Composition and antibacterial effect of mint flavourings in candies and food supplements*

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Abstract

Mint flavourings are widely used in confections, beverages and dairy products. For the first time, mint flavouring composition of mint candies and food supplements (n=45), originating from 16 countries as well as their antibacterial properties, was analyed. The flavourings were isolated by Marcusson`s type micro-apparatus and analysed by GC-MS. The total content of the mint flavouring hydrodistilled extracts was in the range of 0.01-0.9%. Most abundant compounds identified in the extracts were limonene, 1,8-cineole, menthone, menthofuran, isomenthone, menthol and its isomers, menthyl acetate.

The antimicrobial activity of 13 reference substances and 10 selected mint flavouring hydrodistilled extracts was tested on *Escherichia coli* and *Staphylococcus aureus* by broth dilution method. Linalool acetate and (-)-carvone, as most active against both bacteria, had the lowest MIC_{90} values. (+)-Menthyl acetate, (-)-menthyl acetate and limonene showed no antimicrobial activity. Three of the tested extracts had antimicrobial activity against *E. coli* and eight extracts against *S. aureus*. Their summary antimicrobial activity was not always in concordance with the activities of respective reference substances.

Keywords: *Mentha*, *Lamiaceae*, GC-MS, terpenoids, *Escherichia coli*, *Staphylococcus aureus*

Introduction

Essential oils and their components, isolated from the *Mentha* genus have a long history of use as flavour improvers of foods like confectionaries and beverages. *Mentha*-derived flavourings are extensively used as flavour ingredients as reflected in the most recent annual volumes and per capita intakes [1]. Mint flavouring, generally containing some *Mentha* oil or mint isolates, is used to flavour confections such as hard and soft candies, breath mints including the popular extra strong mint tablets, after-dinner mints, chocolates and chewing gum. Besides confections, mint oils and their corresponding isolates are used in both nonalcoholic and alcoholic beverages. They can also be found as flavourings in frozen dairy products such as ice creams and ice lollies, baked goods, icings, toppings, cake frostings, puddings, sauces and chutneys. In addition, spearmint oil is used in the preparation of mint jelly [2]. Mints have also shown to be efficient food constituents, for example in improving the shelf life and safety or organoleptic parameters of flesh foods and dairy products [3-7].

The peppermint flavour is primarily based on menthol, menthone and their isomers, menthyl esters and piperitone [8, 9]. (-)-Menthol not only provides the classic minty note, but also activates the cold-sensitive receptors in the oral cavity to produce a cooling effect [10, 11]. It is also an interesting molecule with a sensation of bitterness. Thus, it stimulates both aroma and taste receptors [11]. Menthofuran adds to peppermint flavour a distinctive mustiness, described as sweet hay-like minty odor, sometimes referred to as lactone-odor [8, 9]. The spearmint flavour is primarily based on R-(-)-carvone, dihydrocarvone, carveol, dihydrocarveol, carvyl- and dihydrocarvyl esters and to a lesser extent on limonene. Carvone is of particular interest because it exists in two enantiomeric forms with different aroma properties. R-(-)-carvone smells like spearmint and is extracted from *Mentha spicata* L. The S-(+)- enantiomer resembles caraway, accounting for 50% of the essential oil in caraway seeds [12]. The concept of a pennyroyal flavour is based on pulegone and its isomers and alcohols. Bergamot or orange mint flavour is based on linalool and linalool acetate [8, 9].

The composition and antimicrobial activity of various mint essential oils is well studied. Mint oils have shown to exhibit growth inhibition activity against a wide range of Gram-negative and Gram-positive bacteria, supporting their medicinal use [13-17]. The peppermint flavouring, isolated from chewing gum and sugar candy, originated from Estonia, has shortly been previously described (Orav and Kann, 2001)[18].

The aim of present study was to investigate for the first time the mint flavouring composition of various mint candies and food supplements (n=45) as well as their antibacterial properties against Gram-negative *Escherichia coli* and Gram-positive *Staphylococcus aureus* to assess their potential as health supporting agents.

Results and discussion

The yield of the mint flavouring hydrodistilled extracts ranged 0.01-0.9% (Table **1S**, Table **2S**, Supporting information). The lowest extract amount was isolated from the chocolate No 40 and respectively the highest from the pastille No 15. In general, the extract content was higher in mint flavoured sugar candies, pastilles and food supplements than in chocolates. From the total of 28 mint flavoured sugar candy, pastille and food supplement samples, 13 contained the extracts more than 0.1%. Among the chocolates, content of >0.1% was isolated only from one sample (No 43).

