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Research Article

In Vitro Antimicrobial Activity of *Mentha piperita* Leaves Extracts

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ABSTRACT

M. piperita (Lamiaceae family) have been used as traditional remedies for the treatment of several diseases. In this work, we aimed to characterize the antimicrobial activity of extracts of *Mentha piperita* leaves. Various extracts (methanolic and aqueous) were analysed for their phenolic content and antimicrobial activity. This was done by the method of diffusion against three Gram+ (*Staphylococcus aureus* ATCC 25923, Methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 43300 and *Bacillus subtilis* ATCC6633) and three Gram- (*Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922 and *Salmonella typhi* ATCC 14028) strains and a yeast (*C. albicans* ATCC 1024). On the other hand, the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of active extracts; were determined by using the dilution method on solid medium. The results indicate that the leaves extracts are rich in polyphénols. On the other side, the Antimicrobial tests exhibited different activities depending on the strain used and the nature of the extract (methanol or aqueous). MRSA was the most sensitive since its growth was inhibited by both extracts with the widest diameter of 23.5mm, whereas Gram-negative bacteria and the yeast *C. albicans* were the most resistant. The results of the MIC and the MBC of active extracts showed that both extracts (methanolic and aqueous) of leaves showed the best activity against SARM (MIC: 8mg/ml; MBC: 10mg/ml). Determination of total phenols and flavonoids suggested that the antibacterial activity is attributed mainly to the richness of this plant in phenolic compounds. These preliminary results may justify the use of this plant in the treatment of certain bacterial diseases and that phenolic compounds may be exploited for therapeutic purposes, especially antibacterial.

INTRODUCTION

Medicinal plants and their derived compounds (phytochemicals) have been considered of pharmacological significance since ancient times. The use of plants in medicine dates back to 60,000 years ago, before the birth of civilization. Today, more than 30% of all medicinal drugs (and their derivatives and analogs) derive from plants, and natural products will continue to possess considerable impact in human medicine. Most synthetic bioactive drugs are structurally similar to the phytochemicals of plants from which they were firstly isolated. In many developing countries, plant materials have an important role in primary care or disease treatment. In addition, due to contraindications in the usage of chemical drugs, there is a growing interest in the utilization of the plant-derived medicinal products [1].

Antimicrobial resistance has been identified as one of the most serious public health threat worldwide. Recently estimated that 700.000 people die from infections globally each year and somberly warned that annual death toll will keep going over 10 million by 2050. Therefore, not surprisingly, it is argued that there is a great need to find effective alternatives to combat with this issue. One of these alternatives is the use of the medicinal plants, which have been widely used and proven as promising alternatives as an ancient practice for thousands of years. Therefore, in recent years, extracts and essential oils originated from plant sources have gained

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more urgency for the search of the antimicrobial properties [2, 3].

The *Mentha* plant grows all year round, mainly in the Mediterranean area, where it forms a dominant part of the vegetation, is a plant commonly used worldwide. The genus of *Mentha* is among the major genera belonging to the *Lamiaceae* family with approximately 240 genera and 7200 species. It has been reported that peppermint has active ingredients such as caffeic acid, carotenes, flavonoids, polymerized polyphenols, tocopherols, tannins, betaine and choline [4-6].

Mint species have been shown to present several virtues and have been used for different purposes, such as in culinary uses to improve aroma and flavor, as well as in cosmetics. Mint species have also been used for medicinal aims, such as in infusions or tinctures for the treatment of intestinal colic, liver disorders, gastritis, and jaundice, as well as for headaches and migraine [6]. On the other hand, *M. piperita* has positive effects on human health such as antibacterial, antifungal, antiviral, antidiabetic, antispasmodic, antiulcer, anti-inflammatory, mildly anesthetic, anticancer, antimutagenic, cytoprotective, hypoallergenic, immuno-modulatory and hepatoprotective effects and it was already known for its wide benefits for human health, especially for digestive and diuretic problems and as a remedy for coughs and colds. Peppermint has several medicinal uses such as treating stomach-aches, chest pains and for treating irritable bowel syndrome [7, 5].

