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IN VITRO EVALUATION OF THE ANTIOXIDANT, ANTIBACTERIAL, ANTIBIOFILM, AND CYTOTOXIC ACTIVITY OF *MENTHA PIPERITA* ESSENTIAL OIL

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ABSTRACT

Nowadays, antibacterial resistance to synthetic antibiotics has become a big issue worldwide. *Mentha piperita* essential oil (EO) and its bioactive components with antibacterial, antibiofilm, and antioxidant properties are recommended for the treatment of microbial infections in traditional medicine. This study aimed to detect phytochemical components, antioxidant and cytotoxic activity; to evaluate antibacterial and antibiofilm effects of *M. piperita* EO on strains.
Gas chromatography/mass spectrometry (GC-MS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) tests were used to determine chemical composition, antioxidant and cytotoxic potentials of EO, respectively. Antibacterial activity was carried out through disc diffusion and minimum inhibitory concentration (MIC) assays. The biofilm biomass of bacteria was determined by the crystal violet (CV) staining method.
Menthone (27.28%), L-menthol (23.71%), and limonene (7.48%) were the main components. *M. piperita* EO was found to have strong antibacterial activity with the MIC-MBC values in the range of 0.125-16.00% (v/v) and moderate antioxidant activity. The peppermint EO showed an antibiofilm effect on all strains and a cytotoxic effect on NIH 3T3 cells. *M. piperita* EO may be used alone or in combination with antibiotics or carrier oils as a therapeutic agent against bacterial infections. Further *in vivo* experiments should be carried out to detect a safe dosage of EO.

Keywords: *Mentha piperita*, Antioxidant, Antibacterial, Antibiofilm, Cytotoxicity

INTRODUCTION

Bacterial infections are treated with synthetic antibiotics **(Wright, 2014)**. Super bacteria have developed resistant to multiple antibiotics due to widespread and indiscriminate use of drugs **(Yang et al., 2020)**. In the worldwide, antibiotic resistant bacteria cause around 700, 000 deaths per year; by 2050, estimated for the number of deaths will be over 10 million **(Aslam et al., 2018)**.
Bacterial populations develop biofilms that adhere to biotic or abiotic surfaces with extracellular polymeric substances consisting of DNA, protein, polysaccharide and free-living forms in the environment. Bacterial biofilms protect the bacteria against the host's immune system and inhibit the antibiotics uptake **(Batoni et al., 2016; Roy et al., 2018)**. Biofilm infections are involved in more than 80% of microbial infections worldwide and have become an important health problem with the increase of antibiotic resistant strains **(Davies, 2003; Koo et al., 2017)**.
Many antibiotics, which can reduce but not destroy biofilms, used in the treatment at non-lethal concentrations due to their toxic and side effects **(Wu et al., 2015)**. Therefore, new and effective therapeutic agents like natural products derived from plants for the treatment of bacterial infections need to be required. Nowadays, 20,000 plants are used in traditional medicine as an alternative therapy agents **(Dhakad et al., 2019)**.
During the last decades, essential oils (EOs) have been reported to exhibit biological activities such as antioxidant, antibacterial and bacterial biofilm inhibition **(El-Zaeddi et al., 2016)**. Gram-negative [Gr (-)] and Gram-positive [Gr (+)] bacteria are more susceptible to EOs of Lamiaceae family and display lower resistance for bacteria and lower toxicity for human compared to other agents **(Nieto, 2017)**. Antioxidants obtained from natural plants reduce the risk of human disease like many skin, chronic and vascular disease, neutralize free radicals and show anti-aging activity **(Ani et al., 2006)**.
Mentha species have been used as medicinal herbs for pharmaceutical preparations in Eastern and Western countries. *Mentha piperita* (commonly identified peppermint) EO is used for the treatment of ulcer, cold, headache, cancer, cough, irritation, inflammation, diabete and the complications of digestive system due to containing aromatic bioactive constituents and terpenoids **(Anwar et al., 2019)**.
The aim of this study was to determine chemical composition, antioxidant capacity, total phenolic content (TPC) and *in vitro* cytotoxic activity of *Mentha piperita* EO; to evaluate its antibacterial and biofilm inhibitory potency against Gr (+) and Gr (-) strains associated with human infections.

