In vitro Antimicrobial and Antioxidant Activity of Two Selected Plants from *Lamiaceae* Species

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Abstract
The aim of the present study was to evaluate antimicrobial and antioxidant activity of *Melissa officinalis* L. and *Mentha piperita* L. from *Lamiaceae* species (The detection of antimicrobial activity was carried out by disc diffusion method and evaluation of minimal inhibition concentration against five Gram-negative bacteria: *Escherichia coli* CCM 3988, *Klebsiella pneumoniae* CCM 2318, *Salmonella enterica* subsp. *enterica* CCM 3807, *Shigella sonei* CCM 1373, *Yersinia enterocolitica* CCM 5671 and five Gram-positive bacteria: *Bacillus thuringiensis* CCM 19, *Clostridium perfringens* CCM 4435, *Haemophilus influenzae* CCM4456, *Listeria monocytogenes* CCM 4699, *Staphylococcus aureus* subsp. *aureus* CCM 2461. Antioxidant activity was determined by DPPH radical scavenging and phosphomolybdenum antioxidant methods. The content of polyphenols and flavonoids was measured by colorimetric methods. Both tested plants showed the highest antimicrobial activity against Gram-negative bacteria confirmed by both methods - disc diffusion and minimal inhibitory concentration. Additionally, both tested plants exhibited high antioxidant activity. The highest content of total polyphenol (37.37 mg GAE/g g) was identified in lemon balm, but flavonoids (33.90 µg QE/g) in peppermint.

Keywords: lemon balm, peppermint thyme essential oil, antimicrobial activity, antioxidant activity, flavonoids, polyphenols

1. Introduction

Lemon balm (*Melissa officinalis* L.) is a perennial herb of the family *Lamiaceae*, cultivated for its characteristic lemon-scented leaves. It is implemented for several purposes in the food, pharmaceutical and cosmetic industries due to its flavouring and therapeutic properties. The most common method for post-harvest processing of medicinal plants is a hot air drying because it allows a quick conservation of the medicinal qualities of the plant material in an uncomplicated manner [1]. Colour of plant has a great influence on their appearance; hence, colour is considered to play a significant role in the acceptability of medicinal, aromatic and spice plants. Additionally, the colour of herb is of prime importance to the consumers who prefer the leaves with a natural green colour. During convective air-drying the discoloration of leaves from bright green to pale green occurs mainly due to loss of chlorophyll, which is sometimes accompanied by browning. The essential oil of *M. officinalis* is a well-known antibacterial and antifungal agent [2].

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M. officinalis contains some phenolic and flavonoid compounds such as rosmarinic acid [3]. Phenolic contents in plants have some antioxidant properties [4].

Mentha piperita L., a medicinally important plant which belongs to the species *Lamiaceae*. Hybrid of *M. spicata* L. (spearmint) and *Mentha aquatic* and commonly known as peppermint. *Mentha piperita* L was cultivated by the ancient Egyptians and documented in the Icelandic pharmacopoeia of the 13th century. It used to be widely grown in areas with a temperate climate, particularly in Europe, North America and North Africa. Nowadays plant is cultivated throughout all regions of the world.

M. piperita is a perennial 50–90 cm high, normally quadrangular and a prototypical member of the mint family [5]. The medicinal parts of plant are the aerial parts of the flowering plant, the dried leaves, the fresh flowering plant and the whole plant. These parts are used for extraction of the *M. piperita* essential oil which posses antimicrobial and antioxidant properties [6]. The antimicrobial activity of EOMP against food-borne pathogens was examined in *in vitro* studies. *Mentha piperita* (peppermint) is one of the most widely consumed single ingredient herbal teas, or tisanes [6]. Tea has been traditionally used for their antiseptic properties for the treatment of infectious diseases [7]. The leaves of *M. piperita* are used in food preparation to enhance taste and appearance.

The purpose of the present study was to evaluate antimicrobial and antioxidant activity of two selected plants from *Lamiaceae* species *Melissa officinalis* L. and *Mentha piperita* L.

2. Materials and methods

2.1 Plant materials

The plant materials used in this experiment consisted of leaves of *Melissa officinalis* L. and *Mentha piperita* L. The plants were collected in Slovakia. The material was initially dried at the room temperature in the dark.

