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Original scientific paper

Chemical Composition and Antibacterial Activity of the Essential Oil of Menthae piperitae L.

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Abstract: The subject of this study was the examination of the chemical composition and antimicrobial activity of peppermint oil (*Menthae piperitae* L.), that was produced from the plant collected from the plantation in the city of Banja Luka. To examine the chemical composition of the *Mentha piperita* essential oil we used the GC-FID method. On it's chromatogram it was clearly shown that the main components of the oil were *menthole* with the content of 43.66%, *menthone* 20.02%, *iso-menthone 7.73%,* following by *1,8-cineole* with a percentage of 6.49% and *menthil-acetate* with 3.31%. The content of the previously mentioned components were all in accordance with the suggestions made by Ph.Eur 8.0. For the purpose of examination antimicrobial activity of the peppermint oil we used agar diffusion method. The results have shown that etheric oil of *Menthae piperitae* reflects very good antimicrobial activity on all bacterial cultures that we used in this study. The inhibiton zones moved from 12 mm for *Pseudomonas aeruginosa* to 37.66 mm for *Staphylococcus aureus*. Such an outcome of our study, and many others from all around the world, indicates the future potential use of peppermint oil in the treatment of bacterial infections, maybe even lowering the use of antibiotics.

Key words: Essential oil, *Menthae piperitae* L., GC-MS analysis, Antimicrobial activity.

INTRODUCTION

Domestic mentha (*Mentha piperita* L.) belongs to the Mentha genus, which contains hundreds of species. It is known as mentha, domestic mentha or mint. It is a perennial herbaceous plant, with strong and pungent odour. It is cultivated around the world in gardens and plantations (Kovačević and Jančić, 2003). The most important type is mint, because it has the most pleasant smell and taste and it has the most curative properties. It is a naturally cultured hybrid of *Mentha aquatic* L. and *Mentha spicata* L. (Iscan *et al*., 2007). The leaf and the essential oil are mostly used as a drug, rarely the flower on the top of the plant. The leaf and fragrant essential oil of mint are prescribed by all pharmacopoeias in the world. Due to their characteristic chemical composition, they are the significant subject of the international trade and they are the most important raw materials for the production of a large number of drugs, prophylactic, hygienic, dietary, cosmetic, perfumery and many other consumer products on all continents (Tucakov, 1996). Modern pharmaceutical researches has shown that the whole plant *M. piperita* has antioxidant, cytotoxic, antiallergic, antiviral and antibacterial properties (McKay and Blumberg, 2006). Essential oil is obtained by hydrodestilation from mossed overgrowth parts of the plant in the flower (*Menthae piperitae herba*), which contains 0.5-4.0% of essential oil. Previous studies have shown that *M. piperite* essential oil has antioxidant and antimicrobial properties (Yadegarinia *et al*., 2006; Zhenliang *et al*., 2014).

The effectiveness of the antimicrobial effect of the essential oil varies from species to species, as well as from different bacteria depending on the structure. Because of their lipophilicity, they easily pass through the membrane of the bacterium. The mechanism of antimicrobial action of essential oils is reflected in the increase in the permeability of the cell membrane, which leads to the release of vital, intracellular

constituents, interruption of cell metabolism and enzymatic kinetics. The antimicrobial effect of essential oil is based on direct action on the cell wall of bacteria, denaturation and coagulation of proteins, changing the permeability of the cytoplasmic membrane for ions of hydrogen (H^+) and potassium (K^+). This leads to changes in numerous, important cells, processes such as electron transport, protein translocation, phase oxidative phosphorylation and other, enzymatic dependent reactions leading to loss of hemiosmotic control and progressively leading to death of the cell (Ramos *et al*., 2017). In some cases isolated individual ingredients exhibit greater antimicrobial activity than the essential oil from which it is isolated. Menthol, thymol and methyl carbacrol lead to an increase in cell membrane, which leads to passive diffusion of ions between expanded phospholipids and "leakage" of intracellular content (Kumara *et al*., 2016).

The expanded use of *M. piperite* in traditional medicine, as well as in various branches of industry, led us to the examination of the biological activity of mint grown in the Republic of Srpska, examining the chemical composition and antimicrobial activity of the essential oil obtained by the hydrodestilation process from parts of the plant that are grown above the ground.