The current study aims to evaluate the *in vitro* antimicrobial activity of peppermint aqueous and methanolic extracts against some common pathogenic bacteria and to determine their minimum inhibitory and bactericidal concentrations.

MATERIAL AND METHODS

Plant Material

Fresh leaves of *M. piperita* were harvested in July 2016, in Setif (Eastern Algeria) (Fig. 1). The leaves were washed with fresh water to remove the soil and dust particles, and subjected to air-drying under shade for three weeks until they were completely dried, then ground into fine particles using an electric grinder. Fig. 2 shows the image of fresh and dry leaves of the plant *M. piperita.*

Preparation of Plant Extracts

Preparation of the Methanolic Extract

The methanolic extract of the *M. piperita* plant is prepared according to the method of Farzana [9]. It is a solid / liquid type extraction (maceration) using a hydroalcoholic mixture (Methanol / Distilled water (70% Methanol; 70:30, V / V). A quantity of 128 g of vegetable powder was introduced into an Erlenmeyer flask containing hydroalcoholic mixture (70% methanol; 70:30, v/v). The set is left for maceration for a week at room temperature and undergoes manual agitation every 24 hours. Then, the extract recovered by filtration (Whattman N°1) is subjected to partial evaporation at 40°C/20min.The filtrate obtained is then placed in glass Petri dishes and put in an oven at 40°C.

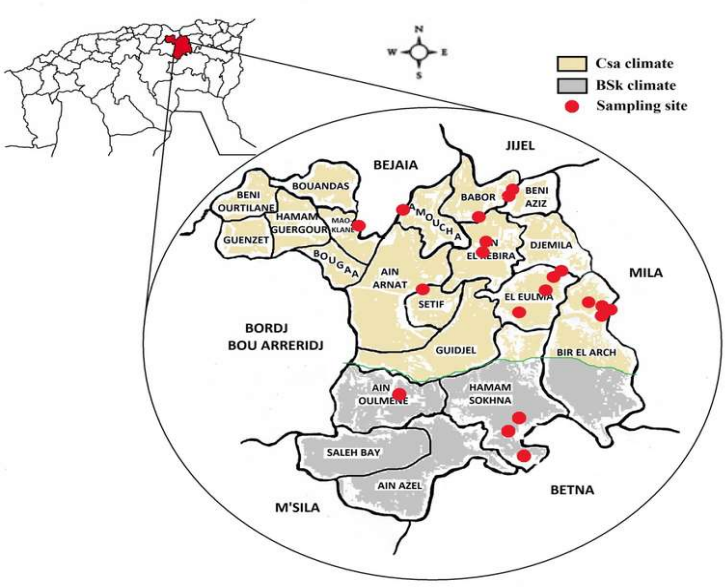

Figure 1: Map of the study region: Setif district in Eastern Algeria [8]

Figure 2: **a)** *M. piperita* fresh; **b)** *M. piperita* dry

Preparation of the Aqueous Extract

The aqueous extract of the *M. piperita* plant is also prepared according to the same method of Farzana [9]. The vegetable powder (140 g) was first extracted with methanol (70 %) by maceration. The filtrate obtained was evaporated under reduced pressure in a rotatory evaporator at 40°C/30 min to get methanol extract. The fraction obtained was re-extracted by mixture of petroleum ether/water (1:1, V/V) and left to settle in a separatory funnel. Aqueous fraction was chosen for the present study and left to evaporate in the oven at 40 °C.

Determination of Total Phenolic Compounds Contents

Determination of Total Phenols Contents

The mixture of phosphotungstic ($H_3PW_{12}O_{40}$) and phosphomolybdic ($H_3PMo_{12}O_{40}$) acids of the Folin-Ciocalteu reagent is reduced during the oxidation of phenolic compounds, to a mixture of blue tungsten oxide (W_8O_{23}) and molybdenum (MO_8O_{23}), the coloration blue produced is proportional to the quantity of phenolic compounds present in the extract [10].