Materials and Methods

Preparation of *Mentha piperita* essential oil

10 mL unit of EO obtained from *M. piperita* leaves by steam distillation method, was purchased from Turkey. 0.5% Tween 80 was used as an emulsifying agent. EO and Tween 80 were aseptically filtered using a 0.45µm pore size steril membrane filters and stored in a dark at 4°C until bioassays.

Chemical analysis of *Mentha piperita* essential oil

The peppermint EO was analysed by gas chromatography coupled to mass spectrometry (GC-MS) using Agilent 6890N GC system coupled to a 5973 MSD (Agilent Technologies, Santa Clara, CA, USA) system equipped with a fused-slica HP-Innowax capillary column (60 m lengthX250 µm inner diameterX0.25 µm film thickness). The carrier gas was hellium (flow rate: 1.7 mL min^{-1}). Split ratio was adjusted to 30:1. The column initial temperature was 60°C for 10 min, followed by an increase of 5°C min^{-1} up to 150°C for 20 min and then increased up to 250°C and maintained for 30 min. The temperature of injector was 250°C. The MS ionization energy was set at 70 eV. The components of peppermint EO was identified by comparision of their mass spectra with National Institute of Standards and Technology (NIST) Library.

Bacterial strains and cultures

A total of seven clinical standard bacterial strains obtained from American Type Culture Collection (ATCC) were used as test microorganisms to determine the antibacterial and antibiofilm properties of peppermint EO. Four of them were Gr (+) (*Staphylococcus epidermidis* ATCC 12228, *Enterococcus faecalis* ATCC 29212, *Bacillus subtilis* ATCC 6633, and *Cutibacterium acnes* ATCC 6919) and three were Gr (-) (*Escherichia coli* ATCC 25922, *Proteus vulgaris* ATCC 13315, and *Pseudomonas aeruginosa* ATCC 10145). *C. acnes* was stored at -80°C in a sterile beaded tubes containing skimmed milk and 10% glycerol (v/v, SIGMA). The other bacteria were stored at -80°C in brain heart infusion broth (BHI) (BİOKAR, France) with 20% glycerol. Each bacterium was refreshed in BHI at 37°C for 24h aerobically, except *C. acnes* (anaerobically, gas generating kit H_2/CO_2, BD, Germany) after inoculated on brain heart agar (BHA) (BIOKAR, France) for controlling the purity. In antibacterial and antibiofilm activity assays,

bacterial suspensions were diluted with BHI and their turbidity equalized to $1.5x10^8$ CFU mL^{-1} (0.5 McFarland).

Antioxidant activity

Antioxidant properties of *M. piperita* EO was determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging and Folin Ciocalteu assays.

2,2-diphenyl-1-picrylhydrazyl free radical scavenging assay

DPPH antioxidant assay kit (DOJINDO, Japon) was used to determine antioxidant potential of *M. piperita* EO (Shimamura et al., 2014). Trolox standard solutions at different concentrations (0, 40, 60, 80 and 100 µg mL^{-1}) were used to create a standard curve. The inhibition ratio (%) was calculated following formula:
DPPH scavenging activity (%)= $(A_{Control} - A_{EO})/A_{Control} \times 100$

The DPPH result was reported as IC_{50}. Antioxidant capacity of EO was expressed as the Trolox equivalent antioxidant capacity (TEAC) and calculated as follows:
TEAC: IC_{50}Trolox/IC_{50} EO

Total phenolic content of *M. piperita* essential oil

Folin Ciocalteu method was used to determine TPC of peppermint EO **(Slinkard and Singleton, 1977)**. Initially, 0.04 mL EO was mixed with 0.8 mLof 0.5 N phenol reagent (MERCK, Germany) and 1.36 mL distilled water. Reaction mixture was incubated for 3 min. Then, 0.8 mL of 10% Na_2CO_3 (ISOLAB, Germany) was added and vortexed. Finally, the mixture incubated at room temperature for 30 min and blue colour was seen. Its absorbance was measured at 760 nm using a spectrophotometer (Shimadzu Spectrophotometer UV-1800). The TPC of EO was calculated as gallic acid equivalents (mg GAE 100 g^{-1} sample).