The mint flavouring hydrodistilled extracts were found to be similar in composition to essential oils of *Mentha* plants [15, 19, 20-23]. For the identification purpose, 78 compounds were identified by GC-MS in the extracts isolated from sugar candies, pastilles and food supplements (Table **1** and in more detailed Table **1S**, Table **2S**, Supporting information). In the extracts isolated from chocolate, 86 compounds were determined. The identified compounds included mainly monoterpenoic and sesquiterpenoic hydrocarbons, terpenoic alcohols, ketones, aldehydes, ethers and esters. Most abundant compounds were limonene, 1,8-cineole, menthone, menthofuran, isomenthone, menthol and its isomers, menthyl acetate (Table **1**). The greatest variation was found in the percentage content of limonene (0.07%-66.40%; Table **1**). The lowest limonene content (0.07%) was found in the extract of food supplement No 28. The highest content was detected in the extracts of pastille No 12 (66.40%) and sugar candy No 2 (57.36%). High limonene content was also in the extracts of pastille No 16 (11.34%) and chocolates No 44 (9.27%), No 30 (8.18%) and No 35 (8.14%). Pastilles No 12 and No 16 as well as sugar candy No 2 were produced by the same Finnish company and could therefore have contained the same mint flavouring rich in limonene. Addition of limonene to a reduced-fat chocolate has been found to reduce chocolate viscosity, therefore facilitating the production of chocolate and make the chocolate softer [24, 25]. Ray et al. [26] observed that with the addition of limonene to cocoa butter the isothermal crystallization of chocolate starts earlier at temperatures from 17 to 20 °C. Similar effect has been shown at 17 °C by Rigolle et al. [27]. Limonene enhances the transformation to more stable cocoa butter polymorphic form β_V [26, 27]. The latter is the desired form for chocolate manufacture as the melting temperature of this form is high enough (34–36 °C) to maintain the chocolates in solid form at room temperature and low enough for the chocolate to melt in the mouth [28]. Form β_V also provides the desired chocolate snap, gloss and shelf life [29]. According to Rigolle et al., after adding limonene, form β_V was formed after 50 min [27]. In contrast, in pure cocoa butter this transition occurs in a time span of few days [30]. However, as the effects of limonene are versatile as well as concentration and temperature dependent, the chocolate manufacturers have to search for a optimum between physical and organoleptic properties. Thus, the concentration on limonene may vary greatly between chocolates, not to mention other types of sweets. Menthone and menthol were major components in most of the extracts (Table **1**). Highest menthone content (25.90%) was found in the extract of chocolate No 45, lowest (1.25%) in the extract of pastille No 19. Chocolate extracts were richer in menthone than extracts of candies or food supplements. Highest and lowest menthol content were detected both in the extracts distilled from pastilles, respectively 83.01% (No 28) and 17.48% (No 12). Relatively high content of menthofuran (7.22%) was found in the extract isolated from the sugar candy No 9. Highest menthyl acetate content (6.15%) was detected in the extract isolated from chocolate No 45. Highest 1,8-cineole content (5.44%) was in the chocolate No 39 extract. The flavouring content in one mint flavoured sugar candy and chewing gum product originating from Estonia was earlier analysed by Orav and Kann [18]. Menthole, menthone and menthyl acetate were, according to them, the main compounds found.

The preliminary antimicrobial susceptibility tests were performed with four candy flavouring extracts and with all the reference substances except mixture of menthone isomers, (-)-carvone, linalool and linalool acetate (Table **3S**, Supporting information). All the flavouring extracts showed to a certain extent bactericidal or bacteriostatic activity on the test bacteria. Among the reference substances, limonene was the only compound not having antimicrobial activity. The preliminary susceptibility experiments encouraged the further studies with clinically important bacteria *Escherichia coli* and *Staphylococcus aureus*.

The reference substances were tested in the concentration range of 0.0625-8.0 mg/mL (n = 3-5) against potentially pathogenic bacteria *E. coli* and *S. aureus* (Table **2**). Reference substances studied were those that constitute the majority of the mint flavouring hydrodistilled extracts or commonly used mint flavourings. *E. coli* was inhibited by 1,8-cineole and menthofuran with MIC_{90} values of 5.0 mg/mL and 7.0 mg/mL, respectively. Racemic menthone and (-)-menthone demonstrated antibacterial activity against *E. coli* with MIC_{90} values of 7.0 mg/mL and 4.0 mg/mL, respectively. Linalool, linalool acetate and (-)-carvone showed higher antibacterial activity with equal MIC_{90} values of 1.0 mg/mL. Racemic menthol, (+)-menthol and (-)-menthol were found to be inactive against *E. coli*. For most of the reference substances, MIC_{90} values against *E. coli* were also minimal bactericidal concentrations (MBC).

Racemic menthol and menthol enantiomers inhibited *S. aureus* by ≥90% at concentrations of 1.5 mg/mL and 1.0 mg/mL, respectively. MIC_{90} values for racemic menthone and (-)-menthone were 2.5 mg/mL. Menthofuran and linalool showed antibacterial activity on *S. aureus* at concentration of 2.0 mg/mL. For linalool acetate and (-)-carvone the MIC_{90} value was equally 1.0 mg/mL.