The total phenol content of *Mentha piperita* extracts is estimated by the Folin-Ciocalteu method, adapted by Kähkönen *et al.* [11], adopted by Singleton *et al.* (1965). A volume of 0.5 mL of extract is added to 2.5 mL of Folin-Ciocalteu reagent after 5 min, 2 mL of sodium carbonate (Na_2CO_3, 7.5%) is added to the mixture. After incubation for 30 min at 40°C and in the dark, the absorbance is measured at 765 nm against a blank without extract. The results are expressed in µg EGA/g of dry extract.

Determination of Total Flavonoids Contents

Flavonoids are a class of plant phenolic compounds with significant chelating properties [12]. The flavonoid content is estimated by the colorimetric method Queltier Deleu *et al.* (2000) *In* Djeridane *et al.* [13]. The latter is based on the formation of flavonoid-metal complexes such as aluminum in the form of aluminum chloride ($AlCl_3$). The binding of oxygen atoms present on carbons 4 and 5 of flavonoids with aluminum chloride forms yellowish complexes [10].

The aluminum trichloride method is used to quantify the flavonoids in the different extracts of the Mentha piperita plant. 1.5 mL of extract is mixed with 1.5 mL of aluminum chloride solution ($AlCl_3$) at 2 %. After incubation at room temperature for 15 min, the absorbance of the mixture is measured at 430 nm with a spectrophotometer. The results are expressed in µg EQ/g of dry extract.

Antimicrobial Activity Test

Microorganisms

The antimicrobial activity of extracts was studied against six strains of pathogenic bacteria, obtained from the American Type Culture Collection, namely: *Staphylococcus aureus* ATCC 25923, methicillin resistant *Staphylococcus aureus* (MRSA) ATCC 43300, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 25922, *Salmonella typhi* ATCC 14028, *Pseudomonas aeruginosa* ATCC 27853 and one yeast: *Candida albicans* ATCC1024. Mueller–Hinton agar and Sabouraud Dextrose agar were used for bacteria and fungi, respectively.

Screening for Antibacterial Activity

The screening of antibacterial activity of aqueous and methanolic extracts of *M. piperita* leaves was carried out with agar disc diffusion method [14].

The extracts were dissolved in distilled water to final concentrations of 1, 50, 100 and 200 mg/ mL and sterilized by filtration through a 0.22 µm membrane filter. The bacteria inoculum was prepared by suspending colonies during 24 hours culture. The cell density of each inoculum was adjusted at 10^8 CFU/mL suspension with a spectrophotometer (DO=0.08-0.1/ λ= 600 nm) [15]. The Wattman paper discs N°3 of 6 mm diameter were each impregnated with 20 µL of extract equal to 0.02mg,1mg, 2mg and 4mg by disc, respectively. The plates were held for 3 hours at 4 °C for diffusion of extract into the agar [16, 17] and then incubated at 37 °C for 24 hours.

Under the same conditions, discs impregnated with distilled water are used as a negative control and four antibiotics; Penicillin (P), Oxacillin (OX), Amoxicillin (AMX), Cefotaxime (CTX), reputed to be active on the strains studied, are used as standards (positive controls).

The activity is determined by the measurement of the inhibitory zone diameter in mm. The antibacterial activity is considered starting from a diameter of 6 mm or higher, and is classified as follows [18]:

Very sensitive: diameter ≥ 20 mm;
Sensitive enough: diameter between 15-19 mm;
Sensitive: diameter between 09-14 mm;
Resistant: diameter ≤ 08 mm.

Screening for Antifungal Activity

The antifungal activity was tested by disc diffusion method [19]. The *C. albicans* suspension was prepared in saline solution (0.9 % NaCl), adjusted to a concentration of 1-5 * 10^6 CFU/mL corresponding to 0.12 to 0.15 absorption at 600 nm [15]. One hundred microliter of suspension was placed over agar in Petri dishes and dispersed using a sterile swab. Then, the sterile paper discs (6 mm diameter) were placed on agar to load 20 µL equal to 0.02 mg, 1 mg, 2 mg and 4 mg by disc, respectively. And the plates were held for 3 h at 4 °C for diffusion of extracts into the agar [16, 17] and then incubated at 37 °C for 48 hours.