MATERIALS AND METHODS

Plant material

Plant material which was used for essential oil isolation has been collected, in June 2016., near to the Banja Luka, northwestern part of the Republic of Srpska, Bosnia and Herzegovina. The harvest is realized in cooperation with the family company d.o.o "Natural Herbs", Banja Luka.

Essential oil isolation

The essential oil was produced within this company by hydrodestilation process on semiindustrial scale. The mint seeds used in the sowing process, for basil harbs growing which was used for production of the essential oil tested in this study, is obtained by their own reproduction. But, the first seed for sowing in this area were purchased in the Institute "Dr Josif Pančić", Belgrade, Republic of Serbia. A boiler with capacity of 2000 liters has been used in the hydrodistillation process, the temperature was 102 °C, pressure of 0.5 bar, and the hydrodistillation process time was 1.5 hours. The yield of obtained essential oil, with characteristic pleasant aroma, was 1.5%.

Reagents and chemicals

Ethanol, 96 % (Centrohem, Zemun, Serbia), 1.1-diphenyl-2-picrylhydrasil (DPPH radical), butylated hydroxytoluene (BHT) (Sigma Chemical Company, St. Louis, USA).

GC-MS and GC-FID analysis

GC-MS analysis of the essential oils obtained from mint (*Menthae piperitae* L.) was performed on Agilent Technologies 7890B gas chromatograph, equipped with weakly polar, silica capillary column, HP-5MS (5 % diphenyl- and 95 % dimethyl-polysiloxane, 30 m x 0.25 mm, 0.25 μm film thickness; Agilent Technologies, USA) and coupled with inert, selective 5977A mass detector of the same company. Three μl of the sample dissolved in diethyl ether in the concentration of 1000 ppm was injected in 5:1 split mode. Helium was used as the carrier gas, at a constant flow rate of 1 ml/ min. The oven temperature was programmed from 50 °C for 2.25 minutes and then increased to 290 °C at the rate of 4 °C/min. Temperatures of the MSD transfer line, ion source and quadruple mass analyzer were set at 300 °C, 230 °C and 150 °C, respectively. The ionization voltage was 70 eV and mass range m/z 35-650.

GC-FID analysis was carried out under identical experimental conditions as GC-MS. The temperature of the flame-ionization detector (FID) was set at 300 °C. Data processing was performed using MSD Chem-Station, MassHunter Qualitative Analysis and AMDIS_32 softwares (Agilent Technologies, USA). Retention indices of the components from the analyzed samples were experimentally determined using a homologous series of n-alkanes from C8-C20 as standards. The percentage composition of particular component in the essential oil was determined on the basis of automatically integrated peak areas of the GCFID signal.

Antimicrobial activity

In order to investigate antimicrobial activity of the mint essential oil difeferent cultures of *Staphylococcus aureus* WDCM 00032, *Listeria monocytogenes* WDCM 00020, *Salmonella enterica* WDCM 00030, *Escherichia coli* WDCM 00013 and *Pseudomonas aeruginosa* WDCM 00024 (BCCM™/LMG Bacteria Collection, Belgium) have been used.

The referent cultures were sown in a nutritive broth and incubated at 37 °C/18h. Petri dishes with the appropriate substrate (Müeller - Hinton agar) were sown with 0.1 ml of bacterial suspension in a concentration of 10^5 cell/ ml. The effects of basil essential oil on inhibition of pathogenic bacteria growth was determined by agar diffusion method (Kirby-Bauer, 1996) on a solidsterile broth (Müeller-Hinton-agar (MHA)). Paper disks of 9 mm diameter were placed on the surface of a solid-sterile broth where previously pure bacterial culture was sown. Twenty microlitres of essential oil was dripped on the discs by a micropipette. As a control, one paper disk with 20 µl of 96 % ethanol was used. The ability of growth and reproduction of each culture depends on its sensitivity to the investigated essential oil. So, if the effect exists, clear transparent zone was formed around the paper disks indicating that there is no growth of microorganisms. Petri dishes were incubated for 24 hours at 37 °C. Every measurement was done after three repetitions. averaged values calculations. Amoxicillin (30 µg/disc), Doxycycline (30 µg/disc), Ciprofloxacin (5 µg/disc), Streptomycin (10 µg/disc) and Gentamicin (10 µg/disc) (Bioanalyse) were used as reference standards.

