Comparative Analysis of Historical Peppermint Oil from Bulgaria and a Commercial Oil of North American Origin

Chemical composition, olfactory analysis and antibacterial activity

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The production of peppermint oil in Bulgaria dates back to the middle of the 18th century, when it was carried out in a very primitive way by water distillation of local varieties of wild mint, pennyroyal and field mint. The derived oil has been used only in traditional medicine.

The first trials to introduce peppermint as a crop culture in Bulgaria were recorded in 1905, but were unsuccessful. The industrial cultivation of peppermint commenced after 1923, and by 1938, Bulgaria ranked third in the world in terms of peppermint oil production. During the Second World War, production dropped substantially, but the country managed to regain its position during the 1950s. Bulgarian peppermint oil has gained world popularity under the name Bulgaro-Mitchum oil (after the Mitchum region, England). Its recognition is especially due to its rich and pleasant odor, sweet-peppery taste and extraordinarily high menthol content, which makes it highly praised on the international market.[1,2]

Currently, the production of peppermint oil in Bulgaria has dropped significantly due to the replacement of peppermint by other crops; it is only being grown to obtain mint leaves for tea.

Although the basic physical and chemical indices and composition of Bulgarian peppermint oil has been the subject of investigation by many researchers, it is most comprehensively summarized by Georgiev and Stoyanova.[1-6]

The quality of peppermint oils of today's market is addressed under the ISO/DIS 856:2003, where strict organoleptic as well as physicochemical data and a chromatographic profile differentiates oils originating from the United States and other regions.[7] The United States is the most important producer of peppermint in the world. In the year 2000, Bulgaria produced about 25 tons of peppermint oil, whereas the United States produced 3,170 tons.[8]

The primary objective of the present study was to compare the chemical composition of the historical peppermint oil with that of a sample of peppermint Mitchum type from the market, and to evaluate the antibacterial properties of the historical peppermint oil sample.

Experimental

Essential oil samples: The historical peppermint oil sample was consigned by a family from Bania, located near the "Valley of Roses" in Bulgaria. They inherited about 5 L peppermint oil from their parents 50 years ago. The oil was stored in dark glass demijohn sealed with wax and was opened for the first time in January 2008. The commercial sample of peppermint oil (802224) was purchased from Kurt Kitzing Co.

GC analysis: GC-FID and GC/MS analyses were carried out simultaneously using a Finnigan ThermoQuest TraceGC with a dual split/splitless injector, a FID detector and a Finnigan Automass quadrupol mass spectrometer. One inlet was connected to a 50 m x 0.25 mm x 1.0 µm SE-54 fused silica column (CS Chromatographie Service, Germany); the other injector was coupled

At a Glance

The chemical compositions of a more than 50-year-old peppermint oil from Bulgaria and a recently produced commercial oil were investigated by GC/FID and GC/MS. Menthol (40.9%; 38.0%), menthone (21.8%; 23.4%), neomenthol (3.3%; 3.2%) and 1,8-cineole (6.5%; 5.3%) were determined as the major compounds, respectively. Using olfactory evaluations, a characteristic odor with strong menthol and menthone notes of peppermint oil was found. The chemical composition of Bulgarian historical peppermint oil corresponded to all criteria stated in ISO 856:2006. In addition, the oil demonstrated antibacterial activity against *Bacillus cereus, Staphylococcus aureus, Staphylococcus epidermidis, Escherichia coli* and *Salmonella abony,* but was inactive against *Citrobacter diversus, Pseudomonas aeruginosa* and *Pseudomonas fluorescens.* The historical peppermint oil was probably obtained from an almost distinct old local population of the Bulgaro-Mitchum type. The commercial peppermint oil used as reference was obtained from an American cultivation of the Mitchum type.

to a 60 m x 0.25 mm x 0.25 µm Carbowax 20M column (J & W Scientific, USA). The two columns were connected at the outlet with a quartz Y connector and the combined effluents of the columns were split simultaneously to the FID and MS detectors with a short (ca. 50 cm) 0.1 mm ID fused silica restrictor column as a GC/MS interface. The carrier gas was helium 5.0 with a constant flow rate of 1.5 mL/min. Injector temperature was 230°C, FID detector temperature 250°C, GC/MS interface heating 250°C, ion source at 150°C, EI mode at 70 eV, scan range 40–300 amu. The following temperature program was used: 46°C for 1 min; 46–100°C with rate of 5°C/min; 100–230°C with rate of 2°C/min; 230°C for 13.2 min. Identification was achieved using the Finnigan XCalibur 1.2 software with mass spectra correlations through the NIST 2005, Adams EO, MassFinder, the authors' own library and by using the retention indices of reference compounds. Quantification was done through peak area calculations of the FID chromatogram.[9-13]