Nystatin, clotrimazon and amphotericin were used as standards and distilled water as a control.

Minimum Inhibition Concentration (MIC)

The MIC of extracts was determined using agar dilution method that has been already described by Oyeleke *et al.* [20]. One milliliter of different concentrations of extract was added to 14 mL of nutrient agar to make the final concentration ranging from 20 mg/mL to 0.05 mg/mL. Standardized inocula approximately containing 1×10^4 CFU/mL [21, 22] were spotted on solidified plates containing various concentrations of the extract. The lowest concentration of extract inhibiting any visible bacterial or fungal growth after an incubation period of 24 h and 48 h at 37 ºC, respectively, was taken as the minimum inhibitory concentration [20, 23].

Minimum Bactericidal Concentration (MBC) and Minimal Fungicidal Concentration (MFC)

In order to verify that the extracts were able to kill bacteria and yeast cells, the plates were evaluated for MBC and MFC. Briefly, samples were taken from the nutrient agar plates that showed no visible growth after 24 hours incubation and sub cultured into tubes containing nutrient broth. The least concentration that did not produce growth after 24 hours was regarded as the minimal bactericidal and fungicidal concentration [20].

Statistical Analysis

Experiments were carried out in triplicate and expressed as the mean ± standard deviation, data were analysed and compared using the one-way ANOVA and Tukey Multiple Comparison with 95 % confidence limits ($P < 0.05$), using Graphpad prism 5 Demo Software.

RESULTS AND DISCUSSION

Methanolic and aqueous extracts of the leaves of *M. piperita* reach the yield of 15.08 % and 12.64 %, respectively (Table 1). The extraction rate of phenolic compounds depends on temperature, time, solubility and the number of extraction cycles including solvent polarity and volume of solvent [24, 25].

Table 1: Extraction rate of phenolic compounds

Extracts	Rate (%)
Methanolic	15.08
Aqueous	12.64

Determination of Total Phenolic Compounds and Total Flavonoids Contents

Results of the quantitative determination of total phenol and flavonoids in *M. piperita* leaves aqueous and methanolic extract are summarized in Fig. 3. The total polyphenol was determined as gallic acid equivalent in micrograms per gram (µg GAE/g), while the total flavonoid was calculated as quercetin.

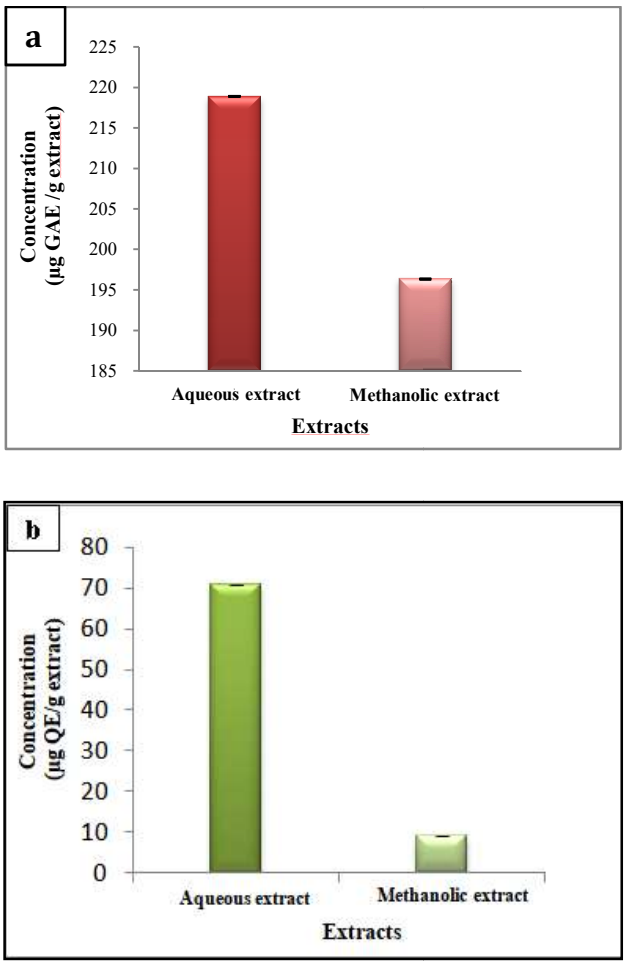

Figure 3: a) Total phenolic compounds contents; **b)** Total flavonoid contents in the aqueous and methanolic extract of *M. piperita* leaves.

