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The antimicrobial and antibiofilm activities of *Mentha x piperita* L. essential oil

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ABSTRACT

In this study, the aerial parts of *M. x piperita* were collected from Uşak province, Turkey, and the antimicrobial and antibiofilm activities were investigated. Essential oil from the aerial parts of this plant was obtained with the hydrodistillation method. *In vitro* antimicrobial activities of the essential oil on various antibiotic resistant bacteria; *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, and the yeast *Candida albicans* were tested by disc diffusion and microplate dilution methods. The effect of different concentrations of the essential oils on biofilm-forming ability of the test microorganisms were determined by microplate biofilm method. The essential oils of *M. x piperita* had antimicrobial activity against the tested bacteria, except for *P. aeruginosa* MU 187, and the inhibition zones were between 10-15 mm. As a result of the MIC test, the essential oil was most effective against *S.aureus* MU 38 and 1 µg/ml concentration of essential oil was found to inhibit this strain. The essential oil of *M. x piperita* was found to have different proportions of antibiofilm activity against all Gram- positive test strains. To our knowledge, this is the first study of the antibiofilm activity of the essential oil of *M. x piperita* collected from Turkey. The results showed that the essential oil can be used as an antibiofilm agent against the biofilm ability strains. This activity is an important topic in the medical field as well as in the food industry.

Key words: *Mentha x piperita*, Lamiaceae, essential oil, antimicrobial activity, antibiofilm activity

Introduction

A constant concern of man over time was the study and use of medicinal plants in order to cure various diseases (Andro et al., 2013). Frequently, aromatic herbs also present pharmacological activities which allow them to be considered as medicinal plants (Aburjai & Natsheh, 2003). The genus *Mentha* (*Lamiaceae*) is one of the most important sources of essential oil production. Some members of this genus are also used as herbal teas and condiments both in fresh and dried form due to their distinct aroma (Baser et al., 1999). *Mentha* is among the plants which have often been used for their therapeutic properties (Andro et al., 2013).

Mentha x piperita (peppermint oil) is commercially the most important mint species. Peppermint oil is one of the most popular and widely used essential oil, with use in various medical condition such as, to relieve skin irritation, sun burn, sore throat, fever, muscle aches and in nasal congestion and also in perfumery and as flavouring agent (Kumar et al., 2011).

The discovery of anti-infective agents, which are active not only against planktonic microorganisms but also against microbial biofilms, represents an important goal (Projan & Youngman, 2002). The alternative to the chemical antibiofilm agents is natural source (Carneiro et al., 2011). In the recent past, there has been an increased interest in the therapeutic properties of some medicinal plants and natural compounds, which have demonstrated antibiofilm activities (Lee et al., 2013; Namasivayam & Roy, 2013; Zaichang et al., 2013; Abraham et al., 2012; Saharkhiz et al., 2012).

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The *in vitro* antimicrobial (Tsai et al., 2013; Sujana et al., 2013; Bassole et al., 2010; Jirovetz et al., 2009; İşcan et al., 2002; Marotti et al., 1994), antioxidant (Tsai et al., 2013; Dorman et al., 2003; Marotti et al., 1994), antifungal (Moghtader, 2013; Freire et al., 2012; Jakowienko&Wojcık-Stopczynska, 2010; Sokovic et al., 2009; Marotti et al., 1994) activities and the chemical composition of the essential oil of *M. x piperita* (Tsai et al., 2013; Orav et al., 2013; Freire et al., 2012; Bassole et al., 2010; Jakowienko&Wojcık-Stopczynska, 2010; Jirovetz et al., 2009; Sokovic et al., 2009) were investigated. To our knowledge, there are no studies about the antibiofilm activity of the essential oil of *M. x piperita*.

Materials and Methods

Plant material

The aerial parts of *M. x piperita* were collected from Uşak, Turkey in May-July 2012. The plant sample was identified by Dr. Mehtap Dönmez Şahin and the voucher specimen has been deposited in the Herbarium of Faculty of Education, University of Usak under acquisition number 1110. Samples were air-dried at room temperature for 2-4 days.

Extraction of essential oil

One hundred grams of dried plant was used to hydro-distillation for 4 h using a Clevenger apparatus. The essential oil was stored in dark vials at 4 °C.