Olfactory evaluation: The samples and reference compounds were olfactorily evaluated by two professional perfumers and one aroma chemist. One droplet of each essential oil sample or a reference compound was placed on a commercial odor strip. Furthermore, due to the unavailability of some reference compounds, evaluations using the GC-split sniffing method were performed. Terms for odor impressions were compared to published data and the aforementioned private database of reference aroma compounds.[14]

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 isolates), *Staphylococcus epidermidis* (clinical isolate), *Pseudomonas aeruginosa* ATCC 9627, *Pseudomonas aeruginosa* (clinical isolate), *Pseudomonas fluorescens* (food spoilage strain, isolated from minced meat), *Salmonella abony* ATCC 6017 and *Salmonella 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°C for 24 hr on a rotary shaker at 220 rev/min. Inoculums were prepared by diluting overnight cultures by adding sterile MHB to achieve absorbance, corresponding to 0.5 McFarland turbidity standard (1.0/1.5 x 10^8 CFU/mL).

Disc diffusion method: Disc diffusion method was carried out as described by Sacchetti et al. in accordance with NCCLS recommendations.[15,16] The Petri dishes (d = 90 mm), containing solidified MHA (Mueller-Hinton Agar) were inoculated by spreading 100 µL of bacteria inoculum. Sterile paper discs Whatman 1 (d = 5 mm, NCIPD, Bulgaria) were soaked with 10 µL of undiluted essential oil and placed on the inoculated surface of Petri dishes. Petri dishes were incubated at 37°C for 24 hr. The growth inhibition zone diameter (IZ, mm) was measured to the nearest millimeter.

Serial broth dilution method: Serial broth dilution method was carried out in accordance with NCCLS recommendations.[17] A stock solution to be tested was prepared by diluting peppermint oil sample in DMSO (Sigma-Aldrich Co.). Stock solution was then added to culture broth to reach final oil concentrations ranging from 3.28–0.01% (v/v). Serial dilutions were inoculated with 100 µL of bacteria inoculum, prepared as listed above. The samples were then incubated at 37°C for 24 hr and the absorbance was read at 680 nm (Camspec, UK). Control samples

Chemical composition and olfactoric evaluation of an historical peppermint oil from Bulgaria and a commercial oil of North American origin **T-1**

Compound	RI	%#		Odor description°
		Historical oil	Commercial oil	
(Z)-3-hexenol	858	0.1	nd	fresh, green, grasslike
hexanol	869	tr	nd	herbal, green, mild woody
α-thujene	927	0.1	tr	herbal, green, weak earthy
α-pinene	936	0.9	0.7	pinelike, warm, herbal
camphene	955	tr	tr	camphoraceous, fresh
sabinene	973	0.4	0.5	fresh, fruity-spicy
β-pinene	977	1.2	1.1	resinous-piney, dry
myrcene	990	0.1	0.2	weak citrus and limelike
3-octanol	992	0.2	0.2	cheesy, minty, herbal
α-phellandrene	1001	tr	tr	citruslike, weak peppery
p-cymene	1025	0.6	nd	citruslike
limonene	1028	1.9	2.6	lemonlike, fresh
1,8-cineole	1034	6.5	5.3	eucalyptuslike, fresh
γ-terpinene	1059	0.5	0.4	citruslike, herbal, terpenic
cis-sabinene hydrate	1068	0.5	0.3	spicy, weak fruity
terpinolene	1088	0.1	0.2	sweet-piney, terpenous
linalool	1095	0.6	0.3	fresh-floral
isopulegol	1148	nd	tr	herbal, slight liquorice note
menthone	1152	21.8	23.4	minty
isomenthone	1159	4.5	3.7	fresh, cool minty
neomenthol	1163	3.3	3.2	minty
menthofuran	1169	1.2	3.7	fresh-minty, herbal, slightly cool
menthol	1173	40.9	38.0	cool minty
terpinen-4-ol	1179	1.8	2.7	fruity, floral, terpenous
isomenthol	1185	0.7	0.5	minty
α-terpineol	1189	0.3	0.4	floral, lilaclike
neoisomenthol	1191	0.2	0.5	minty
pulegol*	1220	0.1	0.1	herbal, minty-camphoraceous
pulegone	1235	1.1	1.9	herbal-minty, camphoraceous
carvone	1243	nd	0.2	herbal, spicy, minty
isopulegone*	1252	nd	tr	herbal, soft minty
piperitone	1255	0.6	0.6	herbaceous, minty
neomenthyl acetate	1277	0.1	0.3	tender minty, discreet fruity
isomenthyl acetate	1294	0.2	0.2	ethereal, fruity, soft minty
menthyl acetate	1296	2.9	4.2	ethereal, nearly odorless
carvacrol	1298	0.1	n.d.	spicy, herbaceous
neoisomenthyl acetate	1325	0.2	0.2	fresh, tingly minty
β-bourbonene	1388	0.3	0.2	green-woody, spicy
β-caryophyllene	1411	1.3	1.7	dry, woody-spicy
(E)-β-farnesene	1455	0.1	0.2	mild, warm-balsamic
germacrene D	1487	0.3	0.9	woody-spicy, weak herbal
mentholactone	1508	0.2	nd	sweet-hay, coumarin, powdery,
bicyclogermacrene	1514	0.2	nd	woody, minty, haylike
caryophyllene oxide	1608	0.1	0.1	warm-spicy, woody
globulol	1618	0.4	n.d.	herbal, balsamic, soft wood
Total		**96.6**	**98.7**	