From results in Fig. 3, aqueous extract from *M. piperita* was found to be richer in phenols and flavonoids.

Total phenolic content and total flavonoid content of *M. piperita* leaf extract is significantly higher in aqueous extract (218.9±0.02 µg GAE/g extract and 70.86±0.01 µg QE/g extract, respectively), than methanolic extract (196±0.005 µg GAE/g extract and 9.23±0.01 µg QE/g extract, respectively). Total phenolic content is affected by the polarity of the solvent used for extraction which is being maximum in polar solvents, therefore total phenolic compounds of *M. piperita* is polar and extracted well with polar solvents.

As can be seen from the extract yields, total phenol and total flavonoid content data in (Table 1 and Fig. 3), the solvents had different quantitative and qualitative extraction efficiences. Quantitatively, the use of methanol (MeOH) produced the greatest extract yields; however, qualitatively, the distilled water (H_2O) solvent isolated extract with the highest total phenol and total flavonoid contents this result is agree with those obtained by Dorman *et al.* [26].

It seems likely that any disagreements in value for total phenolics may originate from the different geographical origins, agro-climatic (climatic, seasonal and geographical) variations, extraction procedures and physiological conditions of the plants [27]. The comparison of the chemical content of biologically active compounds in different species of the same genus, such as phenolics, should be performed for plants that are grown under the same environmental conditions and harvested at the same phenological stage. For example, plants of Mediterranean origin, in general, possess higher amounts of phenolics in comparison to the same species grown under continental conditions [28]. In addition, Ravn *et al.* [29] reported higher levels of rosmarinic and caffeic acids during spring than summer and winter, and noted a loss of polyphenols during sample preparation.

Antimicrobial Activity

The results obtained from antimicrobial assay are presented in Table 2 and 3:

The leaf extracts (aqueous and methanolic) of *M. piperita* were tested against six undesirable bacteria and a yeast, in order to estimate their antimicrobial potentials (Table 2, 3). The results of antibacterial activity, assessed by the presence or absence of inhibition zone, MIC and MBC values, showed that both extracts had great potential against Gram-positive bacteria (*S. aureus*, *SARM* and *B. subtilis*) while, *E. coli, S. typhi, P. aeruginosa* and *C. albicans* were resistant.

Results in Table 2 indicate that the methanolic and aqueous extracts possess high to moderate inhibitory activities against the tested bacteria even zero against some strains. Both extracts showed variable inhibitory activity against all Gram-positive bacteria at 200 mg/mL with

inhibition zone diameters ranging from 8.00–23.5 mm (Fig. 4) this inhibition was high compared to Oxacillin. An extract is considered active when it reveals a zone of inhibition greater than or equal to 9 mm [18]. Furthermore, the assayed extracts indicate no activity against tested Gram-negative and fungi *C. albicans* (Table 2).

