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CHEMICAL COMPOSITION AND ANTIMICROBIAL ACTIVITY OF BULGARIAN PEPPERMINT OILS

V. Gochev[1], A. Stoyanova[2], T. Girova[1], T. Atanasova[2]
[1]“Paisii Hilendarski”- University of Plovdiv, Department of Biochemistry and Microbiology, Biological Faculty,24 Tzar Asen Street, 4000 Plovdiv, Bulgaria
[2]University of Food Technology, Department of Essential Oils, 26 Maritza Boulevard, 4002 Plovdiv, Bulgaria

ABSTRACT

Chemical composition of historical peppermint oil sample (more than 50 years old) and four fresh oils obtained from three confirmed Bulgarian cultivars “Kliment – 63”, “Sofia 35-A” and “Zefir” and newly selected variety from local population was investigated by GC and GC/MS. Chemical composition of Bulgarian peppermint oils corresponded to all criteria stated in ISO 856:2006 and the main components were menthol (35.2 – 46.2%) and menthone (8.7 – 25.9%). Antimicrobial activity of the studied peppermint oil samples was evaluated against pathogenic and provisionally pathogenic bacteria. All of the tested peppermint oils demonstrated higher activity against Gram-positive bacteria and weaker against Gram-negative bacteria. Historical peppermint oil sample was absolutely comparable and even exceeded the studied fresh peppermint oil samples according to its chemical composition, antimicrobial activity and odor characteristics.

Keywords: peppermint oil, chemical composition, antibacterial activity

INTRODUCTION

The production of peppermint oil in Bulgaria dates back to the middle of the 18th century, carried out in a very primitive way by water distillation of local varieties of wild mint, pennyroyal and field mint, while the derived oil has been used only in the traditional medicine. The first trials to introduce peppermint as a crop culture in Bulgaria are recorded in 1905, but with no success. The industrial cultivation of peppermint begins after 1923, and as soon as 1938 Bulgaria has already occupied the third place in the world in terms of peppermint oil production. During the Second World War production has dropped substantially but managed to regain its positions during the 50s. Bulgarian peppermint oil has gained world popularity under the name Bulgaro-Mitcham oil (after the Mitcham region, England). Its recognition is due,

most of all, to its rich and pleasant odor, sweet-peppery taste and high menthol content, which makes it highly praised on the international market [1,2].

Currently, the production of peppermint essential oil is significantly reduced due to the replacement of peppermint by other crops and growing the plant for the purpose of obtaining mint leaves for teas.

Along with the local population, which is nearly extinct, a number of selected confirmed cultivars are grown nowadays in Bulgaria – "Kliment-63", "Sofia 35-A", "Zefir", "Maritza-1" and "Tundzha". These are highly productive and more insect and disease resistant, but the obtained oil deviates in olfactory nuances from that of the local variety, appreciated as the Bulgaro-Mitcham type. The selected cultivars contain essential oil in the ranges: 0.4 – 0.8 % in the fresh raw material and up to 1 – 2 % in the dried raw material [1]. The basic physical and chemical indexes and composition of Bulgarian peppermint oil has been a subject of investigation for many researchers [1-6], but most comprehensively summarized by Georgiev and Stoyanova [1].

Primary objective of the present study was to compare the chemical composition and the antibacterial properties of historical peppermint oil sample and fresh oils obtained from three confirmed Bulgarian *M. x piperita* cultivars ("Kliment-63", "Sofia 35-A" and "Zefir"), and newly selected variety from local population, according to ISO 856:2006 criteria [7].

MATERIALS AND METHODS

Essential oil samples: The historical peppermint oil sample was kindly consigned to us by an old family from small town Bania, located near the "Valley of Roses" in Bulgaria. Before 50 years they inherited about 5 L peppermint oil from their parents. The oil was stored in dark glass demijohn sealed with wax and was opened for the first time during January this year. The fresh peppermint oil samples from confirmed cultivars and newly selected variety from local population were purchased from The Institute of Roses and Aromatic Plants (IRAP), Kazanlik, Bulgaria.

Analysis of essential oils

GC analysis: GC/FID analyses were carried out using a GC-14A with split/splitless-injector, FID and C-R6A-Chromatopac integrator (Shimadzu, Japan), a GC-3700 with FID (Varian, Germany) and C-R1B-Chromatopac integrator (Shimadzu). The carrier gas was hydrogen (flow-rate: 1.0 mL/min); injector temperature, 250°C; detector temperature, 320°C. The temperature programme was: 40°C/5 min to 280°C/5 min, with a heating rate of 6°C/min. The columns were 30 m x 0.25 mm bonded DB-5MS fused silica, with a film thickness of 0.50 μm (J & W Scientific, USA) and 30 m x 0.32 mm bonded Stabilwax, with a film thickness of 0.50 μm (Restek, USA). Quantification was achieved using peak area calculations, and compound identification was carried out partly using correlations between retention times [8-12].