RESULTS AND DISCUSSION

Qualitative and quantitative essential oil composition

The chemical composition of essential oil of mint is shown in Table 1 and the GC-FID chromatogram in Figure 1. The GC-MS analysis identified 40 components, which makes 92.65% of the total oil mixture. All identified components, as well as their percentage representation in the examined oil are given in Table 1.

The most represented compounds are monoterpenes, which contain oxygen, 86.74% of the total components of the oil. Monoterpen hydrocarbons are represented with 2.66%, sesquiterpenic which contain oxygen with 2.48%, and sesquiterpenic hydrocarbons with 0.53%. Based on the obtained results, it can be seen that menthol is the most represented component (43.66%), then menthone (20.02%), iso-menthone (7.73%), 1.8-cineole (6.49%) and menthyl acetate (3.31%). Structural formulas of the basic components of the examined essential oil are given in Figure 2.

Menthol and menthone are basic components of the essential oil of mint (Iscan *et al*., 2002). Derwich *et al.* (2010) identified 29 components (58.61%) of the essential oil of Moroccan leaf originating from Morocco, using the gas chromatography, where the highest content of menthoes (29.01%), menthol (5.58%), mentyl acetate (3.34%), mentho furan (3.01%), 1.8-cineole (2.40%), iso-menthone (2.12%) and limonene (2.10%). Menthol (36.24%) and menthone (32.42%) are the basic components of the essential oil

originating from Iran (Behnal *et al*., 2006), while menthone (44.10%), menthol (29.5%), menthyl acetate (3.8%) and mentho furan (0.9%) are basic components of the essential oil *Mentha piperita* L. originating from Turkey (Arldogan *et al*., 2002). Soković *et al.* (2009) examined the essential oil of domestic mint origins from the territory of the Republic of Serbia and obtained results that also show the highest content of menthol (37.04%). Menthyl acetate is represented with 17.40% and a menthone with 12.70%. The essential oil of *Mentha piperita* L. leaves from Korea has a significantly different chemical composition, where the basic component is linalyl acetate (28.2%) (Seun-Ah *et al*., 2010). The reasons for the differences in the content of certain components of essential oils are generally genetic, but they may also be a technological nature. Some of the components are synthesized during the desilation process from their precursors (Stanojević *et al*., 2016).

Comparing the obtained results with the chromatographic profile of the European Pharmacopoeia (Ph Eur 8), it can be concluded that the composition of the examined essential oil of domestic mint which originates from the Republic of Srpska, meets the regulations regarding to all isolated components (European Pharmacopeia, 2013).