(+)-Menthyl acetate, (-)-menthyl acetate and limonene were found to be inactive towards both of the bacteria. These compounds have the highest partition coefficients logP, respectively 3.60 (for acetates) [31] and 3.40 [32] between *n*-octanol and water phases. A hypothesis can be raised that these substances as well as hexane (4.15) are too hydrophobic to be absorbed by bacterial cells by passive diffusion through cellular membranes, and hence, to have the possibility for evoking of antibacterial effect. LogP values of all other studied and active reference substances are between 2.23 (carvone) and 3.56 (menthofuran). LogP value of menthol is 3.20 [33].

The results of the antimicrobial activity of menthol and menthone on *E. coli* and *S. aureus* are consistent with the results of previous studies. Menthol and menthone show higher antimicrobial activity on Gram-positive *S. aureus* than on Gram-negative *E. coli* [13, 15, 16]. However, the MIC values determined by Hussain et al. [15] and Reddy et al. [16] are much lower. Also, İşcan et al. reported (-)-menthol to inhibit the growth of *E. coli* at 1.25 mg/mL [13]. The antibacterial activity of carvone has been studied by Hussain et al. [15]. Contrary to the results of the present study, the MIC values were reported to be higher for *E. coli* [15]. Whereas in the study by Moro et al. [17] only (-)-carvone presented antibacterial activity against *S. aureus* with the MIC value of 2.5 mg/mL.

The antimicrobial activity of 10 mint flavouring hydrodistilled extracts was tested at the concentrations of 1.0-4.0 mg/mL (n = 1-3; Table **2**). Antimicrobial activity of extracts with a diverse qualitative chemical composition was studied. They represented the most commonly consumed classes of mint flavoured candies – pastilles, sugar candies and chocolates. Also, an extract isolated from a food supplement, was tested.

Three extracts inhibited the growth of *E. coli*. The MIC_{90} value for the extract No 10 was 2.0 mg/mL and for the extracts Nos 25 and 31, 4.0 mg/mL. The results show rather high variation in activity if comparison is based on the composition of the hydrostilled extracts. The active extract No 10 was found to be diverse in compounds, whereas extract No 9 had rather similar composition but was not active. The active sample No 25 had high content of menthone and its isomers. This stands also for flavouring extract No 31.

Eight mint flavouring extracts were antibacterial against *S. aureus*. The MIC_{90} values for most of the extracts were 1.0 mg/mL. The MIC_{90} for the extract isolated from the sugar candy No 26 was 1.5 mg/mL. The extract isolated from chocolate No 36 inhibited the growth of *S. aureus* at the concentration of 4.0 mg/mL. Mint flavouring extracts No 11 and No 31 showed no activity towards

S. aureus. Surprisingly, extract No 11 showed no activity although its main constituent was menthol (63.80%). However, the hydrodistilled extract was less diverse in compounds. Also, it contained lower amount of menthone compared to other tested extracts. The other non-active extract (No 31) was rich in antimicrobially active compounds such as menthol and menthone. Thus, the other ingredients apart from essential oil components may influence the activity.

When taking into consideration the mint flavoring hydrodistilled extract yield in the antimicrobially active extracts, the amount of the sweets to be consumed to obtain the *in vitro* MIC_{90} value would be in the range of 0.3 to 6.7 g. As a comparison, from the studied sweets, the average weight of a single sugar candy, pastille and food supplement was respectively 3.5, 1.5 and 0.6 g. Regarding chocolates, one small bar weighed 35 g and one row of a 100 g bar about 20 g. Thus, the amounts of the sweets to be eaten are moderate. The no-observed-adverse effect (NOAEL) level for peppermint oil is 200 mg/kg bw/day [1, 34]. Also, in a short-term oral toxicity study with rats NOAEL was concluded for consumption of a 28-g box of mint lozenges containing 0.4% peppermint oil [35]. Therefore, the moderate consumption of mint sweets can be considered safe.

Confectionary products or ingredients have been reported to be contaminated with foodborne pathogens *Salmonella* Typhimurium [36], *Listeria monocytogenes* [37] and verocytotoxin producing *Escherichia coli* O157:H7 [38]. In a study by Karagözlü et al [39], mint oil had antimicrobial effect against *S.* Typhimurum, *E. coli* O157:H7. and *L. monocytogenes* [40]. Therefore, the mint flavoring hydrodistilled extracts may also have a role in the preservation of the sweets.

The present study is the first to determine the mint flavouring composition used in various mint flavoured products with different matrix components. Many of the selected mint flavouring hydrodistilled extracts showed antimicrobial activity against potentially pathogenic *E. coli* and *S. aureus*. Some antimicrobial activity results were comparable with the extract composition and antimicrobial activity of the tested reference substances.

In conclusion, mint flavouring in candies and food supplements was proved to have antimicrobial effect against *E. coli* and *S. aureus* and therefore may also prevent related bacterial infections and diminish food contamination.

