in their appropriate agar slants at 4 °C throughout the study and used as stock cultures.

2.7.2. Preparation of standard inoculums

The microorganisms were inoculated into Muller Hinton broth (MHB) supplemented with 5% defibrinated sheep blood and incubated at 37 °C for 12–15 h. The turbidity of the resulting suspension was diluted with MHB to match with 1 McFarland turbidity standard. This level of turbidity is equivalent to approximately 3.0×10^8 CFU/mL, equivalent to 0.5 Macfarland standards.

2.7.3. Agar diffusion method

The protocols used in this study were based on guidelines of CLSI, formerly known as NCCLS (CLSI, 2006) with slight modification. Briefly, 200 μL fresh overnight cultures of the indicator strains of bacteria (~10^8 CFU/mL) were added onto Muller Hinton agar (MHA) containing 5% defibrinated sheep blood. The MHA was vigorously mixed and poured over Petri plates with previously dried correspondent agar medium on the surface of which the sterile tubes (7 mm diameters) were placed. After solidification of the MHA, the tubes were removed and the obtained wells were filled with 10 μL of the *M. piperita* extracts and oil. In order to accelerate diffusion of the essential oil into agar, plates were incubated at 4 °C for 1 h and were then incubated at 37 °C. After 24–48 h of incubation, the antibacterial activity was evaluated by

 of the zone of inhibition (clear) of growth against the ind

To establish the nature of inhibitory activity of the oil, samples were taken from the clear zones with a loop and surface-plated onto appropriate agar and incubated under optimal conditions for up to 48 h.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined by the broth twofold macro dilution method in Muller Hinton broth supplemented with 5% defibrinated sheep blood for bacterial strains according to a modification of the procedures reported earlier (Mazzanti et al., 2000; NCCLS, 2000). MIC was defined as the lowest concentration of *M. piperita* essential oil that allows no more than 20% growth of the bacteria, which is seen as the decreased number of colonies after removing the loop with 10 μL of each dilution on MHA and incubation at 37 ± 1 °C for 18 h. MBC was defined as the lowest concentration of the peppermint oil that allows no growth of microorganisms. Gentamycin served as positive control while pet. ether, chloroform, ethyl acetate, ethanol and water were included in every experiment as negative controls. All determinations were carried out in triplicate.

2.7.4. Antibiogram test

With the application of agar medium as per manufacturer's instructions, the test was performed. A solution of 10 μg/mL of gentamycin in the form of standard antibiotic discs was used in order to provide a positive control while pet. ether, chloroform, ethyl acetate, ethanol and water were included in every experiment as negative controls for the sensitivity of the indicator organism.

2.7.5. Statistical analysis

The resultant zones (clear) or suppression (diffuse) around the discs were measured in mm. The antibacterial activity of oil and plant extracts were indicated by clear zones of growth inhibition. All the experiments were conducted in triplicate and the data are presented as mean values ± standard deviation.

3. Results and discussion

The essential oil and different extracts of *M. piperita* were explored for antioxidant activity by evaluating their antioxidant capacity, DPPH free radical scavenging activity and reducing power, and the results are given in Table 2. Chloroform extract and peppermint oil showed almost equal antioxidant potency (about ~90%). Aqueous extract exhibited the least potency among all. In DPPH free radical scavenging activity and reducing power absorbance of both chloroform extract exhibited similar trends as observed in

showed the antioxidant capacity, DPPH scavenging activity and reducing power in between those of chloroform and aqueous extracts. The IC_{50} (μg/mL) of peppermint oil by using DPPH scavenging method was found to be 15.2 ± 0.9 while for positive control BHT it was 6.1 ± 0.3.

Table 2. The results of antioxidant screening of oil and leaves extracts of *M. piperita*.