Table 1. Chemical composition of *Mentha piperitae* L. essential oil

No.	RT min.	Compound	RI^{exp}	RI^{lit}	Method of identification	Composition %
1	9.80	α-Thujene	925.4	924	RI, MS	tr
2	10.04	α-Pinene	932.0	932	RI, MS	0.68
3	10.56	Camphene	946.8	946	RI, MS	0.06
4	11.41	Sabinene	970.8	969	RI, MS	0.25
5	11.54	β-Pinene	974.5	974	RI, MS	0.63
6	12.01	Myrcene	987.6	988	RI, MS	0.08
7	12.14	3-Octanol	991.3	988	RI, MS	0.23
8	12.53	α-Phellandrene	1002.2	1002	RI, MS	tr
9	12.96	α-Terpinene	1014.6	1014	RI, MS	tr
10	13.24	*o*-Cymene	1022.3	1022	RI, MS	0.45
11	13.39	Limonene	1026.6	1024	RI, MS	0.45
12	13.49	1.8-Cineole	**1029.4**	**1026**	**RI, MS**	**6.49**
13	14.49	γ-Terpinene	1057.6	1054	RI, MS	0.06
14	14.78	*cis*-Sabinene hydrate	1065.7	1065	RI, MS	2.16
15	14.98	*cis*-Linalool oxide (furanoid)	1071.4	1067	RI, MS	0.10
16	15.56	*trans*- Linalool oxide (furanoid)	1087.7	1084	RI, MS	0.09
17	15.91	*trans*-Sabinene hydrate	1097.8	1098	RI, MS	0.43
18	16.08	2-Methyl butyl-2-methyl butyrate	1102.6	1100	RI, MS	0.07
19	16.23	2-Methyl butyl isovalerate	1106.7	1103	RI, MS	0.17
20	16.79	3-Octanol acetate	1122.4	1120	RI, MS	0.15
21	16.93	α-Campholenal	1126.5	1122	RI, MS	tr
22	**17.98**	Menthone	1156.1	1148	RI, MS	20.02
23	18.32	Iso-Menthone	1165.6	1158	RI, MS	7.73
24	18.72	Menthol	**1177.0**	**1167**	**RI, MS**	**43.66**
25	18.82	Terpinen-4-ol	1179.7	1173	RI, MS	0.22
26	19.01	iso-Menthol	1184.9	1179	RI, MS	0.57
27	19.17	neoiso-Menthol	1189.6	1184	RI, MS	0.15
28	19.24	α-Terpineol	1191.5	1188	RI, MS	0.24
29	19.47	Myrtenal	1198.0	1195	RI, MS	0.18
30	19.91	Verbenone	1210.8	1204	RI, MS	0.06
31	21.06	Carvone	1244.7	1239	RI, MS	0.08
32	21.42	Piperitone	1255.4	1249	RI, MS	0.74
33	22.10	neo-Menthyl acetate	1275.5	1271	RI, MS	0.27
34	**22.74**	Menthyl acetate	1294.3	1294	RI, MS	3.31

35	23.24	iso-Menthyl acetate	1309.4	1304	RI, MS	0.13
36	25.50	α-Copaene	1379.7	1374	RI, MS	tr
37	25.80	β-Bourbonene	1389.1	1387	RI, MS	0.32
38	25.97	β-Elemene	1394.5	1389	RI, MS	0.21
39	31.61	Spathulenol	1583.3	1577	RI, MS	0.34
40	31.79	Caryophyllene oxide	1589.7	1582	RI, MS	1.87
		Total identified (%)	92.65			