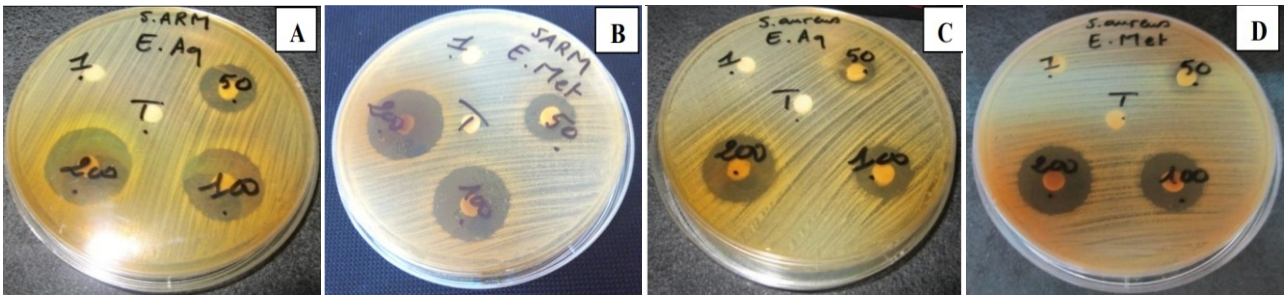

Figure 4: Activity of *M. piperita* leaf extracts on *MRSA* and *S. aureus* at 1 mg/mL, 50 mg/mL, 100 mg/mL and 200 mg/mL. A) Aqueous extract on *MRSA*. B) Methanol extract on *MRSA*. C) Aqueous extract on *S. aureus*. D) Methanolic extract on *S. aureus*. T: negative control (distilled water).

Table 2: Antimicrobial activities of methanolic and Aqueous extracts, pure phenolic compounds, standards and control

M. piperita	**Concentrations (mg/mL)**	**Zone of inhibition (mm)**						
		Bacterial strains						**Fungul strain (yeast)**
		S.aureus	***MRSA***	***B.subtilis***	***E.coli***	***S.typhi***	***P.aeruginosa***	***C.albicans***
Methanolic extract	1	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
	50	9.5±0.7^{a}	14.5±0.7^{b}	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
	100	15.5±0.7^{d}	20.5±0.7^{d}	8.0±0.0^{b}	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
	200	**19.0±0.0^{f}**	**22.5±2.1^{e}**	9.5±0.7^{c}	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Aqueous Extract	1	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
	50	10.5±0.7^{b}	17.5±2.1^{c}	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
	100	14.5±0.7^{c}	21.0±0.0^{d}	6.5±0.7^{a}	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
	200	**18.5±0.7^{ef}**	**23.5±0.7^{f}**	8.0±0.0^{b}	7.0±0.0^{a}	0.0±0.0	0.0±0.0	0.0±0.0
Pure phenolic compound								
Tannic acid	100	13.8±0.3^{c}	14.0±0.0^{b}	10.0±0.6^{c}	-/-	18.0±0.0^{a}	12.0±0.0^{a}	18.8±1.0^{c}
	200	17.3±0.6^{e}	18.0±0.0^{c}	16.5±0.5^{e}	12.0±0.0^{b}	**20.3±0.6^{b}**	13.0±0.0^{a}	**25.0±0.0^{d}**
Galic acid	100	-/-	-/-	-/-	-/-	-/-	-/-	-/-
	200	-/-	-/-	-/-	-/-	-/-	-/-	-/-
Quercetin	100	-/-	-/-	-/-	-/-	-/-	-/-	-/-
	200	-/-	-/-	-/-	-/-	-/-	-/-	-/-
Standards								
Penicillin		48.0±0.0^{g}	0.0±0.0	34.0±0.0^{g}	0.0±0.0	0.0±0.0	0.0±0.0	NT
Oxacillin		16±0.0^{de}	0.0±0.0	14.0±0.0^{d}	0.0±0.0	0.0±0.0	0.0±0.0	NT
Amoxicillin		0.0±0.0	0.0±0.0	28.0±0.0^{f}	17.0±0.0^{c}	0.0±0.0	0.0±0.0	NT
Cefotaxime		0.0±0.0	8.0±0.0^{a}	28.0±0.0^{f}	40.0±0.0^{d}	22.0±0.0^{c}	24.0±0.0^{b}	NT
Amphotericin		NT	NT	NT	NT	NT	NT	15.67±0.29^{b}
Clotrimazon		NT	NT	NT	NT	NT	NT	44.67±0.58^{e}
Nystatin		NT	NT	NT	NT	NT	NT	9.17±0.29^{a}
Control		-/-	-/-	-/-	-/-	-/-	-/-	-/-

-/-: No zone of inhibition; NT: Not Tested. In the same column, means followed by the same letters are not significantly different ($p < 0.05$).