Sample	Antioxidant capacity at 734 nm (%)	DPPH free radical scavenging activity (%)	Reducing power (absorbance 700 nm)
Peppermint oil	89.4 ± 6.3	92.6 ± 6.8	0.9 ± 0.3
Pet. ether extract	73.6 ± 8.2	71.3 ± 9.1	0.6 ± 0.5
Chloroform extract	91.2 ± 5.6	91.8 ± 5.8	0.8 ± 0.3
Ethyl acetate extract	87.8 ± 6.6	84.9 ± 4.2	0.8 ± 0.1
Ethanol extract	76.2 ± 4.5	74.8 ± 5.2	0.7 ± 0.1
Aqueous extract	69.8 ± 5.2	70.3 ± 6.1	0.4 ± 0.3

The antibacterial activity of the *M. piperita* oil and different extracts was assessed using the agar well diffusion method by measuring the diameter of growth inhibition zones at different concentrations. The results of antimicrobial activity of the peppermint essential oil by the MHA well diffusion method are presented in Figure 1, Figure 2, Table 3. Both Gram +ve bacterial species (*S. aureus* and *S. pyogenes*) tested were sensitive to peppermint essential oil with the inhibition zone 17.2 and 13.1 mm, respectively. The inhibition zone for Gram –ve bacteria ranges from 5.1 to 12.4 mm. *S. aureus* was found to be the most sensitive, followed by *S. pyogenes. and K. pneumoniae* which were found to be more sensitive to essential oil when compared to *E. coli.* Thus it is effective against Gram +ve and Gram –ve bacteria and more effective against Gram +ve organisms when compared to Gram –ve. The lipopolysaccharides present in the outer membrane of Gram –ve bacteria might be responsible for their enhanced resistance to antibacterial substances (Iscan et al., 2002). The peppermint oil (10 μL) exhibited greater zone of inhibition against *S. aureus, S. pyogenes,* and *K. pneumonia* than the positive control gentamycin (10 μL of 10 μg/mL concentration) as shown in Table 3 and Fig. 3. However, [illegible]ved lesser zone of inhibition (5.1 ± 0.4 mm) against *E. coli* t[illegible]

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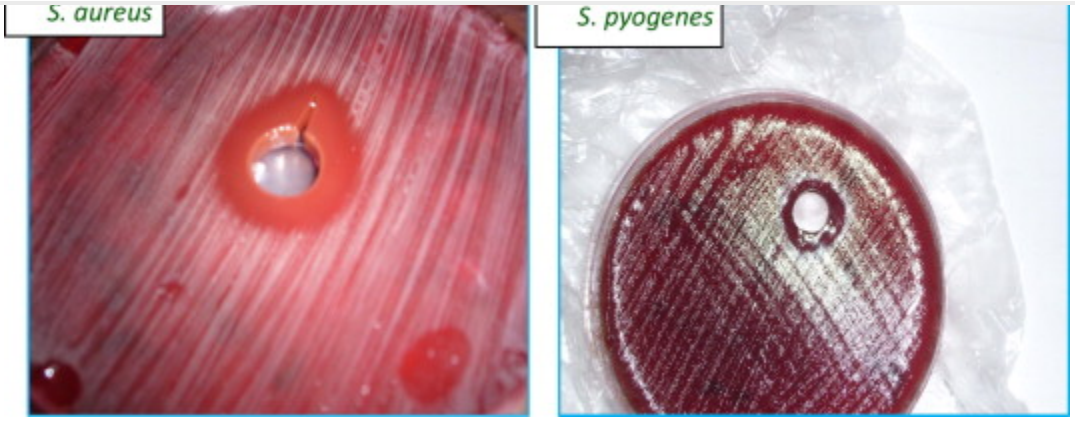

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Figure 1. Inhibitory zone of *M. piperita* oil (1 μL) on Gram +ve bacterial strain.

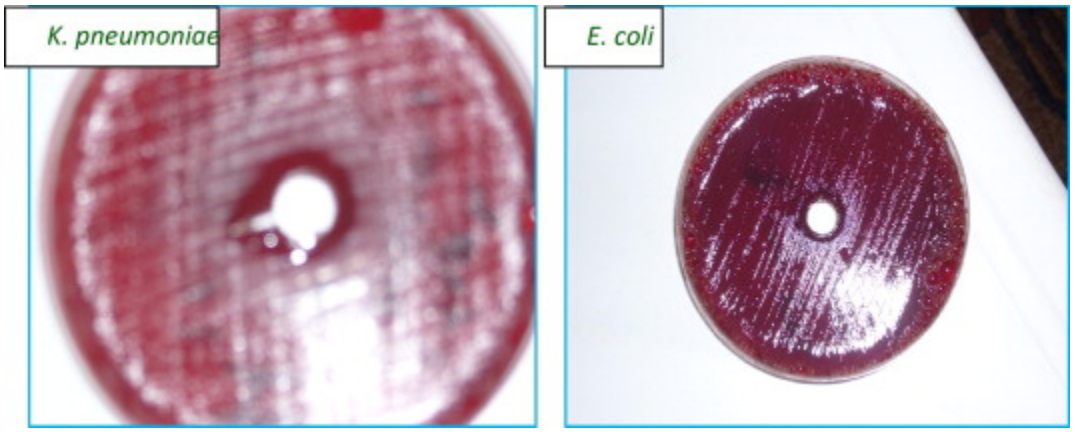

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Figure 2. Inhibitory zone of *M. piperita* (1 μL) on Gram –ve bacterial strains.

