VOLATILE COMPOUNDS AND ANTIBACTERIAL EFFECT OF COMMERCIAL MINT CULTIVARS - CHEMOTYPES AND SAFETY

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Highlights

- The chemical composition of volatile fractions of mint cultivars was determined.
- Three chemotypes dominated by *trans*-piperitenone oxide, carvone, menthol were found.
- The levels of menthone, pulegone and menthofuran in mint cultivar oils were safe.

- The essential oils of mint cultivars have shown a varied microbiological activity.

***Keywords*:** *Mentha* spp.; Essential oil; Headspace volatile compounds; GC-MS; Antimicrobial activity; PCA

ABSTRACT: The taxonomy of *Mentha* species is complicated due to the possibility of hybridization, and the introduction of many cultivars of mint used mainly for industrial purposes is a big challenge for their correct identification. Multidirectional studies of new mint cultivars give the possibility to determine their chemical composition and safety in use. An analysis of the essential oils (EOs) obtained by hydrodistillation from the leaves of fifteen commercial mints was performed using the GC-MS method. Additionally, the headspace volatile compound profiles of analyzed mint leaves were obtained by headspace solid-phase microextraction (HS-SPME/GC-MS). The essential oil yields were found to vary from 2.0 to 26.5 mL/kg. The GC-MS of EOs, as well as for the headspace compounds, showed large variation in their chemical composition. The principal component analysis (PCA) enabled distinction of three groups of chemotypes among fifteen mint cultivars, characterized by the abundance of *trans*-piperitenone oxide, carvone or menthol and related compounds. The use of these essential oils seems to be safe with respect to menthone, pulegone and menthofuran levels. The majority of isolated essential oils inhibited the growth of *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*, but the effectiveness of their antibacterial activity varies. The obtained results indicate a variability of chemical composition and antimicrobial properties within the analyzed mint cultivars.

1. INTRODUCTION

The genus *Mentha* from the Lamiaceae family consists of aromatic herbs, which are found mostly in temperate and sub-temperate areas of the world (Salehi et al., 2018). Since ancient times, various species of mint have been widely used as food products, in numerous

cosmetics, and also in medical treatment (Mahendran and Rahman, 2020). Recently, there has been a growing demand for plants with certain organoleptic characteristics, mainly for food and cosmetic purposes. The creation of new cultivars, including from medicinal plant species, e.g. mints, thymes and sages, makes it possible to obtain taxa with desired characteristics such as color, taste or aroma (Nayak et al., 2020). One of the methods of obtaining them is inter-species hybridization. The genus *Mentha* exhibits a wide range of morphological and phytochemical diversity and ability to easily hybridize varieties cultivated and wild among themselves, resulting in a variety of forms and interspecific hybrids (Nayak et al., 2020). Hybridization of the genus *Mentha* can also occur naturally. One of the best known hybrids is *Mentha* × *piperita* L., composed of *Mentha aquatica* L. and *Mentha spicata* L., which in turn is a combination of *Mentha suaveolens* Ehrh. and *Mentha longifolia* L. (Mahendran and Rahman, 2020). According to the latest statistics of the Food and Agriculture Organization of the United Nations from 2018, the world leader in peppermint production was Morocco with 92.5% of the world total production (98 704 tonnes), while Europe's participation was only 0.3% (FAOSTAT, 2018). The possibility of interbreeding different species of the genus *Mentha* results in the fact that their taxonomy is complicated, and thus their chemical composition is heterogeneous. Therefore, the chemical composition and biological properties of individual hybrids can significantly vary from one another, depending on many factors, both environmental and genetic. The heterogeneous botanical systematics of the constantly emerging new mint cultivars creates problems with their proper identification, and thus with the appropriate determination of their chemical profile and possible use for therapeutic purposes.