GC-MS analysis: For GC/MS measurements a GC-17A with QP5050 (Shimadzu), split/splitless-injector and HP-Compaq data system (GCMSsolution-

software), a GC-HP5890 with HP5970-MSD (Hewlett-Packard, USA) and ChemStation software on a HP-Pentium, a GCQ (Finnigan-Spectronex, Germany-USA) and Gateway-2000-PS75 data system (Siemens-Nixdorf, Germany, GCQ-software) were used. The carrier gas was helium (flow-rate: 1.0 mL/min); injector temperature, 250°C; interface-heating at 300°C, ion-source-heating at 200°C, EI-mode was 70 eV, and the scan-range was 41-450 amu. For other parameters, see description of GC/FID, above. Mass spectra correlations were done using Wiley, NBS, NIST and our own library as well as published data [8-11].

Physical-chemistry analysis: Acid value of the studied peppermint oils was determined according to ISO 1242:1999 procedure [13]. Ester value, before and after acetylation, were determined according to ISO 709:2003 procedure [14]. Determination of relative density at 20 °C was carried out according ISO 279:1998 reference method [15]. Optical rotation was determined according to ISO 592:1998 procedure [16] and miscibility in ethanol was determined according to ISO 875:1999 procedure [17].

Antimicrobial testing procedures

Test microorganisms and preparation of test inoculum: *Bacillus cereus* ATCC 11778*, Citrobacter diversus* (clinical isolate) *Escherichia coli* ATCC 8739, *Staphylococcus aureus* ATCC 6538, *Staphylococcus aureus* (clinical isolate), *Staphylococcus epidermidis* (clinical isolate), *Pseudomonas aeruginosa* ATCC 9627, *P. aeruginosa* (clinical isolate), *Pseudomonas fluorescens* (food spoilage strain, isolated from minced meat), *Salmonella abony* ATCC 6017 and *S. abony* (clinical isolate) were used as test microorganisms. Test strains were obtained from culture collections of The National Bank of Industrial Microorganisms and Cell Cultures (NBIMCC, Bulgaria), Department "Biochemistry and Microbiology", University of Plovdiv, Bulgaria and Clinic of Infectious Diseases, Medical University of Plovdiv, Bulgaria. Bacteria were maintained on Nutritional Agar (NA), National Center of Infectious and Parasitic Diseases (NCIPD), Bulgaria. Overnight bacteria cultures were prepared by inoculating about 2 mL of Mueller-Hinton Broth (MHB, NCIPD, Bulgaria) with 2-3 colonies selected from NA. Broths were incubated at 37^{o}C for 24 h on a rotary shaker 220 rev/min. Inoculums were prepared by diluting overnight cultures by adding sterile MHB to achieve absorbance, corresponding to 0.5 McFarland turbidity standard (1.0/1.5x10^{8} CFU/mL).

Serial broth dilution method: Serial broth dilution method was carried out in accordance with NCCLS recommendations [18-20]. A stock solution to be tested was prepared by diluting rose oil sample in DMSO (Sigma-Aldrich Co.). Stock solution was then added to culture broth to reach final oil concentrations ranging from 3.28% (v/v) to 0.01% (v/v). Serial dilutions were inoculated with 100 μL of bacteria inoculum, prepared as listed above. The samples were then incubated at 37^{o}C for 24 h and the absorbance was read at 680 nm (CAMSPEC, UK). Control samples of inoculated broth without oil and without DMSO and inoculated broth with DMSO, were also incubated under the same conditions. For the broth dilution method the mean absorbance of the duplicate samples was compared with the mean absorbance

of the broth samples containing DMSO without oil to give a measure of the overall reduction in growth. The concentration of DMSO in the broth dilution assay was kept at concentration to ensure that the effect on bacterial and yeast growth was minimal. Minimal inhibitory concentration (MIC) was defined as the lowest concentration which resulted in a reduction of > 90% in the observed absorbance. To determine minimal bactericidal concentration (MBC), 100 μL of each dilution showing no growth was spread on MHA. The inoculated Petri dishes were incubated at 37^{o}C for 24 h. The colony forming units were counted and compared to control dishes. MBC was defined as the lowest concentration that killed > 99.9% of the initial inoculum. Each experiment was performed in duplicate.

RESULTS AND DISCUSSION

A comparison of the historical peppermint oil with fresh peppermint oils from confirmed Bulgarian cultivars and newly selected variety from local population (see Table 1) revealed qualitative similarities but quantitative differences. Historical peppermint oil characterized with the lowest content of menthylacetate (0.15%), but the highest content of 1,8-cineol (6.47%). According to its chemical composition historical peppermint oil differs from all of the peppermint oils used in the present study. Based on the differences in chemical composition and odor descriptions we can assume that probably this historical oil was obtained from an old extinct local population of *M. x piperita* Bulgaro-Michum type.