Antibacterial activity of *M. piperita* essential oil "*in vitro*"

Disc diffusion method

The antibacterial activity of peppermint oil was determined against seven strains using Kirby Bauer disc diffusion assay described by Clinical & Laboratory Standards Institute (CLSI, 2012). Each bacterial suspension was adjusted to 0.5 Mc Farland turbidity units. 100 µl of each inoculum suspension was spread on BHA plates. Aseptically, steril 6 mm diameter blank paper discs (OXOID) were impregnated with 20 µl EO dissolved in Tween 80 and incubated at room temperature for 15-20 minutes. After incubated under aerobic condition (except *C. acnes* strain) at 37 °C for 24 h, inhibition zone diameters (IZD) were measured in mm with a ruler and evaluated as follows: Not sensitive: <8 mm; sensitive: 9-14 mm; very sensitive: 15-19 mm; extremely sensitive: >20 mm (Singh et al. 2014). Vancomycin, gentamicin and ampicillin were used as positive controls. Blank disc impregnated with steril Tween 80 was used as a negative control.

Microdilution method

The MIC of EO was carried out by microdilution broth using 96 well plates (CLSI, 2012). Two fold dilution series (32%-0.03125% v/v) of EO containing 0.5% Tween 80 in BHI were prepared. Then, bacterial inoculum (20 µl) added to all wells. After *C. acnes* and other bacteria were incubated at 37°C for 72h and for 24h under anaerobic and aerobic conditions, respectively; 20 µl of 0.2 mg mL^{-1} iodonitrotetrazolium chloride (INT dye, SIGMA-ALDRICH) solution as a growth indicator was added to each well to define MIC. The growth control and sterility control were put into 11th and 12th wells, respectively. 10 µl of inoculum from four negative wells was transferred on BHA and incubated at 37C for 24h. The EO concentration, in which bacteria did not grow, was recorded as MBC **(Bouyahya et al., 2017)**.

Biofilm inhibitory activity of *M. piperita* essential oil

Biofilm formation assay

Seven strains were screened by crystal violet (CV) assay using 96-well plates for detection of biofilm formation according to the **Stepanovic et al. (2007)** with slight modifications. Firstly, strains were grown in TSB and incubated at 37°C for 24h. 20 µl of each bacterial suspension was adjusted to 0.5 Mc Farland with NaCl solution and 180 µl of TSB were added to each well and incubated at 37°C for 24h. Negative control wells contained 200 µl of TSB. After incubation, the contents of the wells were washed three times with 250 µl of phosphate-buffered saline (PBS; Sigma-Aldrich) to remove non-adherent cells. 250 µl of 95% ethyl alcohol was added to each well for 15-20 min to fix adherent cells. Then, removed the contains and each plate was dried at room temperature for 40 min in an inverted position. Afterwards, each well was stained with 0.5% CV (MERCK, France) for 15-20 min. The excess stain was discarded and washed three times with PBS. Then the plates were air dried for 50 min. The dye was solubilized in 250 µl of ethanol-acetone (80:20) mixture for 10-15 min. Adherent biofilms were measured at 570 nm using microtiter plate reader (Thermo Scientific). Biofilm formation was interpreted as follows: OD570 ≥ 1: Highly positive; 0.1 ≤ OD570 < 1: Low grade positive; OD570 < 0.1: Negative.

Biofilm inhibition assay

The influence of peppermint EO on bacterial biofilm was evaluated using 96-well polystyrene microtiter plate method **(Jadhav et al., 2013)**. Briefly, 20 µl of the EO concentrations (equalized to 0.25MIC, 0.5MIC, 1MIC, and 2MIC) were pipetted into sterile wells. Then, 20 µl of bacterial suspension (0.5 McFarland) and 160 µl of TSB were added into each well. Following incubation at 37°C for 24h, CV staining method was used to quantify the biofilm biomass. TSB (180 µl)+inoculum (20 µl) and only TSB (200 µl) were used as a positive (biofilm formation) and a negative control, respectively. Inhibition of biofilm was calculated using the following formula:

% Inhibition= $100-(OD_{570\ sample}/OD_{570\ control} \times 100)$

Cytotoxicity assay

Cytotoxic activity of *M. piperita* EO was evaluated on 3T3 cell line derived from mouse embryonic fibroblasts. Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) and cultivated at 37°C in the 5% CO_2 atmospheric in 96 well-plates. After then, two fold serial dilutions (0.0625-2% v/v) of EO were added. The absorbance was read at 570 nm using microplate reader (HTX Synergy, BioTek, USA). Viability of cells were measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay after 24, 48, 72 and 96 hours of treatment with EO and their IC_{50} (half maximal inhibitory concentration) values were calculated.