Materials and methods

Mint flavoured candies and food supplements

Mint flavoured candies and food supplements (n=45, Table **3**) were purchased from food markets, candy shops or retail pharmacies in Estonia and in Finland. The samples were collected during October 2012-April 2013 and originated from 16 countries. Samples Nos 11, 20 and 28 were marketed as food supplements. The candies and food supplements were stored at room temperature, protected from moisture and direct sunlight.

Chemicals and instruments

Organic solvents and reagents used were of analytical grade. Menthol racemic (purity ≥98.0% by GC), (+)-menthol (99.0%), (-)-menthol (99.0%), (-)-menthone (90.0%) were purchased from Sigma-Aldrich Chemie GmbH. 1,8-cineole (99.0%), menthone mixture of isomers (98.0%), menthofuran (95.0%), (+)-menthyl acetate (99.0%) and (-)-menthyl acetate (98.0%) were obtained from Alfa Aesar GmbH and Co KG. Limonene (95.0%), linalool (95.0%) and linalool acetate (95.0%) were from

Haarmann & Reimer GmbH. (-)-Carvone (≥99.0%) was obtained from Fluka Sigma-Aldrich Chemie GmbH. LiChrosolv *n*-hexane and SeccoSolv dimethyl sulfoxide (DMSO) were from Merck Millipore. Iso-Sensitest Agar and DST-Agar were from Oxoid Ltd. Plate Count Agar, Mueller Hinton II agar (MHA) and Mueller Hinton II broth (MHB) were obtained from Becton Dickinson. Ciprofloxacin hydrochloride was purchased from ICN Biomedicals Inc. Cuplaton anti-foam agent was produced by Orion Pharma. Nunclon Delta Surface 96-well microplates were obtained from Thermo Scientific. Petri dishes were produced by Heger Plastics. Multiskan GO microplate spectrophotometer was made by Thermo Fisher Scientific.

Isolation of mint flavouring

Hydrodistillation was used for mint flavouring isolation with a Marcusson's type micro-apparatus with *n*-hexane as a trap (300 µL) [41]. For the isolation procedure, 50 g of mint flavoured candy or food supplement and water 100 mL or 120 mL (for chocolates) was used. The isolation was carried out in the presence of 150 mg of anti-foam agent. Distillation time was 2 h. The amount of the isolated mint flavouring solvent extract from the distillation water (referred to as mint flavouring hydrodistilled extract) was determined gravimetrically after evaporating *n*-hexane in nitrogen flow.

Gas chromatography

The mint flavouring hydrodistilled extracts were analysed using an Agilent 7890A chromatograph, combined with Agilent 5975C TAD Mass Selective Detector (GC/MSD) with the Triple-Axis High Energy Diode (HED) Electron Multiplier (EM) detector on DB-5 capillary column [poly (5%-diphenyl-95%-dimethylsiloxane) (30 m × 0.25 mm, film thickness 0.25 µm)]. The oven temperature was programmed from 50 °C to 240 °C at 2 °C/min with the injector temperature of 300 °C. The carrier gas was helium, with a split ratio of 1:30 and flow rate of 1.3 mL/min. The identification of the oil components was accomplished by spectra using commercial spectral libraries NIST 11, Scientific Instrument Services Inc. and FFNS 2 Wiley Library. Identification of compounds was confirmed by retention indices (RI) of reference standards and library data. The composition of the oils was calculated as the percentage from peak areas using normalization method without correction factors. The relative standard deviation of percentages of oil components in three repeated GC analyses of a single oil sample did not exceed 5%.

Antimicrobial susceptibility testing

Preliminary susceptibility testing by agar diffusion method

Preliminary antimicrobial susceptibility testing was performed by modified agar well-diffusion method [42, 43]. Bacterial strains were obtained from the reference strains collections of the Estonian Veterinary and Food Laboratory. Gram-negative bacteria were represented by *Yersinia ruckeri* (NCIM 13282). Gram-positive bacteria were *Bacillus cereus* (ATCC 11778), *Bacillus subtilis* (BGA), *Bacillus pumilus* (CN607) and *Micrococcus luteus* (ATCC 9341). Cultures from the solid medium were subcultivated into liquid media. Incubated bacterial suspension was mixed with sterilized Iso-Sensitest Agar (*Bacillus cereus, Micrococcus luteus*), Plate-count agar (*Bacillus subtilis, Yersinia ruckeri*) or DST-agar (*Bacillus pumilus*) to obtain final density of 10^6 colony-forming units (CFU)/mL and then poured into Petri dishes for the solidification at the room temperature. Wells

were made into agar gel (5 mm in diameter) and filled with 30 µL of extract or reference substance dissolved in *n*-hexane (10% v/v). After 24 h incubation at 30 °C for *Bacillus cereus* and *Yersinia ruckeri* or at 37 °C for *Micrococcus luteus*, *Bacillus subtilis* and *Bacillus pumilus*, the radius of the inhibition zone was measured. Commercial peppermint oil (*Oleum Mentha × piperita* L., Solnetšnogorsk, Russian Federation) was used as a positive and *n*-hexane as a negative control.