2.2 Microbial strains

Ten strains of microorganisms were tested in this study, including five Gram-negative bacteria: *Escherichia coli* CCM 3988, *Klebsiella pneumoniae* CCM 2318, *Salmonella enterica* subsp. *enterica* CCM 3807, *Shigella sonei* CCM 1373, *Yersinia enterocolitica* CCM 5671 and five Gram-positive bacteria: *Bacillus thuringiensis* CCM 19, *Clostridium perfringens* CCM 4435, *Haemophilus influenzae* CCM4456, *Listeria monocytogenes* CCM 4699, *Staphylococcus aureus* subsp. *aureus* CCM 2461. All tested strains were collected from the Czech Collection of microorganisms. The bacterial suspensions were cultured in the nutrient broth (Imuna, Slovakia) at 37°C.

2.3. Preparation of plant extracts

After drying, the plant materials were crushed, weighed out to 10 g and soaked separately in 100 mL of ethanol p.a. (99.9%, Sigma, Germany) during 14 days at room temperature. Then, ethanolic plant extracts were filtered through the Whatman No. 1 filter paper. The obtained extracts were subjected to evaporation under reduced pressure at 40 °C in order to remove the ethanol (Stuart RE300DB rotary evaporator, Bibby Scientific Limited, UK, vacuum pump KNFN838.1.2KT.45.18, KNF, Germany). For the antimicrobial assays, the crude plant extracts were dissolved in dimethylsulfoxid (DMSO) (Penta, Czech Republic) to 102.4 mg/mL as stock solution, while for chemical analysis ethanol was used as solvent. Stock solutions of plant extracts were stored at -16 °C in refrigerator until the experiments were initiated.

2.4 Disc diffusion method

Antimicrobial activity of each plant extract was determined by a disc diffusion method. Briefly, 100 µl of the bacterial suspension were grown in 10 ml of fresh media until they reached a count of approximately 10^5 cells/ml. Then, a quantity of 100 µl of the microbial suspension was spread onto Mueller Hinton agar plates. The extracts were tested using 6 mm sterilized filter paper discs. The diameters of the inhibition zones were measured in millimeters. All measurements were to the closest whole millimeter. Each antimicrobial assay was performed in at least triplicate. Filter discs impregnated with 10 µl of distilled water were used as a negative control.

2.5 Microbroth dilution method

MICs were determined by the microbroth dilution method according to the Clinical and Laboratory Standards Institute recommendation [8] in Mueller

Hinton broth (Biolife, Italy). Briefly, the DMSO plant extracts solutions were prepared as serial two-fold dilutions obtaining a final concentration ranging between 0.5-512 μg/ml. After that, each well was inoculated with microbial suspension at the final density of 0.5 McFarland. After 24 h of incubation at 37 °C, the inhibition of microbial growth was evaluated by measuring the well absorbance at 450 nm in an absorbance microplate reader Biotek EL808 with shaker (Biotek Instruments, USA). The 96 microwell plates were measured before and after experiment. Differences between both measurements were evaluated as growth. Measurement error was established for 0.05 values of absorbance. Wells without plant extracts were used as the negative controls of growth. Pure DMSO was used the negative control. This experiment was done in eight-replicates for a higher accuracy of the MICs of used medical plant extracts.

2.6 Radical scavenging activity

Radical scavenging activity of extracts was measured using 2,2-diphenyl-1-picrylhydrazyl (DPPH) [9]. An amount of 0.4 ml of sample was mixed with 3.6 ml of DPPH solution (0.025 g DPPH in 100 ml methanol). Absorbance of the reaction mixture was determined using the spectrophotometer Jenway (6405 UV/Vis, England) at 515 nm. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (10-100 $mg.L^{-1}$; R^2=0.989) was used as the standard and the results were expressed in mg/g of Trolox equivalents.

2.7 Reducing power

Reducing power of extracts was determined by the phosphomolybdenum method of Prieto et al. [10] with modifications. The 1 ml of mixture of sample, monopotassium phosphate (2.8 ml, 0.1 M), sulfuric acid (6 ml, 1 M), ammonium heptamolybdate (0.4 ml, 0.1 M) and distilled water (0.8 ml) was incubated at 90°C for 120 min, then rapidly cooled and monitored for absorbance at 700 nm using the spectrophotometer Jenway (6405 UV/Vis, England). Trolox (10-1000 mg/l; R^2=0.998) was used as the standard and the results were expressed in μg/g of Trolox equivalents.