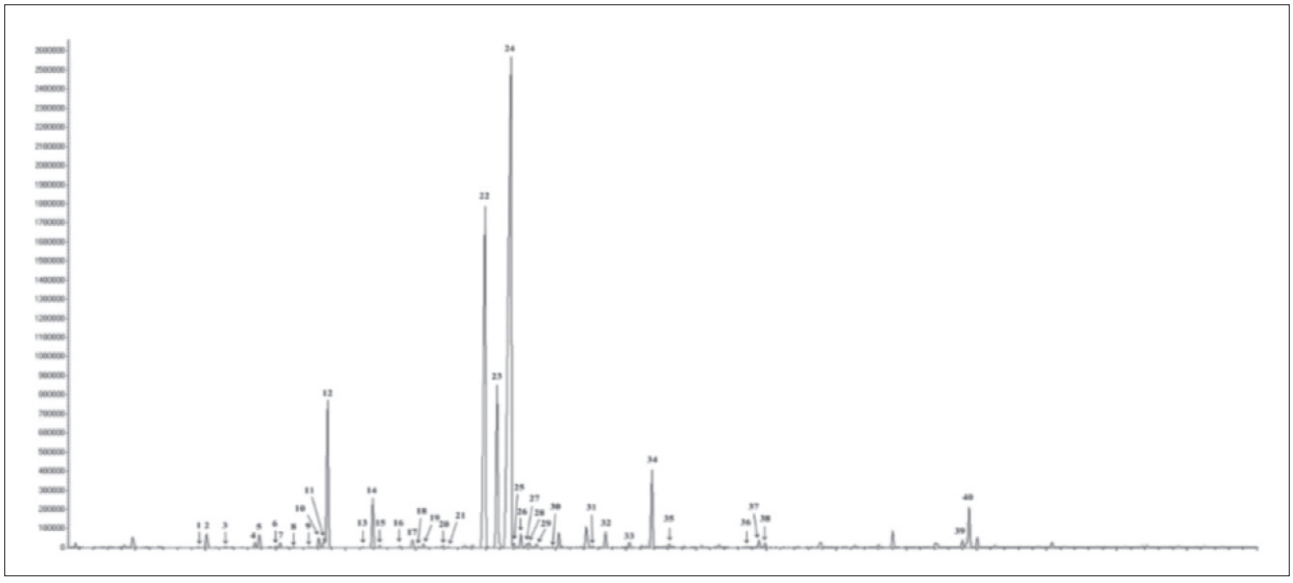

Figure 1. GC-FID chromatogram of *Mentha piperitae* L. essential oil

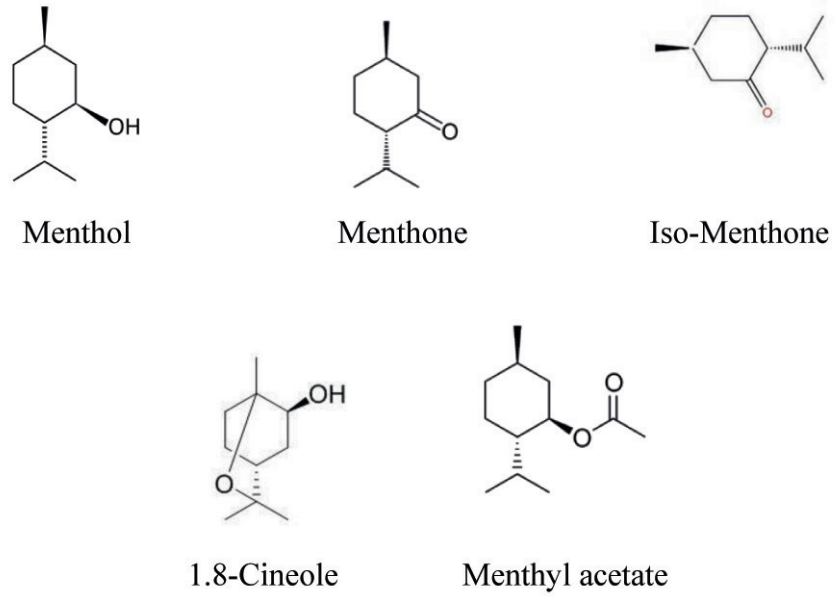

Figure 2. Structures of major components of peppermint essential oil

Antimicrobial activity

The influence of the essential oil of domestic mint on the growth of various types of microorganisms was examined. Essential oil has a strong influence on all tested bacterial strains (Table 2). The essential oil of mint showed good antimicrobial activity with zones of inhibitions in diameter of 12.00 mm (for *P. aeruginosa*) to 37.66 mm (for *S. aureus*). From table 2 it can be noticed that the essential oil of mint has

a stronger antimicrobial effect on *L. monocytogenes* and *S. aureus* than all commercial antimicrobial agents that were tested.

It has greater impact on *S. enterica* than doxycycline, streptomycin and gentamicin, and on *E. coli* greater than streptomycin. At the same time, essential oil has the same antimicrobial effect on *E. coli* as gentamicin. The results also showed that ciprofloxacin is an antimicrobial agent with the strongest inhibitory effect on the tested pathogens. It has stronger antimicrobial effect on *P. aeruginosa*, ciprofloxacin and gentamicin than the examined essential oil. The comparison of the antimicrobial activity of the essential oil mint with the most effective antimicrobial drugs is shown in Figure 3.

Table 2. Antimicrobial activity of selected antibiotics and *Mentha piperitae* L. essential oil

Microorganism	Inhibition zone (mm) Antibiotic					
	Essential oil	A	D	C	S	G
Listeria monocytogenes WDCM 00020	32.33±0.20	13	28	30	28	26
Pseudomonas aeruginosa WDCM 00024	12.00±0.31	-	-	34	-	18
Escherichia coli WDCM 00013	21.00±0.25	-	26	31	20	21
Salmonella enterica WDCM 00030	27.33±0.37	-	25	36	22	23
Staphylococcus aureus WDCM 00032	37.66±0.26	14	35	35	15	22