Broth microdilution method

Antimicrobial assays were performed by the broth microdilution method following the guidelines of the Clinical and Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST).
Clinical control strains of *Escherichia coli* (Gram-negative, ATCC 25922) and *Staphylococcus aureus* (Gram-positive, ATCC 25923) were obtained from Microbiologics Inc. (St. Cloud, MN, USA) and used for the antimicrobial screening. Bacterial strains were grown on MHA. Media were prepared in MilliQ water, according to the manufacturer's instructions, and autoclaved at 121 °C for 15 min. Prior to the assay, bacterial suspensions were prepared in MHB from fresh slant cultures and incubated at 37 °C for 16-20 h at 100 rpm.
Bacterial suspensions were prepared as described above and diluted with MHB to obtain a final inoculum of 5×10^5 colony-forming units (CFU)/mL in the assay (determined on the basis of absorbance values at 620 nm previously calibrated against plate counts). Assays were carried out by the tube dilution method combined with absorbance measurement at 620 nm in 96-well microtiter plates.
245 µL of MHB and 250 µL of bacteria suspension were added to the tube, followed by test samples dissolved in 5 µL of DMSO. Ciprofloxacin was used as a positive control. The tubes were incubated at 37 °C for 24 h at 100 rpm and observed for turbidity at 4, 8 and 24 h. Absorbance was measured at 620 nm with a Multiskan GO microplate spectrophotometer by transferring the incubated samples to 96-well microtiter plates. The antimicrobial activity of the samples was calculated from the absorbance values by comparing to untreated controls and expressed as the percentage inhibition of growth. Compounds were assayed at final concentrations of 0.0625-8.0 mg/mL (n = 1-5). The MIC_{90} was defined as the lowest concentrations that showed ≥90% inhibition of growth.

Determination of minimal bactericidal concentration (MBC)

Samples with ≥90% inhibition of growth were further tested for minimal bactericidal concentration (MBC). 50 µL samples from the MIC assay were plated on fresh MHA plates and incubated for 24 h at 37 °C. Concentration at which 99.9% of the initial bacterial inoculum had been killed was considered as MBC.

Supporting information

Relative total content (%; w/w), percentage composition (%) of mint flavouring hydrodistilled extracts as well as retention indices of compounds are available in Supporting information (Table 1S, sugar candies, pastilles and food supplements; Table 2S, chocolates).
Inhibition zones (mm) of reference substances and mint flavouring hydrodistilled extracts (10%; v/v in n-hexane) determined during preliminary susceptibility testing by agar diffusion method are available in Supporting information, Table 3S.

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Conflict of Interest

The authors declare no conflict of interest.

References

1 Cohen SM, Eisenbrand G, Fukushima S, Gooderham NJ, Guengerich FP, Hecht SS, Rietjens IMCM, Bastaki M, Davidsen JM, Harman CL, McGowen MM, Taylor SV. FEMA GRAS assessment of natural flavor complexes: Mint, buchu, dill and caraway derived flavoring ingredients. Food Chem Toxicol 2020; 135:110870

2 Hayes JR, Stavanja MS, Lawrence BM. Mint. The genus Mentha. Medical and Aromatic Plants – Industrial Profiles. London, New York: CRC Press, Taylor & Francis Group; 2006: 422-483

3 Kanatt SR, Chander R, Sharma A. Chitosan and mint mixture: A new preservative for meat and meat products. Food Chem 2008; 107: 845-852

4 Najeeb AP, Mandal PK, Pal UK. Efficacy of leaves (drumstick, mint and curry leaves) powder as natural preservatives in restructured chicken block. Int J Food Sc Tech 2015; *52*: 3129-3133

5 Shahdadi F, Mirzaie H, Kashaninejad M, Khomeiri M, Ziaiifar AM, Akbarian A. Effects of various essential oils on chemical and sensory characteristics and activity of probiotic bacteria in drinking yoghurt. Agric Communic 2015; 3: 16-21

6 Verma A, Ansari R, Broadway AA. Preparation of herbal ice cream by using aloe vera with mint flavor. J Pharmacogn Phytochem 2018; 7:391-394

7 Ibrahim OAE, Mohamed AG, Bahgaat WK. Natural peppermint-flavored cheese. Acta Sci Pol Technol Aliment 2019; 18:75-85

8 Arctander S. Perfume and Flavor Chemicals (Aroma Chemicals), 2nd edition. S. Arctander, Montclair, New Jersey, 1969

9 Tucker AO. Mint. The genus *Mentha*. Medical and Aromatic Plants – Industrial Profiles. London, New York: CRC Press, Taylor & Francis Group; 2006: 519-522