2.8 Total polyphenol content

Total polyphenol content extracts was measured by the method of Singleton and Rossi [11] with Folin-Ciocalteu reagent. A quantity of 0.1 ml of each sample was mixed with 0.1 ml of the Folin-Ciocalteu reagent, 1 ml of 20% (w/v) sodium carbonate, and 8.8 ml of distilled water. After 30 min the absorbance at 700 nm was measured in darkness using the spectrophotometer Jenway (6405 UV/Vis, England). Gallic acid (25-300 mg/l; R^2=0.998) was used as the standard and the results were expressed in μg/g gallic acid equivalents

2.9 Total flavonoid content

Total flavonoids were determined using the modified method of Willett [12]. An amount of 0.5 ml of sample was mixed with 0.1 ml of 10% (w/v) ethanolic solution of aluminium chloride, 0.1 ml of 1 M potassium acetate and 4.3 ml of distilled water. After 30 min the absorbance at 415 nm was measured in darkness using the spectrophotometer Jenway (6405 UV/Vis, England). Quercetin (0.5-20 mg/l; R^2=0.989) was used as the standard and the results were expressed in $\mu g.g^{-1}$ quercetin equivalents.

2.10 Statistical analysis

Differences in absorbance before and after the analysis were expressed as a set of binary values. These values were assigned to exact concentrations. The following formula was created for this specific experiment: value 1 (inhibitory effect) was assigned to absorbance values lower than 0.05, while value 0 (no effect or stimulant effect) was assigned to absorbance values higher than 0.05. For this, statistical evaluation the Probit analysis in Statgraphics software was used.

3. Results and discussion

In our study, two medicinal plants of *Lamiaceae* species (*Melissa officinalis* L. and *Mentha piperita* L.) were tested against 10 different strains of Gram-positive and Gram-negative bacteria with disc-diffusion and MIC methods.

The results of disc diffusion method showed that peppermint exhibited the highest antibacterial activity with 7.5 mm zone of inhibition and lemon balm exhibited the highest antibacterial activity with 8.2 mm zone of inhibition against *Shigella sonei* CCM 1373 (Figure 1).

Minimal inhibition concentration of *Mellisa officinalis* ranged from 3.2 in MIC50 resp. 3.41 in MIC 90 to 27.36 resp. 66.13 µg/ml and MIC of *Mentha piperita* from 7.06 resp. 9.04 to 28.96 resp. 44.33 µg/ml.

The best antimicrobial activity according to MIC was found against *Shigella sonei* CCM 1373 and *Yersinia enterocolitica* CCM 5671.

In comparison, peppermint oil was found to be strongly effective against *Enterococcus faecium* ATCC 10541, *Salmonella choleraesuis, Staphylococcus aureus* and *Bacillus subtilis* in previous study [13].

The antibacterial (against *E. coli* and *S. aureus*) of peppermint were reported by Rasooli et al. [14], whereas the antibacterial activity of different extracts against the same bacterial strains was reported by Priya et al. [15].

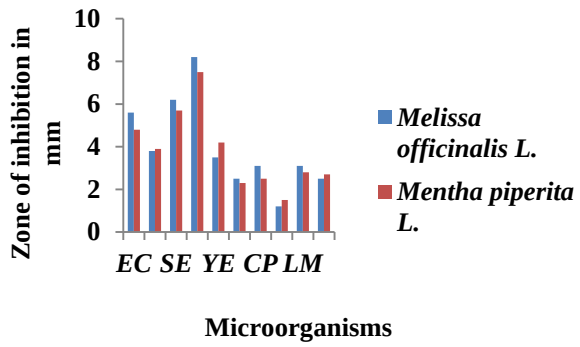

Figure 1 Antibacterial activity of *Lamiaceae* species detected by disc diffusion method

EC-Escherichia coli CCM 3988, *KP-Klebsiella pneumoniae* CCM 2318, *SE-Salmonella enterica* subsp. *enterica* CCM 3807, SS-*Shigella sonei* CCM 1373, *YE-Yersinia enterocolitica* CCM 5671, *BT-Bacillus thuringiensis* CCM 19, *CP-Clostridium perfringens* CCM 4435, *HI-Haemophilus influenzae* CCM4456, *LM-Listeria monocytogenes* CCM 4699, *SA-Stapylococcus aureus* subsp. *aureus* CCM 2461

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 [7]. 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.

The results of the antimicrobial activities of *M. officinalis* performed by disk method and MICs against human pathogenic *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, Klebsiella pneumoniae, Salmonella enterica* and *Listeria monocytogenes* showed that the essential oil exhibited a strong activity against all the strains tested with very low MICs. The inhibition zones ranged between (17 and 18 mm) for the Gram-positive bacteria, and (14-21 mm) for the pathogenic Gram-negative bacteria. Generally, the essential oils were more active against Gram-positive bacteria than against Gram-negative. *Melissa officinalis* oil is more active against the Gram-negative ones [16].