The active constituents of *Mentha* species leaves include flavonoids (eriocitrin, hesperidin, diosmin, luteolin and their glycosides), phenolic acids (derivatives of caffeic acid, e.g. rosmarinic acid), terpenoids, and volatile compounds (Fecka and Turek, 2007; Mahendran and Rahman, 2020; Nilo et al., 2017). Some of the plants of this genus are well known for the

presence of essential oils (EOs) characterized by a wide variety of components. The main groups of natural compounds in the EOs are monoterpenes (menthol, menthone, menthyl acetate, 1,8-cineole, menthofuran, isomenthone, neomenthol, limonene) and sesquiterpenes (β-caryophyllene), whereas other groups, such as aldehydes, aromatic hydrocarbons, miscellaneous compounds, lactones and alcohols, are present with a smaller proportion (Beigi et al, 2018; Anwar et al., 2019; Mahendran and Rahman, 2020). The European Union assessment report of *Menthae piperitae aetheroleum* defines its well-established therapeutic oral use as a herbal medicinal product for the symptomatic relief of minor spasms of the gastrointestinal tract, flatulence and abdominal pain, especially in patients with irritable bowel syndrome and in cutaneous application for the symptomatic relief of mild tension type headache (EMA, 2020).

In recent years, there has been an increasing need of new medications to treat bacterial infections. Multidrug resistant pathogens become a global problem and lack of new antibiotics leads to limited therapeutic options. The Centers for Disease Control and Prevention has drawn up a list of the most dangerous and resistant superbugs that cause severe infections and high mortality in hospital patients because of their antibiotic resistance. These include, among others, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and bacteria of the Enterobacteriaceae family (HHS, 2019). The World Health Organization (WHO) published in 2017 a similar list of pathogens requiring increased research and development of new antibiotics (WHO, 2017). Peppermint essential oil and menthol have demonstrated growth inhibition of Gram-positive and Gram-negative bacteria (Desam et al., 2019; Mittal et al., 2018). Other biological properties of peppermint include antioxidant, antiviral, insecticidal and anti-inflammatory properties (Da Silva Ramos et al., 2017; Salehi et al., 2018).

The aim of the study was to evaluate using gas chromatography and mass spectrometry the chemical profiles of EOs obtained by hydrodistillation and headspace obtained by headspace solid phase microextraction of the leaves of fifteen different *Mentha* species or cultivars cropped in Poland. Also, microbiological tests of the obtained mint essential oils against *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* strains were performed. Ten of the investigated taxa were not studied under this concept to determine the profile of volatile compounds present in their leaves and antimicrobial activity. This is particularly interesting in terms of assessment of their chemotaxonomic affinity, presence of potentially toxic compounds and in search for new plant substances with activity against pathogens.

2. MATERIALS AND METHODS

2.1. Plant samples

The leaf samples of fifteen commercial mints were obtained in homogeneous environmental conditions from the Botanical Garden of Medicinal Plants, Wroclaw Medical University, Botanical Garden, Wroclaw University and Ogrody Ziolowe in Wrocław, Poland in late July/August during flowering. The following taxa were used for the analysis: *Mentha* × *piperita* L. (**M1**), *Mentha* × *piperita* 'Mitcham' (**M2**), *Mentha* × *piperita* var. *citrata* 'Eau de Cologne' (**M3**), *Mentha* × *piperita* 'Kümmel' (**M4**), *Mentha arvensis* 'Thai' (**M5**), *Mentha arvensis* ssp. *haplocalyx* var. *piperascens* (**M6**), *Mentha suaveolens* 'Jokka' (**M7**), *Mentha suaveolens* 'Calixte' (**M8**), *Mentha suaveolens* 'Variegata' (**M9**), *Mentha spicata* 'Russian' (**M10**), *Mentha spicata* 'Dionysos' (**M11**), *Mentha spicata* var. *crispa* 'Persian' (**M12**), *Mentha spicata* 'Moroccan' (**M13**), *Mentha spicata* 'Strawberry' (**M14**), *Mentha spicata* 'Nanah' (**M15**). The identity of all plant materials was confirmed by Małgorzata Stachura (Botanical Garden of Medicinal Plants, Department of Biology and Pharmaceutical Botany, Wroclaw Medical University, Poland) and by Jolanta Kochanowska (Botanical Garden, Wroclaw University,

Poland). Voucher specimens were placed in the Department of Pharmacognosy and Herbal Medicines, Wroclaw Medical University, Poland. The leaves used for the analyses were previously dried in the shade, ~~and~~ in air at room temperature of 22 ± 2°C and the humidity of 40-45%.