10 Eccles R. Menthol and related cooling compounds. J Pharm Pharmacol, 1994; 46: 618-630

11 Salles C. Odour-taste interactions in flavour perception. In: Etievant P, Voileey A, editors. Flavour in Food. Woodhead Publishing Series in Food Science, Technology and Nutrition. 2006: 345-363

12 Parker JK. Introduction to aroma compounds in food. In: Parker JK, Elmore JS, L. Metheven L, editors. Flavour Development, Analysis and Perception in Food Beverages Woodhead Publishing Series in Food Science, Technology and Nutrition; 2015: 3-30

13 İşcan G, Kįrįmer N, Kürkcüoğlu M, Başer KHC, Demįrcį F. Antimicrobial screening of *Mentha piperita* essential oils. J Agric Food Chem 2002; 50: 3943-3946

14 Mkaddem M, Bouajila J, Monia E, Lebrihi A, Mathieu F, Romdhane M. Chemical composition and antimicrobial and antioxidant activities of *Mentha* (*longifolia* L. and *viridis*) essential oils. J Food Sci, 2009; 74: M358-M363

[15] Hussain AI, Anwar F, Nigam SP, Ashraf M, Gilani AH. Seasonal variation in content, chemical composition and antimicrobial and cytotoxic activities of essential oils from four *Mentha* species. J Sci Food Agric 2010; 90: 1827-1836

[16] Reddy DN, Al-Rajab AJ, Sharma M, Mary Moses M, Reddy GR, Albratty M. Chemical constituents, in vitro antibacterial and antifungal activity of *Mentha* × *piperita* L. (peppermint) essential oils. J King Saud Univ Sci 2019; 31:528-533

[17] Moro IJ, Gondo GDGA, Pierri EG, Pietro RCLR, Soares CP, Sousa de DP, Santos dos AG. Evaluation of antimicrobial, cytotoxic and chemopreventive activities of carvone and its derivatives. Braz J Pharm 2017; 53, e00076. Available from: http://www.scielo.br/scielo.php?script=sci_arttext&pid=S198482502017000400602&lng=en&nrm=iso. Accessed January 13, 2020

[18] Orav A, Kann J. Determination of peppermint and orange aroma compounds in food and beverages. Proc Estonian Acad Sci Chem 2001; 50: 217-225

[19] Mimica-Dukić N, Božin B, Soković M, Mihajlović B, Matavulj M. Antimicrobial and antioxidant activities of three *Mentha* species essential oils. Planta Med 2003; 69: 413-419

[20] Orav A, Kapp K, Raal A. Chemosystematic markers for the essential oils of *Mentha* spp. leaves cultivated or growing naturally in Estonia. Proc Estonian Acad Sci Chem 2012; 62: 175-186

[21] Kapp K, Hakala E, Orav A, Pohjala L, Vuorela P, Püssa T, Vuorela H, Raal A. Commercial peppermint (*Mentha* × *piperita* L.) teas: Antichlamydial effect and polyphenolic composition. Food Res Int 2013; *53*: 758-766

[22] Sousa Barros de A, Morais de SM, Ferreira PATF, Viera ÍGP, Craveiro AA, Santos Fontenelle dos RO, Menezes de JESA, Silva da FWF, Sousa de HA. Chemical composition and functional properties of essential oils from *Mentha* species. Ind Crops Prod 2015; 76: 557-564

[23] Shabani B, Rezaei R, Charehgani H, Salehi A. Study on antibacterial effect of essential oils of six plant species against Pseudomonas syringae pv. syringae Van Hall 1902 and Pseudomonas fluorescens Migula 1894. J Plant Physiol 2019; 101:671-675

[24] Beckett S. Preparation of chocolate with limonene to reduce fat content. U.S. Patent 6200625; 2001

[25] Do T, Vieira J, Hargreaves J, Wolf B, Mitchell J. Impact of limonene on the physical properties of reduced fat chocolate. J Am Oil Chem Soc 2008; 85:911-920

[26] Ray J, MacNaughtan W, Chong P, Vieira J, Wolf B. The effect of limonene on the crystallization of cocoa butter. J Am Oil Chem Soc 2012; 89:437-445

[27] Rigolle A, Goderis B, Van Den Abeele K, Foubert I. Isothermal crystallization behavior of cocoa butter at 17 and 20 °C with and without limonene. J Agric Food Chem 2016; 64:3405-3416

[28] Stapley AGF, Tewkesbury H, Fryer PJ. The effects of shear and temperature history on the crystallization of chocolate. J Am Oil Chem Soc 1999; 76:677-685

[29] Beckett ST. Science of chocolate, 2nd edition. Cambridge: The Royal Society of Chemistry; 2008