Statistical analysis

Statistical analyses were performed by one way ANOVA and post hoc Tukey test using SPSS 19 version. $p \leq 0.05$ value was considered statistically significant. All experiments were repeated three times. MTT results were plotted using GraphPad Prism version 6.0.

RESULTS

Chemical Characterization

16 components, representing 92.95% of the total compounds of *M. piperita* EO, were identified by GC/MS and are presented in Table 1. Oxygenated monoterpenes (menthone 27.28%, L-menthol 23.71%, menthyl acetate 5.42%, neo-menthol 4.02%, 1.8-cineole 5.12%, α-terpineol 3.85%) followed by monoterpene hydrocarbons (α-pinene 2.82%, β-pinene 3.66%, myrcene 0.41%, limonene 7.48%, δ-3-carene 0.56%, p-cymene 0.91%, terpinolene 0.56) and monoterpene ketone (d-piperitone 7.03%).

Table 1 The phytochemical composition (%) of *M. piperita* EO identified by-GC-MS

Components of *M. piperita* EO	Retention time (RT)	Composition (%)
α-pinene	5.745	2.82
cyclohexanol	25.073	9.81
β-pinene	7.707	3.66
Myrcene	10.162	0.41
Limonene	11.735	7.48
delta-3-carene	14.167	0.56
p-cymene	14.648	0.91
Terpinolene	15.575	0.56
Menthone	21.863	27.28
menthyl acetate	24.616	5.42
L-menthol	26.366	23.71
α-terpineol	27.568	3.85
d-piperitone	28.793	7.03
Eugenol	46.554	0.07
Thymol	47.984	0.05
Total		93.62 %

Antioxidant activity and TPC

The radical scavenging capacity and TPC of *M. piperita* EO were measured by DPPH and Folin Ciocalteu assay, respectively. The IC_{50} (105.67 µg mL^{-1}) and the TPC (0.198 mg GAE l^{-1}) value of EO are shown in Table 2.

Table 2 DPPH-radical scavenging capacity (IC_{50}) and TPC (mg GAE l^{-1}) of *M. piperita* EO

EO	DPPH IC_{50} (µg mL^{-1})	TPC (mg GAE l^{-1})
M. piperita	105.67±3.21	0.198±0.035

Antibacterial activity of *M. piperita* essential oil against bacterial strains

Disc diffusion and MIC-MBC results of *M. piperita* EO against pathogenic strains are presented in Table 3 and Table 4. The greatest inhibitory activity with IZD value of 57.50 mm was observed in *B. subtilis* strain. The other bacterial strains except *S. epidermidis* were sensitive to EO with IZDs ranging from 14.25 mm to 46.00 mm. The MIC-MBC values of this oil were in the range of 0.125-16.00% (v/v).

Table 3 IZD values for *M. piperita* EO against bacterial strains

Bacteria	IZD (mm)				
	***M. piperita* EO**	**GEN**	**AMP**	**VAN**	**p value**
E. coli	31.75±1.258	18.25±0.957	17.25±0.500	-	p≤0.05
P. aeruginosa	46.00±4.320	14.75±0.500	21.50±0.577	19.25±0.500	p≤0.05
P. vulgaris	38.50±2.860	16.50±0.577	18.50±0.577	14.75±0.500	p≤0.05
E. faecalis	14.25±0.500	14.00±0.816	20.50±0.500	14.25±0.500	p:1.00 (VAN) p≤0.05 (AMP) p:0.937 (GEN)
B. subtilis	57.50±2.380	13.50±0.577	22.25±0.500	24.75±1.893	p≤0.05
S. epidermidis	10.25±0.500	15.50±0.577	20.75±0.957	18.00±0.816	p≤0.05
C. acnes	33.25±1.500	27.00±0.816	11.50±0.577	24.50±0.577	p≤0.05

IZD in mm (Mean±SD: Standard Deviation); -: ≤6mm (IZD); p≤0.005: Statistically significant value; GEN: Gentamicin; AMP: Ampicillin; VAN: Vancomycin

Table 4 MIC and MBC values of *M. piperita* EO on some bacterial strains

Strains	***E. coli***	***P. aeruginosa***	***P. vulgaris***	***E. faecalis***	***B. subtilis***	***S. epidermidis***	***C. acnes***
MIC (v/v %)	0.125	0.125	0.50	0.25	0.25	4.00	4.00
MBC (v/v %)	0.50	0.125	1.00	2.00	0.25	16.00	8.00

Biofilm inhibitory activity of *M. piperita* essential oil

Biofilm formation of tested strains were evaluated by crystal violet assay for their abilities to produce biofilm on polystyrene plates. All strains were found to be biofilm producers with OD_{570nm} values ranging from 0.296 to 0.948 (low grade positive) after 24h of incubation. As presented in Table 5, *M. piperita* EO at different concentrations (2MIC, MIC, 0.5MIC and 0.25MIC) exhibited dose-dependent antibiofilm activity on all strains with a percentage of inhibition ranging from 8.45% to 86.50%.