Table 1 MIC of *Melissa officinalis* L. and *Mentha piperita* L essential oils in µg/ml

Tested microorganisms	*M. piperita*		*M. officinalis*	
	MIC 50	MIC 90	MIC 50	MIC 90
E. coli	28.96	44.33	15.26	34.62
K. pneumoniae	12.78	13.59	27.36	66.13
S. enterica	12.78	13.59	3.20	3.41
S. sonei	8.53	9.54	3.20	3.41
Y. enterocolitia	7.06	9.04	3.20	3.41
B. thuringiensis	28.96	44.33	15.26	34.62
C. perfringens	12.78	13.59	27.36	66.13
H. influenza	12.78	13.59	13.75	35.24
L. monocytogenes	18.53	19.54	13.75	35.24
S. aureus	17.06	19.04	13.75	35.24

Table 2 Antioxidant activity and content of polyphenols and flavonoids of *Melissa officinalis* L. and *Mentha piperita* L essential oils

Indicator	Unit	*Melissa officinalis* L.	*Mentha piperita* L.
DPPH	mg TEAC/g	7.10	6.62
FOMO	mg TEAC/g	274.61	184.47
Polyphenols	mg GAE/g	37.37	25.81
Flavonoids	µg QE/g	28.96	33.90

The antioxidant properties were evaluated by two differential method - DPPH and phosphomolybdenum method and the results on each plant extract are summarized in Table 2. The highest activity by DPPH method was found in *Melissa officinalis* L. (7.10 mg TEAC/g). DPPH is

a protonated radical having the characteristic absorption maxima at 517 nm which decreases with the scavenging of the proton radical by plant extracts. *Melissa officinalis* L. showed the highest antioxidant activity (reducing power) - 274.61 mg TEAC/g with phosphomolybdenum method. Albayrak et al., (2013) [17] also determined antioxidant activity of *Melissa officinalis* L. extracts (methanolic, infusion and decoction) by phosphomolybdenum method and found higher activity with compare to another plant from *Lamiaceae* family with the best results in methanolic extract – 190.19 mg ascorbic acid per g of sample. Antioxidant activity of the plant extract is often associated with the content of polyphenol compounds. The highest content of total polyphenol (Table 2) was found in *Melissa officinalis* L. (37.37 mg GAE/g) and flavonoid content was found in *Mentha piperita* L. (37.37 µg GAE/g). Large amount of polyphenolic compounds was detected in *Melissa officinalis* L and *Mentha piperita* L. by HPLC method. In *Melissa officinalis* L among the flavonoids is predominant luteolin and rosmarinic acid among the phenolic acid. In *Mentha piperita* L. among the flavonoids predominant is luteolina and diosmin; rosmarinic acid and chlorogenic acid are predominant in the group of phenolic acid [18-19].
Medicinal plants are the main sources of natural antioxidants. *Melissa officinalis* belongs to the family of *Lamiaceae*, a large group of medicinal plants. *M. officinalis* is a perennial plant that grows in all over the Mediterranean region. The leaves of *M. officinalis* have been used in folk medicine especially in Turkey and Iran for the treatment of some disease [20]. Also, the leaves of *M. officinalis* are often used as herbal teas. *M. officinalis* contains some phenolic and flavonoid compounds such as rosmarinic acid [3]. Phenolic compounds of the plants have antioxidant properties [4].
The model of scavenging stable DPPH free radicals can be used to evaluate the antioxidative activities in a relatively short time. The absorbance decreases as a result of a color change from purple to yellow as the radical is scavenged by antioxidants through donation of hydrogen to form the stable DPPH-H molecule [21]. The effect of antioxidants on DPPH radical scavenging was achieved by the hydrogen-donating ability. Antioxidant activities of essential oils from aromatic plants are mainly attributed to the presented active compounds. Constituents of essential oils may interact and to develop synergy among the cimponds. Different antioxidant activities of the plant may be related to variable chemical constituents and origin. Antioxidant activity of *M. piperita* has been reported previously and is in agreement with our results [22-25].

4. Conclusions

In conclusion, our results indicate that *Lamiaceae* plants are rich in bioactive compounds and can be used intensively in pharmacy, medicine and food industry. Benefits of consumption of plants exhibiting the antioxidant activity should be emphasized among the consumers, who could selected the plants rich in bioactive compounds for promotion of healthy life-style *Lamiaceae* plants showed evident antimicrobial activity against Gram-positive and Gram-negative bacteria with better antimicrobial activity against Gram-negative bacteria.

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