[30] van Malssen K, van Langevelde A, Peschar R, Schenk H. Phase behavior and extended phase scheme of static cocoa butter investigated with real-time X-ray powder diffraction. J Am Oil Chem Soc 1999; 76:669-676

[31] https://pubchem.ncbi.nlm.nih.gov/compound/Menthyl-acetate. Accessed January 13, 2020

[32] https://pubchem.ncbi.nlm.nih.gov/compound/Limonene#section=Synonyms. Accessed January 13, 2020

[33] Prasanthi D, Lakschmi PK. Terpenes: Effect of lipophilicity in enhancing transdermal delivery of alfuzosin hydrochlorideJ Adv Pharm Tech Res 2012; 3: 216-223

[34] Serota DG. 28-Day oral toxicity study in rats: compound B100. Unpublished report. Hazelton Laboratories America Inc., Vienna, VA, USA

[35] Thorup I, Würtzen G, Carstensen J, Olsen P. Short term toxicity study in rats dosed with peppermint oil. Toxicol Lett 1983; 19: 211-215

[36] Kapperud G, Gustavsen S, Hellesnes I, Hansen AH, Lassen J, Hirn J, Jahkola M, Montenegro MA, Helmuth R. Outbreak of *Salmonella typhimurium* infection traced to contaminated chocolate and caused by strain lacking the 60-megadalton virulence plasmid. J Clin Microbiol 1990; 28: 2597-2601

[37] Pearson LJ, Marth EH. Behavior of Listeria monocytogenes in the presence of cocoa, carrageenan, and sugar in a milk medium incubated with and without agitation. J Food Prot 1990; 53: 30-37

[38] Baylis CL, MacPhee S, Robinson AJ, Griffiths R, Lilley K, Betts RP. Survival of Escherichia coli O157:H7, O111:H- and O26:H11 in artificially contaminated chocolate and confectionary products. Int J Food Microbiol 2004; 96: 35-48

[39] Karagözlü N, Ergönül B, Özcan D. Determination of antimicrobial effect of mint and basil essential oils on survival of *E. coli* O157:H7 and *S. typhimurium* in fresh-cut lettuce and purslane. Food Control 2011; 22: 1851-1855

[40] Evrendilek GA, Balasubramaniam VM. Inactivation of *Listeria monocytogenes* and *Listeria innocua* in yogurt drink applying combination of high pressure processing and mint essential oils. Food Control 2011; 22: 1435-1441

[41] Bicchi C, D`amato A, Nano GM, Frattini C. Improved method for the analysis of small amounts of essential oils by microdistillation followed by capillary gas chromatography. J Chrom A 1983; 279: 409-416

[42] Pikkemaat MG, Oostra-van Dijk S, Schouten J, Rapallini M, Egmond HJ van. A new microbial screening method for the detection of antimicrobial residues in slaughter animals: The Nouws antibiotic test (NAT-screening). Food Control 2008; 19: 781-789

[43] Raudsepp P, Anton D, Roasto M, Meremäe K, Pedastsaar P, Mäesaar M, Raal A, Laikoja K, Püssa T. The antioxidative and antimicrobial properties of the blue honeysuckle (*Lonicera caerulea* L.), Siberian rhubarb (*Rheum rhaponticum* L.) and some other plants, compared to ascorbic acid and sodium nitrite. Food Control 2013; 31: 129-135

Table 1 Main compounds and their percentage composition (%) identified in the mint flavouring hydrodistilled extracts.

Compound	Sugar candies	Pastilles	Food supplements	Chocolates
α-Pinene	nd-0.5	tr-2.05	nd-tr	nd-0.43
β-Pinene	nd-1.48	tr-2.19	nd-tr	tr-0.66
Limonene	0.21-57.36	0.30-66.40	0.07-0.77	0.32-9.27
1,8-Cineole	nd-3.11	nd-3.16	0.30-1.44	nd-5.44
Isopulegol	0.11-1.38	0.06-1.30	0.19-1.90	tr-1.89
Menthone	6.90-20.78	1.25-18.76	3.49-13.70	12.77-25.90
Menthofuran	nd-7.22	nd-5.48	nd	nd-9.93*
Isomenthone	nd-9.94	nd-8.13	1.33-6.43	3.40-9.93 *
Neomenthol	0.86-5.54	nd-7.47	1.96-6.39	nd-5.25
Menthol	20.35-75.58	17.48-83.01	55.33-83.01	30.64-62.63
Isomenthol	nd-0.88	nd-5.69	0.05-1.02	nd-17.75
α-Terpineol	nd-2.98	nd-1.06	0.26-1.98	0.07-1.24
Pulegone	0.47-1.64	nd-1.87	0.45-1.51	0.53-2.28
Piperitone	0.19-1.17	0.05-1.31	0.28-0.67	0.11-0.86
Menthyl acetate	1.74-5.70	0.69-5.39	2.07-5.36	1.98-6.15
(E)-β-Caryophyllene	0.20-1.96	nd-0.95	0.08-2.04	0.22-1.58

tr - traces, <0.05%

nd - not detected

* - Menthofuran and isomenthone were occasionally partially separated and could be detected by MS but not quantified.