Bacterial Strain and Culture Conditions

The antimicrobial activity of the essential oil was individually tested to a group of multiple antibiotic resistant bacteria including *Staphylococcus aureus* (MU 38, MU 40, MU 46, MU 47), *Staphylococcus epidermidis* (MU 30), *Pseudomonas aeruginosa* (MU 187, MU 188, MU 189), *Pseudomonas fluorescens* (MU 180, MU 181) and *Candida albicans* ATCC 10239 were provided from Culture Collection of Mugla Sitki Kocman University (MUKK).

The *Staphylococcus* strains were incubated at 37±0.1°C for 24–48 h, *P. aeruginosa* and *P.fluorescens* strains were incubated at 30±0.1°C for 18-24 h, and *C. albicans* were incubated at 30±0.1°C for 24-48 h. Inocula were prepared adjusting the turbidity of the medium to match the 0.5 McFarland standard dilutions. The strains were maintained in their appropriate agar slants at 4°C throughout the study and used as stock cultures.

Disc diffusion assay

The antibacterial activity was based on the disc diffusion method (CLSI, 2007). Each bacterial suspension (0.1 mL) was spreaded on a Mueller-Hinton agar plate. Sterile 6 mm paper discs (Schleicher and Schuell) were impregnated with 20 µL of essential oil. The discs were allowed to dry and were then placed on the inoculated agar. The plates were incubated at appropriate temperature for performing the microorganisms, as mentioned above. At the end of the incubation period, diameters of growth zones around the disks were measured. The experiments were replicated for three times.

Assessing of minimum inhibitory concentration (MIC)

The minimum inhibitory concentration of the essential oil of *M. x piperita* ssp. *hirsuta* was determined using tube dilution method (Rota et al., 2004). The MIC was defined considering the lowest essential oil concentration with no visible growth. Mueller-Hinton Broth (MHB) was used to be test medium and the density of microorganism was adjusted to 5×10^5 colony-forming units (CFU)/mL. Cell suspensions (100 µL) were inoculated into the wells of 96-well microtitre plates (Nunc F96 MİKroWell™ plates; NunclonTM Δ, Denmark) in the presence of essential oil with different final concentrations (0.05-25 µL/mL). The essential oil was dissolved in DMSO (Sigma, USA) and serially diluted 2-fold in MHB to give final concentrations. Negative controls (bacteria+MHB), positive controls (bacteria+MHB+ essential oil), vehicle controls (bacteria+MHB+DMSO) and media controls (MHB) were included. At the end of the incubation the MIC value was determined.

Effect of essential oil on bacterial biofilm formation

The effect of *M. x piperita* essential oil concentrations (25, 5, 1, 0.5, 0.1, and 0.05 µl/ml) on biofilm-forming ability of bacteria was tested with a microplate biofilm assay (Meritt et al., 2005). Bacterial strains were inoculated at Tripticase soy broth (TSB) and grown up to stationary phase. Cultures diluted to 1:100 in fresh TSB with 0.5% glucose, and 200 µl of each dilution pipetted to four wells in a sterile flat bottom microtiter plate. After incubation at 37ºC for 48 h, the wells were washed with distilled water twice to remove the planktonic bacteria. The remaining bacteria were subsequently stained with 125 µl of 0.1% crystal violet solution (Sigma Chemical Co.) at room temperature. Wells were washed once again to remove the crystal violet solution that is not specifically staining the adherent bacteria. The

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plates were air-dried, and 200 µl of 95% ethanol and 33% glacial acetic acid (Sigma Chemical Co.) were added to each Gram-negative/*C.albicans* and Gram-positive bacteria wells, respectively.

Biofilm stains solubilized at room temperature. After shaking and pipetting of wells, 125 µl of the solution from each well transferred to a sterile tube and the volume was made up to 1 mL with distilled water. Finally, optical density of each well measured at 550 nm wavelength (Thermo Scientific Multiskan FC, Vantaa, Finland). Negative controls (cells+TSB), positive control (cells+TSB+essential oil), vehicle control (cells+TSB+DMSO), and media controls (TSB) were included. Positive controls for essential oil were prepared via serial dilution techniques.

Each strain was tested for biofilm formation in duplicate and the assay was repeated three times. Replicate absorbance readings for each concentration were averaged and the average of the media control was subtracted. This value was then divided by the mean absorbance of the (cell+TSB) and multiplied by 100.

$$Percentage\ inhibition = \frac{OD\ Negative\ control - OD\ Experimental\ x\ 100}{OD\ Negative\ control}$$

Results and Discussion

The antimicrobial activity of the essential oils of *M. x piperita* was evaluated to various multiple antibiotic resistant bacterial strains of *S. aureus*, *S. epidermidis*, *P. aeruginosa*, *P. fluorescens* and the yeast *C. albicans*. The inhibition zones and MIC values are given in Table 1.