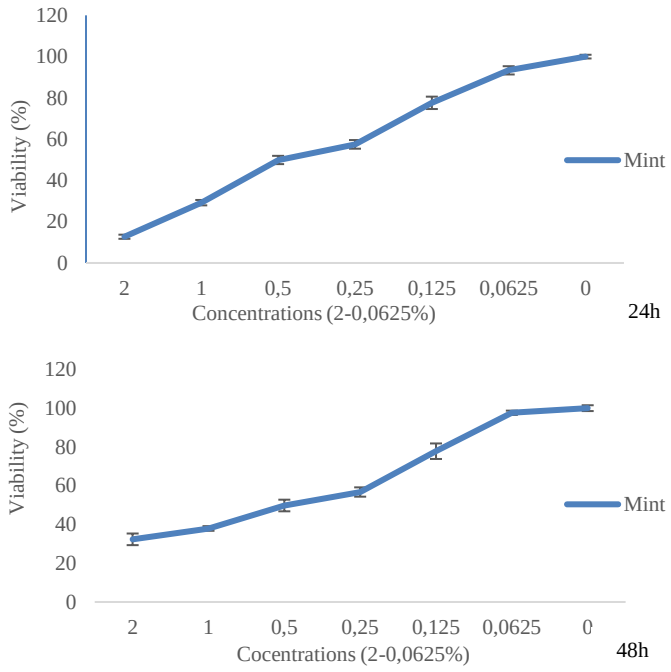
Table 5 Biofilm inhibitory activity of *M. piperita* EO at the 2MIC, MIC, 0.5MIC and 0.25MIC concentration (v/v %)

Bacteria	Optical Density (OD_{570nm})								
	2MIC	**Inhibition (%)**	**MIC**	**Inhibition (%)**	**0.5MIC**	**Inhibition (%)**	**0.25MIC**	**Inhibition (%)**	**Control**
E. coli	0.122±0.003	81.98%	0.142±0.007	79.03%	0.232±0.007	65.74%	0.387±0.002	42.84%	0.677±0.003
P. aeruginosa	0.119±0.013	83.13%	0.137±0.004	80.57%	0.163±0.005	76.88%	0.224±0.006	68.23%	0.705±0.006
P. vulgaris	0.126±0.008	70.97%	0.133±0.009	69.36%	0.148±0.018	65.90%	0.182±0.013	58.07%	0.434±0.006
E. faecalis	0.129±0.014	82.45%	0.152±0.016	79.32%	0.238±0.023	67.62%	0.324±0.009	55.92%	0.735±0.014
B. subtilis	0.117±0.008	60.48%	0.134±0.10	54.73%	0.145±0.006	51.02%	0.271±0.007	8.45%	0.296±0.007
S. epidermidis	0.128±0.009	86.50%	0.145±0.006	84.71%	0.454±0.07	51.90%	0.714±0.017	24.69%	0.948±0.018
C. acnes	0.150±0.009	81.11%	0.441±0.019	44.46%	0.545±0.024	31.37%	0.668±0.009	15.87%	0.794±0.022

Data are presented as mean ± SD; The difference was statistically significant ($p<0.01$) when compared with control.

Cytotoxic activity of *M. piperita* essential oil

The cytotoxic effects of *M. piperita* EO was tested on NIH 3T3 cells using MTT assay after 24h, 48h, 72h and 96h exposure of different concentrations [0.0625-2 % (v/v)] of EO are presented in Figure 1. The IC_{50} values were 0.40 %, 0.40 %, 0.60 % and 0.30 % for 24h, 48h, 72h and 96h, respectively.