Table 3: MIC and MBC of methanolic and Aqueous extracts and pure phenolic compounds against the tested microbial strains

M. piperita Extracts	MIC et MBC/MFC (mg/mL)						
	Bacterial strains						Fungal strains
	MRSA	*S. aureus*	*B. subtilis*	*E. coli*	*S.typhi*	*P.aeruginosa*	*C.albicans*
Methanolic Extract	8/10	9/10	10/12	16/16	NT	NT	NT
Aqueous Extract	8/10	9/10	12/12	>20/>20	NT	NT	NT
Pure phenolic compound							
Tannic acid	0.3/0.3	0.4/0.4	0.3/0.6	>20/>20	<50µg/3	0.9/0.9	0.1/5

Antimicrobial activity of pure phenolic compounds showed that tannic acid has more impact on *S.typhi* (20.3±0.6 mm) (Table 2). While Galic acid and Quercetin have showed no inhibitory effect against all bacteria (Gram-positive and Gram-negative) and fungi. On the other hand, tannic acid showed significant activity against *C. albicans* (25.0±0.0 mm) compared to standards nystatin (9.17±0.29 mm) and amphotericin (15.67±0.29 mm).

Among the fourth different types of antibiotics used in the study, Cefotaxime has a broad spectrum of activity on almost of species of human pathogenic bacteria and the widest zone of inhibition was observed against *E. coli* (40.0±0.0 mm) (Table 2). Whereas the minimum zone of inhibition was exhibited against *MRSA* (8.0±0.0 mm).

The MICs results show that both extracts have high value of MIC/MBC on all tested species. These results are not in good agreement with those of the antibiogram for some tested species (Table 3); since *MRSA* showed a high MIC (8 mg/mL) and has a largest zone of inhibition with both aqueous and methanolic extracts (23.5±0.7 mm) (22.5±2.1 mm), respectively. Also, the best activity was obtained with tannic acid with MIC value of <50 µg/mL against *S. typhi* and MBC 0.3 mg/mL against MRSA (Table 3).

In fact, *M. piperita* extracts were most efficient against Gram-positive (MRSA, *S. aureus, B. subtilis*) (Fig. 4) than Gram-negative bacteria (*E. coli, P. aeruginosa, S. typhi*). There is evidence in the literature that gram-positive bacteria are more sensitive to plant extracts than gram-negative bacteria, because of hydrophobic lipopolysaccharide in the outer membrane permeability barrier in Gram-negative bacteria which provides protection against different agents [30, 31]. In addition, the periplasm contains enzymes that destroy foreign molecules introduced from the outside [32, 33].

Mint extracts were found to contain a wealth of compounds, collectively named terpenoids and polyphenols, which include phenolic acids, flavones, and flavanols, in their free forms or as glycoconjugates [6]. Olennikov and Tankhaeva [34] reported that the total phenolic compound contents of different *Mentha* species ranged from approximately 6% to 12%. This indicates that the phenolic compounds such as phenolic acids, flavonoids, and tannins play a significant role in the biological activities of the *M. piperita* extracts**.** Previous studies with the same plant reported that this polyphenols are a source of antimicrobial agent, but there are often large variations in the intensity of the antimicrobial activities against Gram-negative and Gram-positive bacteria. Such differences can be due to the different extract chemical composition.In fact, the effect of chemical components of extract of peppermint on cell membrane integrity of bacteria has been reported [4, 5, 35].

CONCLUSION

In conclusion, our data confirms that the leaves of *M. piperita* contain high levels of polyphenolic compounds, as determined by the total phenolic and total flavonoid content. Also suggest that the aqueous and methanolic extracts of *M. piperita* can be considered as a good source of natural compounds with significant antibacterial activity and that it could be very useful in the detection of new agents of plant origin. Therefore it is essential to research farther by the identification of biologically active compounds, characterization and purification of the extracts of this plant. *M. piperita* leaves could be a possible alternative to chemicals as it can be harnessed as antibacterial agent.

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