Table 2 MIC_{90} values (mg/mL) of reference substances and mint flavouring hydrodistilled extracts.

Sample	*Escherichia coli*	*Staphylococcus aureus*
Menthol, racemic	NA	1.5
(+)-Menthol	NA	1.0
(-)-Menthol	NA	1.0
Menthone, racemic	7.0 *	2.5
(-)-Menthone	4.0 *	2.5
1,8-Cineole	5.0 *	NA
Menthofuran	7.0 *	2.0
(+)-Menthyl acetate	NA	NA
(-)-Menthyl acetate	NA	NA
Limonene	NA	NA
Linalool	1.0 *	2.0
Linalool acetate	1.0 *	1.0
(-)-Carvone	1.0	1.0
No 9. sugar candy	NA	1.0
No 10. pastille	2.0	1.0
No 11. food supplement	NA	NA
No 18. pastille	NA	1.0
No 25. pastille	4.0	1.0
No 26. sugar candy	NA	1.5
No 27. sugar candy	NA	1.0
No 31. chocolate	4.0	NA
No 36. chocolate	not tested **	4.0
No 39. chocolate	NA	1.0

NA - not active

* - minimal bactericidal concentration

** - not tested due to limited sample amount

Table 3 Mint flavoured candies and food supplements studied.

No	Country of origin	Product	Flavouring indicated on package	Marketing place
1	Finland	Pastille	Natural flavourers	Food market
2	Finland	Sugar candy	Flavouring	Food market
3	Sweden	Sugar candy	Natural peppermint oil	Food market
4	Italy	Sugar candy	Essential oil of Piedmontese peppermint, flavours	Candy shop
5	Italy	Pastille	Essential oil of Piedmontese peppermint, flavours	Candy shop
6	Finland	Pastille	-	Pharmacy
7	Finland	Pastille	Natural flavouring (peppermint oil)	Food market
8	Finland	Pastille	Natural flavourings (peppermint oil, menthol)	Food market
9	Belgium	Sugar candy	Natural mint oil	Food market
10	Finland	Pastille	Peppermint oil, menthol, anise oil	Food market
11	Finland	Food supplement	Natural peppermint aroma	Pharmacy
12	Finland	Pastille	Flavourings	Food market
13	Finland	Pastille	Aroma (natural peppermint)	Candy shop
14	Finland	Sugar candy	Peppermint oil	Candy shop
15	Finland	Pastille	Aroma (menthol, mint)	Food market
16	Finland	Pastille	Aroma (peppermint oil)	Food market
17	Finland	Pastille	Peppermint oil	Food market
18	Denmark	Pastille	Aroma, peppermint oil	Food market
19	Finland	Pastille	Aroma	Food market
20	Russian Federation	Food supplement	Peppermint oil	Pharmacy
21	Moldova	Sugar candy	Flavours (mint oil, barberry extract)	Food market
22	Lithuania	Sugar candy	Mint flavour, menthol	Food market
23	Ukraine	Sugar candy	Food aromatizing identical to natural mint	Food market
24	Lithuania	Sugar candy	Flavour and aroma “Mint”	Food market
25	England	Pastille	Flavour and aroma (peppermint oil)	Food market
26	Estonia	Sugar candy	Flavour	Food market
27	Lithuania	Sugar candy	Mint oil	Food market

28	Finland	Food supplement	Peppermint oil, menthol	Pharmacy
29	Finland	Chocolate	Peppermint oil	Food market
30	Finland	Chocolate	Flavouring (mint oil, vanillin)	Food market
31	Switzerland	Chocolate	Peppermint sugar granules (incl. peppermint leave, flavourings)	Food market
32	Bolivia	Chocolate	Peppermint oil	Food market
33	Germany	Chocolate	Natural peppermint oil	Candy shop
34	Finland	Chocolate	Aroma (peppermint, vanillin)	Food market
35	Finland	Chocolate	Aroma (vanillin, peppermint oil)	Food market
36	Spain	Chocolate	Peppermint natural flavour	Food market
37	Finland	Chocolate	Aroma (peppermint oil)	Food market
38	Germany	Chocolate	Natural mint aroma	Food market
39	Finland	Chocolate	Aromas	Food market
40	Finland	Chocolate	Flavourings (peppermint oil, vanillin)	Food market
41	France	Chocolate	Aromas (peppermint oil, vanillin)	Food market
42	Germany	Chocolate	Peppermint oil, natural flavouring	Food market
43	Estonia	Chocolate	Flavourings (peppermint oil, vanillin)	Food market
44	Finland	Chocolate	Flavourings (incl. peppermint oil)	Food market
45	Germany	Chocolate	Peppermint oil, flavourings	Candy shop