Table 3. The results of the antibacterial activity of peppermint oil.

	ssential oil, IZ[a]	MIC (%	MBC (%	Gentar

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aureus	[d]					
Streptococcus pyogenes	13.1 ± 0.7	8.1 ± 0.4	2.1 ± 0.02	0.5 ± 0.01	0.7 ± 0.01	18.9 ± 0.7
Escherchia coli	5.1 ± 0.4	1.9 ± 0.3	–	0.7 ± 0.04	0.9 ± 0.03	19.7 ± 0.3
Klebsiella pneumonia	12.4 ± 0.7	7.3 ± 0.6	1.8 ± 0.03	0.4 ± 0.02	0.8 ± 0.03	21.2 ± 0.6

a

IZ, diameter of inhibition zone (mm) excluding diameter of well (6 mm).

b

Distilled concentration of oil.

c

The final volume filled in the wells was 10 μL (diluted in 75% ethanol). The negative control (75% ethanol) did not show any activity.

d

All values in this table represent the mean ± SD ($n = 3$).

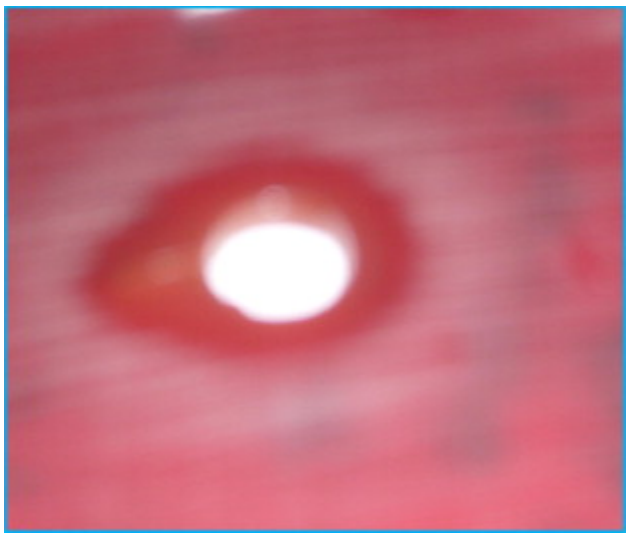

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Figure 3. Inhibitory zone of gentamycin on *S. aureus* strain.

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essential oil of *M. piperita* indicate that *S. aureus* is more susceptible than *E. coli*.

The antibacterial results for different extracts (Fig. 4) indicates that the pet. ether, chloroform and ethyl acetate extracts were found more effective than compared to ethanol and aqueous extracts. Among the organisms tested, pet. ether and ethyl acetate extracts were more effective against *S. aureus* and *K. pneumoniae* when compared to *S. pyogenes* and *E. coli*. Similar trend is observed in case of ethyl acetate and aqueous extracts.