Amoxicillin; D-Doxycycline; C-Ciprofloxacin; S-Streptomycin; G-Gentamicin

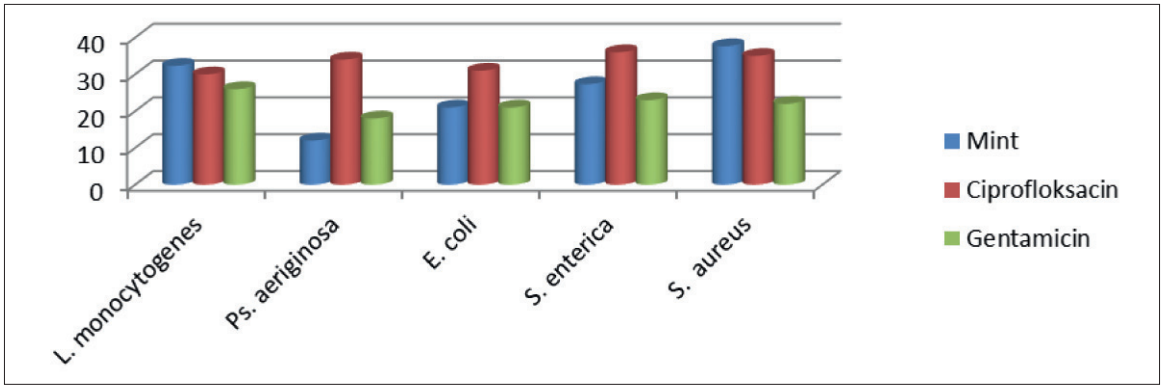

Figure 3. Inhibition zone of mint essential oil and selected antimicrobial drugs on *L. monocytogenes, P. aeruginosa, E. coli, S. enterica* and *S.aureus* (in mm)

A large number of *in vitro* studies have shown good antimicrobial activity of essential oil (Miletić *et al*., 2009; Kalaba *et al*., 2014, Nikolic *et al*., 2013; Mahmmod, 2013; Jakubcova *et al*., 2014; Jakubcova *et al*., 2014b; Beatovic *et al*., 2013; Kizil *et al*., 2010; Agarwal *et al*., 2008; Saharkhiz *et al*., 2012). A large number of previous studies have shown that the resistance of the bacteria depends on a combination of several factors, such as different cellular organisms and the permeability of the cell membrane, which acts as a barrier to antimicrobial agents (Lambert, 2002; Iscan *et al*., 2002).

The obtained results are consistent with the results of other researchers (Iscan *et a*l., 2002; Josh *et al*., 2009; Mishra *et al*., 2011; Mimica-Dukic *et al*., 2003; Rasooli *et al*., 2008). Singh *et al*. (2015) examined the essential oil of *Mentha piperitae* L. showing a stronger antimicrobial effect of gentamicin on *S. enterica* versus essential oil, which is somewhat in contrast to our results. Differences in antibacterial activity in the case of the same strains of bacteria can be attributed to different distillation processes, the geographical origin of the plant, the harvest period (Rasooli *et. al*., 2008; Priya *et al*., 2007). The antibacte-

rial effect of essential oil is closely related to the amount of the presented menthol, but the synergistic effect of other oil components can not be excluded (Iscan *et al*., 2002; Singh *et al*., 2015).

The obtained results provide good information about essential oil, which could be significant for its use in the prevention of the treatment of various diseases caused by the examined pathogens. The essential oil of mint can be good alternative to synthetic antibiotics. However, it also finds a significant application as a raw material in pharmaceutical, food and chemical production.

CONCLUSION

In the obtained essential oil 40 components were identified, or 92.65% of the total oil mixture, and their antimicrobial activity were tested.

Based on the chemical analysis, it is concluded that the analyzed essential oil of domestic mint fulfills the quality requirements prescribed by the European Pharmacopoeia with the regard to all isolated components. The highest content of menthol was 43.66%.

The results of antimicrobial analysis indicate a good antimicrobial effect of the essential oil on all tested bacterial strains. *Staphylococcus aureus* is the most sensitive bacterial strain with an inhibition zone of 37.66 mm.

Taking into account the chemical composition and antimicrobial effect of the essential oil of domestic mint from the Republic of Srpska, it can be concluded that the obtained results can be taken as valuable research references in the examination of the phytomedicine potential of essential oil in the pharmaceutical, food and chemical industry as well as in its use in the form of replacement by synthetic additives which show toxic and carcinogenic effects.

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