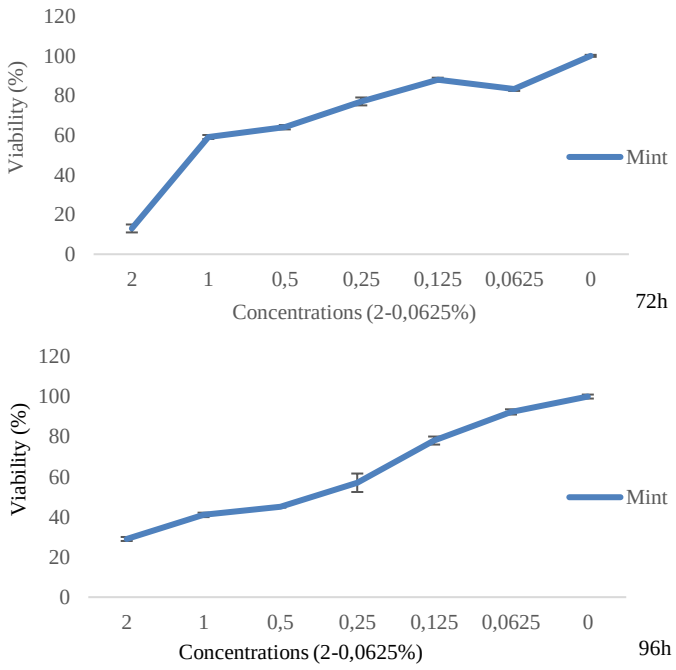


Figure 1 Cytotoxicity/viability of NIH 3T3 cells exposured to different concentrations [2-0.0625 % (v/v)] of EO for 24h, 48h, 72h and 96h. $p<0.05$*

DISCUSSION

Treatments with classical antibiotics against Gram-positive and Gram-negative bacterial infections have become inefficient due to increasing antibacterial resistance **(Vila et al., 2020)**. This global problem has led scientists to develop useful drugs based on ethnobotanical approaches **(Mahendran and Rahman et al., 2020)**. EOs are known safe substances for human health and often used in herbal medicine. The organs (leaf, stem, etc.) of plant and the extraction techniques determine the phytochemical composition and biological potency of EO **(Hoffman et al., 2015)**.

Menthone and L-menthol are found as major components of *M. piperita* EO in this study. Previous studies have reported that the major components of *M.piperita* EO were menthone (28.13% and 25.54%), menthyl acetate (9.51% and 9.68%) and limonene (7.58% and 7.73%) **(Hussain et al., 2010)**, menthol (29.0%), menthone (22.7%) and menthyl acetate (19.2%) **(Kot et al., 2019)**, menthol (38.45%), menthone (21.8%), 1,8-cineole (5.62%), and neo-menthol (4.19%) **(Wu et al., 2019)**. Plant species and age, soil type, season, geographical origin, genetic properties, extraction method and harvest time considerably affect phytochemical composition of EO **(Senthilkumar et al., 2020; Silva et al., 2021)**. Menthol shows beneficial biological properties such as antibacterial and antiinflammatory activities **(Du et al., 2020)**.

The chemical components of EO such as unsaturated terpenes, monoterpenes, monocyclic terpenes, oxygenated monoterpenes and interactions among them are affected its antioxidant ability. **(Clark and Menary, 1984)**. In our study, the IC_{50} and TPC values of *M. pipierita* EO were 105.67 µg mL^{-1} and 0.198 mg GAE l^{-1}, respectively. Smilar and different antioxidant activity and TPC results were reported by previous studies **(Raeisi et al., 2018; Pellegrini et al., 2018; Wu et al., 2019; Bittner et al., 2020)**. Menthol, α-pinene and 1,8-cineole showed strong antioxidant activity in *M. piperita* **(Gharib and Silva, 2013)**. Phenolic content of medicinal plants is associated with its antioxidant potency and can act as a reducing agent in biochemical reactions **(Miguel, 2010)**. The compositions of phenolics and terpenes can vary according to edaphic and climatic conditions **(Riachi and De Maria, 2015)**.