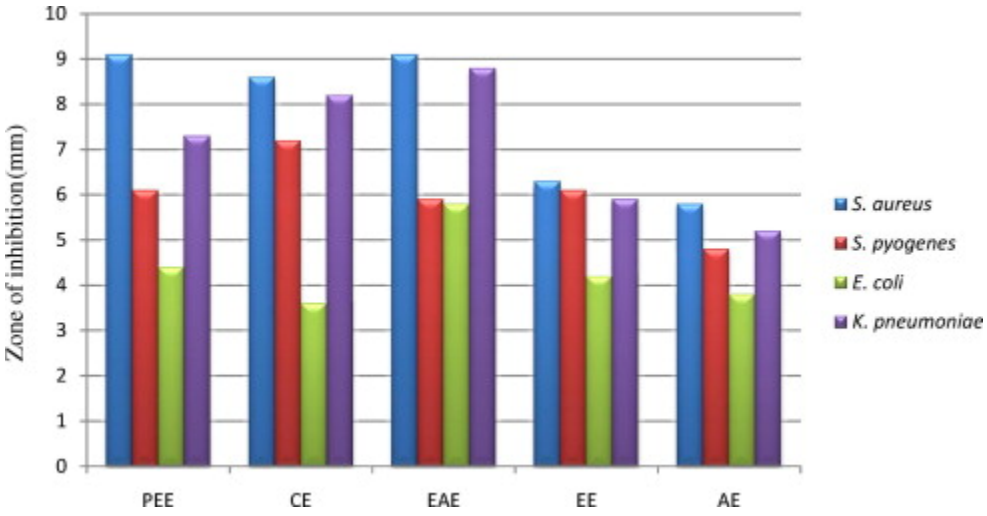

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Figure 4. Antibacterial effect of various extracts (30 μg/mL) of *M. piperita* on *S. aureus*, *S. pyogenes*, *E. coli* and *K. pneumoniae*.

The antibacterial results for different extracts (Fig. 4) indicates that the pet. ether, chloroform and ethyl acetate extracts were found more effective compared to ethanol and aqueous extracts. Among the organisms tested, pet. ether and ethyl acetate extracts were more effective against *S. aureus* and *K. pneumoniae* when compared *to S. pyogenes* and *E. coli*. A similar trend is observed in the case of ethyl acetate and aqueous extracts.

The antibacterial (against *E. coli* and *S. aureus*) and antioxidant activities (DPPH) of peppermint oil were reported by Rasooli et al. (2008), whereas the antibacterial activity of different extracts against the same bacterial strains was reported by Priya et al. (2007). The differences in antibacterial and antioxidant activities with the reported one may be attributed to different procedures followed or a different geographical environment, cultivar type, seasonality, [illegible] the plant, and the method of oil isolation.

fractional distillation under vacuum were tested for their antibacterial and antioxidant activities. The medicinally important constituents are the essential oils, which comprise about 0.64% of the leaves. The major components of peppermint oil which were reported earlier (Clark and Menory, 1980) include menthol (29–48%), menthone (20–31%), menthofuran (6.8%) and menthyl acetate (3–10%) representing nearly 90% of the total essential oils. The antibacterial activity associated with the contribution of the menthol (Iscan et al., 2002). Menthol a non polar terpene can be extracted either by pet. ether or chloroform and the antibacterial activity of these extracts should be more in that case but actually ethyl acetate extract possessed more inhibition. Therefore, antibacterial activity may likely to be associated with a high concentration of menthol (pet. ether and chloroform extracts), phenols or flavanoids (chloroform and ethyl acetate extracts) but a synergistic effect of the other constituents of peppermint oil cannot be ruled out. Finally, it can be concluded that the active chemical compounds present in *M. piperita* should certainly find a place in the treatment of various bacterial infections. The results from the present study are very encouraging and indicate that this herb should be studied more extensively to explore its potential in the treatment of infectious diseases as well.

It was established that the fractions containing the high concentrations of oil inhibited the growth of microorganisms and results were compared with antibiotic gentamycin commonly used therapeutically and they showed less strong inhibition for Gram –ve bacteria and pronounced inhibition for Gram +ve bacteria. Antimicrobial and antioxidant properties of essential oils are of great interest in food, cosmetic and pharmaceutical industries since their possible use as natural additives emerged from the tendency to replace synthetic preservatives with the natural ones. But additional investigations need to be performed in order to confirm the safety of these concentrations (MIC) for human consumption. Furthermore, the MBC/MIC ratio is clearly higher than 1, indicating a bacteriostatic effect of the essential oil. The underlying antimicrobial and antioxidant mechanisms of the essential oils as well as their active components need to be further studied and clarified. Additional in vivo studies and clinical trials would be needed to justify and further evaluate the potential of this oil as an antibacterial agent in topical or oral applications.

Conflict of interest

The authors report no financial or nonfinancial conflict of interest. The authors alone are responsible for the content and writing of the paper.

Acknowledgment

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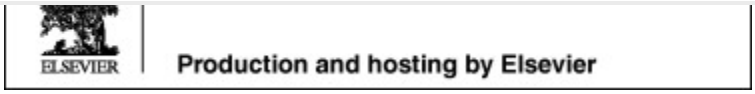

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