° in accordance to published data[21, 22]
nd = not detected
tr = trace (> 0.05%)
RI = retention indices on apolar column and correlation with published data [8-12]
# in relative %-peak area using GC/FID with an apolar column (mean value of three analyses)
* correct isomer not identified

Antibacterial activity of historical peppermint oil from Bulgaria **T-2**

Test microorganism	Source	IZ±SD [mm]	MIC [%] (v/v)	MBC [%] (v/v)
Bacillus cereus	ATCC 11778	48±1.52	0.05	0.1
Citrobacter diversus	Clinical isolate	0	0	0
Escherichia coli	ATCC 8739	25±1.0	0.1	0.2
Pseudomonas aeruginosa	ATCC 9627	0	0	0
Pseudomonas aeruginosa	Clinical isolate	0	0	0
Pseudomonas fluorescens	Raw-smoked pork fillet	0	0	0
Salmonella abony	ATCC 6017	10±0.58	0.2	0.2
Salmonella abony	Clinical isolate	11±0.47	0.2	0.2
Staphylococcus aureus	ATCC 6538	48±1.25	0.05	0.1
Staphylococcus aureus	Clinical isolate	49±1.15	0.05	0.1
Staphylococcus epidermidis	Clinical isolate	44±1.70	0.05	0.1

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°C for 24 hr. 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

Using GC/FID and GC/MS, 38 and 34 compounds, representing 98.01% and 98.40% of the total composition of the historical peppermint oil from Bulgaria and the commercial peppermint oil, respectively, were identified. The results obtained and odor characteristics of pure compounds are presented in **T-1**. As can be seen, the main compounds found at a concentration higher than 3% were menthol (40.9%; 38.0%), menthone (21.8%; 23.4%), neomenthol (3.3%; 3.2%) and 1.8-cineole (6.5%; 5.3%). After comparison of the odor attributes for each single constituent published (see **T-1**) it can be stated that the major and some minor components are responsible for the characteristic odor impression of both peppermint oils. The low content of menthofuran in the historical peppermint oil (1.2%) is noteworthy, while the commercial oil contains 3.7%. Menthofurane is a marker for peppermint oils and important for the odor profile.[8] Both peppermint oil samples were olfactory analyzed as fresh, herbaceous minty, cool and dry fruity, slightly sweet, in the background balsamic-dusty.

A comparison of the historical peppermint oil with fresh peppermint oil from the American market shows differences in menthofurane, menthone, 1,8-cineole and menthyl acetate. Based on the differences in chemical composition and odor descriptions one can assume that this historical oil was probably obtained from an old extinct local population of *M. x piperita* Bulgaro-Mitchum type. It must be confirmed, that compared to older data by Lawrence (1989; 1997), the stability of the historical peppermint oil was given.[18,19] Comparisons with physical data published in ISO/DIS 856:2006, as well as the chromatographic profile, show the conformity.[7]

Antibacterial activity of historical peppermint oil sample was investigated against four Gram-positive and seven Gram-negative bacteria. The results obtained are presented in **T-2.** As can be seen among the used test microorganisms, *C. diversus*, both strains of *P. aeruginosa* and *P. fluorescens* were resistant to historical peppermint oil. Generally, the Gram-positive bacteria seem to be more susceptible to the investigated peppermint oil, compared to the Gram-negative ones. The results obtained are in accordance with data, showing that Gram-negative bacteria are more resistible to various antimicrobials as summarized by Dorman and Dean.[20] For its antimicrobial activity peppermint oil was one of the dominant essential oils used in folk medicine. Antimicrobial activity of historical peppermint oil was probably another reason for its stability during the long storage period. The antibacterial activity of the commercial peppermint oil has been tested previously, but partly using different strains of Gram-positive and Gram-negative bacteria as well as the yeast *Candida albicans*.[14]

On the basis of the objectives of this respective study one can summarize that the studied historical peppermint oil sample from Bulgaria was chemically and microbially stable for a very long storage period. It differs somewhat from the commercial oil, but is absolutely in the line of the ISO/DIS 856:2006 criteria and according to its pleasant and unique odor at a certain extent even exceeds fresh peppermint oils. This old oil was likely obtained from an extinct local population and belongs to the special Bulgaro-Mitchum type.

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