Table 1. *Comparison of the percentage composition of the major components (over 3 %) of historical and fresh peppermint oils from confirmed Bulgarian cultivars (M. x pipperita) and newly selected variety from local population*

Compound	Historical oil	Sofia 35-A	Kliment 63	Zefir	Newly selected variety from local population
1,8-Cineol	6.47	2.60	5.40	5.10	2,4
Menthone	21.75	25.90	23.10	8.70	11,8
Menthol	40,89	35.20	35.70	46.20	45,7
Menthylacetate	0.15*	8.80	15.10	16.80	3,3
β-Caryophyllene	1.25*	3.60	0.60	1.20	3,9

* Both components were at concentrations up to 3 % in historical oil but were placed in the table for better comparison

To verify this assumption physical-chemistry characteristics of historical peppermint oil were compared with characteristics of confirmed Bulgarian cultivars, newly selected local population, Bulgaro-Michum type and ISO 856:2006 criteria. The results obtained have been presented in Table 2.

Table 2. *Comparison of physical-chemistry characteristics of historical and fresh peppermint oils from confirmed Bulgarian cultivars (M. x pipperita), newly selected local population, Bulgaro-Michum type and ISO 856:2006.*

Character	Historical oil	Confirmed Bulgarian *M. x piperita* cultivars [1]	Newly selected variety from local population	Type Bulgaro-Michum [1]	ISO 856:2006
d_{20}^{20}	0,900	0,900÷0,910	0,900	0,900÷0,910	0,898÷0,918
α_D^{20}	-24,6	-16÷-28	-25,8	-16÷-28	-14÷-30
Acid value	1,48	up to 1,50	0,66	up to 1,50	up to 1,50
Ester value	29,5	14÷42	32,8	14÷34	12÷30
Acetyl value	174,5	135÷193	164,4	147÷193	135÷200
P_{70}	1:5	1:5	1:5	1:5	1:5

As to be seen historical oil sample corresponded exactly to all of the criteria stated in ISO 856:2006 and Bulgaro-Michum type. Regardless of long storage period historical peppermint oil was comparable with all fresh oil samples and literature data which mean that it is very chemically stable.

Antimibacterial activity of peppermint oil samples was studied against four Gram-positive and seven Gram-negative bacteria. The results obtained have been presented in Table 3.

Table 3. *MBC of various peppermint oils from Bulgaria*

Test microorganism	Source	1	2	3	4	5
B.cereus	ATCC 11778	0,1	0,2	0.05	0,2	0.05
C.diversus	Clinical isolate	0	0	0	0	0
E.coli	ATCC 8739	0,2	0.4	0.1	0.4	0.1
Ps.aeruginosa	ATCC 9627	0	0	0	0	0
Ps.aeruginosa	Clinical isolate	0	0	0	0	0
Ps.fluorescens	Raw-smoked pork fillet	0	0	0	0	0
S. abony	ATCC 6017	0,2	0,4	0.1	0,4	0.1
S. abony	Clinical isolate	0,2	0,4	0.1	0,4	0.1
S. aureus	ATCC 6538	0,1	0,2	0.05	0,2	0.05
S.aureus	Clinical isolate	0,1	0,2	0.05	0,2	0.05
S.epidermidis	Clinical isolate	0,1	0,2	0.05	0,2	0.05

1 – Historical oil; 2 – Sofia 35- A; 3 – Newly selected variety; 4 – Kliment 63; 5 – Zefir.

As to be seen all of the studied peppermint oils demonstrated antimicrobial activity. Among the used test microorganism *C. diversus*, both strains of *P. aeruginosa* and *P. fluorescens* were resistant to investigated peppermint oils. Generally, the Gram-positive bacteria seem to be more susceptible to the investigated peppermint oil, comparing to the Gram-negative ones. The results obtained are in accordance with data, showing that Gram-negative bacteria are more resistible to various antimicrobials [23]. For its antimicrobial activity peppermint oil was one of the dominant essential oils used in folk medicine. The peppermint oil obtained from

confirmed cultivar Zefir and newly selected variety demonstrated equal antimicrobial activities and characterized with almost equal total content of 1,8-cineole, menthol and β-caryophyllene, 52.5 % and 52 %, respectively. Both oil samples demonstrated the highest antimicrobial activity, followed by historical peppermint. The lowest antimicrobial activity demonstrated both peppermint oils obtained from confirmed cultivars "Sofia 35-A' and "Kliemnt 63" wich characterized with the lowest total content of 1,8-cineole, menthol and β-caryophyllene, 41.4 % and 41.7 %, respectively. Antimicrobial activity of the studied peppermint oil samples decreased in the order of total content of 1,8-cineole, menthol and β-caryophyllene decreasing. It's well known from the literature that these components possessed high antimicrobial activity, which confirmed the results, obtained.

CONCLUSION

On the basis of the carried out researches and the results obtained we can summarized that the studied historical peppermint oil sample from Bulgaria was chemically and microbially stable for a very long storage period. It differs from currently confirmed Bulgarian *M. x piperita* cultivars and newly selected variety from local population. It corresponds to all of ISO 856:2006 criteria and according to its pleasant and unique odor at a certain extent even exceeds fresh peppermint oils. Probably this old oil was obtained from extinct local population and belongs to Bulgaro-Michun type.

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