M. piperita EO showed strong inhibitory activity with IZD ranging from 14.25 to 57.50 mm and and MIC/MBC ranging from 0.125 to 8%(v/v) against both Gram-positive and Gram-negative bacterial strains except *S. epidermidis*. **Marwa et al. (2017)** reported that the MIC values of 0.125% (v/v), 0.50%, and 8.00% against *B. subtilis*, *E. coli* and *P. aeruginosa*, respectively. Previous studies have demonstrated that *M. piperita* EO exhibited strong antibacterial activity with IZD and MIC values of 12.24 mm and 0.50 µl/mL against *E.coli* **(Djenane et al., 2012)**; 6 to 20 mm and 0.50% against *E. coli* and *P. aeruginosa* **(Iseppi et al., 2020)**. In another study, MIC values were found 1.25 mg mL^{-1}, 2.5 mg mL^{-1}, 1.25 mg mL^{-1} and 0.625 mg mL^{-1} against *E. coli*, *P. aeruginosa*, *P. vulgaris* and *S. epidermidis*, respectively **(Iscan et al., 2002)**. **Mattazi et al. (2015)** reported that IZD: 13 mm and MIC: 40 mg mL^{-1} against *E. coli*, IZD: 14 mm and MIC: 40 mg mL^{-1} against *B. subtilis*; IZD:11 mm and MIC: 100 mg mL^{-1} against *P. aeruginosa*. In this study, *M. piperita* EO was found to be more effective on all bacterial strains except *S. epidermidis* when compared to previous results. These broad-spectrum antibacterial activity depends on its chemical composition especially oxygenated monoterpenes and synergy between major and minor components **(Bakkali et al., 2008; Vinda-Martos et al., 2008)**.

In this study, dose-dependent antibiofilm activity of peppermint EO against all strains was detected. A study by **Lagha et al. (2019)** demonstrated that the smilar OD_{570nm} values (ranging from 0.102 to 0.543) were detected in *E. coli* isolates. In a study by **Ben Lagha et al. (2020)** reported that biofilm viability was decreased by 69.1% on *F. nucleatum* ATCC 25586, after incubated with peppermint EO at the 1 % (v/v) concentration. Another study reported that antibiofilm activity was not seen in cinnamomum EO against *S. aureus* ATCC 6538 although it was seen against Gram negative bacteria **(Condò et al., 2020)**. For thymol-carvacrol-chemotype (II) oil from *Lippia origanoides*, the percentage of biofilm reduction of 75%, 73%, and 74% were determined in *E. coli* O33, *E. coli* O157:H7 and *S. epidermidis* ATCC 12228, respectively **(Cáceres et al., 2020)**. These differences attributed to the components of EO and the structure of biofilm. Several factors such as temperature, nutrition, surface features and treatment with sanitizers were affected their compositions **(Marinho et al., 2013; Fernandes et al., 2015)**.

The peppermint EO exhibited cytotoxic effect on cells in a dose dependent manner. Similar cytotoxic effects on HEL 12469 human embryo lung cells of different concentrations (1.0-0.0025 µl mL^{-1}) of oregano, thyme, clove and arborvitae EOs after 24h exposure has been reported by **Puškárová et al. (2017)** and smilar results on HaCaT cells of different concentrations (0.25-0.00 % w/v) of oregano, thyme, clove, cassia, melaleuca, eucalyptus, lavender, lemongrass, clary sage and arborvitae EOs were obtained by **Kozics et al. (2019)**. On the other hand, a study demonstrated that 20 components of EO on normal human conjunctiva cells (WKD) at low cocentrations (0.0078-0.0004 % v/v) showed no cytotoxicity **(Cannas et al., 2015)**. Our results indicated that *M. piperita* EO causes NIH 3T3 cell death at the MIC concentration. The chemical composition of Mentha species is determining by many factors such as harvest season, climatic condition and soil type **(Hussain et al., 2010)**, therefore it is difficult to compare our results with previous data. The synergistic interactions between major and minor components of the EO may be increased its cytotoxicity. Each component of EO may be reduced cytotoxic effects on cells when modified by nanoparticles. Eugenol and carvacrol grafted with chitosan nanoparticles showed less cytotoxic activity against mammalian cells than alone **(Chen et al., 2009)**. *In vivo* experiments (in animal model) are required to detect cytotoxicity of EO. Metabolic pathways play an important role in this assay and affect cytotoxicity rate.

In conclusion, monoterpenes were the major components of *M. piperita* EO. Moderate antioxidant activity was detected in EO. Significant biofilm reduction and antimicrobial activity was observed on Gr (+) and Gr (-) strains after exposure to EO. *M. piperita* EO with antibacterial and anti-biofilm properties may be useful as new natural alternative agents for the cure of microbial infections. The peppermint EO could be used in combination with antibiotics or carrier oils (like vegetable oils) to reduce its cytotoxicity. Further *in vitro* assays with clinical isolates on human cell line and *in vivo* (animal model) assays are required to evaluate cytotoxicity of EO.

Conflict of interest: None.

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