Table 1. *The inhibition zones and MIC values of M. x piperita essential oil*

Microorganism	Inhibition zone (mm)	MIC value (µl/ml)
S.aureus MU 38	13	1
S.aureus MU 40	12	>25
S.aureus MU 46	15	>25
S.aureus MU 47	14	>25
S. epidermidis MU 30	10	>25
P.aeruginosa MU 187	-	>25
P.aeruginosa MU 188	15	>25
P.aeruginosa MU 189	10	>25
P.fluorescens MU 180	10	>25
P.fluorescens MU 181	10	>25
C.albicans ATCC 10239	14	>25

(-) : No activity

In the disk diffusion method performed to determine the antimicrobial activity of essential oil obtained from *M. x piperita*, inhibition zones between 10-15 mm were identified against all tested strains except for *P. aeruginosa* MU 187. In the Gram- positive test strains, the highest inhibition zone was 15 mm against *S. aureus* MU 46 and the lowest inhibition zone was 10 mm against *S. epidermidis* MU 30. In the Gram- negative test strains, 15 mm inhibition zone against *P. aeruginosa* MU 188 and 10 mm inhibition zones was measured against the other 3 strains of *Pseudomonas*. As a result of the MIC test, the essential oil was most effective against *S. aureus* MU 38 and 1 µg/ml concentration of essential oil was found to inhibit this strain. Other test strains used in the study were found to be inhibited at higher values of concentration of 25 µg/ml essential oil.

The essential oil of *M. x piperita* was found to have different proportions of antibiofilm activity against all Gram-positive test strains. The percentage inhibition of biofilm formation against the test bacteria are given in Table 2.

The reduction rate of essential oil in 25 µg/ml concentration was determined as 61.93% against *S. aureus* MU 46 biofilm production. According to the experimental results, the essential oil of *M. x piperita* showed no antibiofilm activity against *P. aeruginosa* MU 187 and *P. fluorescens* MU 181. The highest antibiofilm activity in Gram- negative bacteria was determined at the 25 µg/ml concentration of essential oil with the reduction rate of 84.56% against *P. fluorescens* MU 180 biofilm production. Against of *P. aeruginosa* MU 189 biofilm production, the essential oil in only 5 µg/ml concentration caused a reduction rate of 13.55%. 25 µg/ml concentration of essential oil was found to reduce the *C. albicans* ATCC 10239 biofilm production at the rate of 10.52%.

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Table 2. *The percentage inhibition of biofilm formation of the essential oil of M. x piperita against the test bacteria*

Concentration (µl/ml)	S.a.				S.e.	P.a.			P.f.		C.a.
	MU 38	MU 40	MU 46	MU 47	MU 30	MU 187	MU 188	MU 189	MU 180	MU 181	ATCC 10239
	Inhibiton (%)										
25	-	-	61.93	56.46	-	-	-	-	84.56	-	10.52
5	-	18.66	60.09	51.27	31.97	-	32.25	13.55	20.05	-	-
1	48.4	12.21	51.55	39.18	28.65	-	30.37	-	-	-	-
0.5	42.15	-	28.42	32.57	17.32	-	24.33	-	-	-	-
0.1	31.53	-	25.09	30.05	11.02	-	8.35	-	-	-	-
0.05	22.4	-	23.3	12.46	5.12	-	-	-	-	-	-

S.a.: *S.aureus*, S.e.: *S.epidermidis*, P.a.: *P.aeruginosa*, P.f.: *P.fluorescens*, C.a.: *C.albicans*, (-): No activity

Conclusion

The antimicrobial compounds from plant source have increasing attention in recent years. Although there are many reports on the antimicrobial properties of plant extracts and essential oils, there are very few reports on the antibiofilm activities of these compounds. Isolation and identification of the constituents that exhibit antibiofilm properties might be essential to include as alternatives in the control of biofilms. The present study aimed to determine the antimicrobial and antibiofilm activities of the essential oil of *M. x piperita*. Especially the antibiofilm activity is an important in the medical field as well as in the food industries for inhibition the biofilm formation.

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