2.2. Essential oil hydrodistillation

From the dried leaves of fifteen mint species, EOs for microbiological tests and composition analysis were obtained by hydrodistillation as described in the monograph *Menthae piperitae folium* in the European Pharmacopoeia (Ph.Eur., 2020), without the use of xylene, but with pentane and diethyl ether trap (1 : 2, v/v) in the stack. All the experiments were performed in triplicate and the results are presented as mean values.

2.3. *Headspace solid-phase microextraction (HS-SPME)*

An automated PAL RSI SPME holder (CTC Analytics AG, Zwingen, Switzerland) was used equipped with DVB / CAR / PDMS (Divinylbenzene / Carboxene / Polydimethylsiloxane, 50/30 μm) fiber obtained from Supelco Co. (Bellefonte, PA, USA). Each sample of the leaves was cut in small pieces and each sample (~2 g) was placed in a 20 mL clear screw top vial and hermetically sealed with a cap containing PTFE/silicone septum. The automated PAL RSI experimental procedure involved the following steps: conditioning SPME fiber (according to Supelco Co. instructions), sample agitation (agitation speed: 250 rpm; agitator on time: 5 s; agitator off time: 2 s) and equilibration for 30 min at 40 °C, extraction of volatiles for 40 min without agitation, and injection of SPME fiber in the GC injector for desorption (7 min). All the experiments were performed in triplicate and the results are presented as mean values.

2.4. Gas chromatography and mass spectrometry (GC-MS) analysis

The GC-MS analyses were performed using an Agilent Technologies (Palo Alto, CA, USA) gas chromatograph model 7820A equipped with Mass Selective Detector (MSD) model 5977E

144 (Agilent Technologies). An HP-5MS capillary column (30 m x 0.25 mm, 0.25 μm film
145 thickness, Agilent Technologies, Palo Alto, CA, USA) was used for GC-MS analysis (5%-
146 phenyl-methylpolysiloxane, Agilent J & W GC column) with 0.25 mm internal diameter and
147 0.25 μm coating thickness. The GC conditions were: the split ratio 1:50; oven programmed at
148 70°C for 2 min, then increased at the rate of 3 °C/min to 200°C and held isothermal for 15 min;
149 injector temp. 250 °C; detector temp. 300 °C; carrier gas was helium (velocity: 1 mL/min). The
150 MSD (EI mode) was operated at 70 eV, and the mass range was 30-300 average mass unit
151 (amu). For the EO analysis, 1 μL of diluted essential oil (10 μL of the oil in 1 mL of pentane)
152 was manually inserted with a syringe into the GC injector. The identification of the volatile
153 constituents was based on the comparison of their retention indices (RI) determined relative to
154 the retention times of *n*-alkanes (C_9-C_{25}) with those reported in the literature and their mass
155 spectra with the spectra from Wiley 9 (Wiley, New York, NY, USA) and NIST 17 (D-
156 Gaithersburg) mass spectral libraries. The percentage composition of the samples was
157 computed from the GC peak areas using the normalization method (without correction factors).
158 The average component percentages were calculated from three GC-MS analyses.

159 *2.5. Antimicrobial screening*

160 The antibacterial activity of mints' EOs was checked against reference bacterial strains: Gram-
161 positive *Staphylococcus aureus* ATCC 29213, Gram-negative *Escherichia coli* ATCC 25922
162 and *Pseudomonas aeruginosa* ATCC 27853 with regard to ciprofloxacin. The strains were
163 obtained from the collection of the Department of Pharmaceutical Microbiology and
164 Parasitology, Wroclaw Medical University, Poland. Before experiments, the strains were plated
165 on Columbia Agar (*Becton Dickinson*) culture medium with 5% sheep blood (*Pro Animali*) and
166 incubated at 37°C for 24 h. Bacterial suspensions in 0.9% NaCl with a density of 0.5 on the
167 McFarland scale were prepared from pure cultures. All suspensions were diluted 100-fold in
168 cation-adjusted Mueller Hinton Broth (CAMHB) (*Becton Dickinson*) medium before adding to

test tubes. The tests were carried out using the microdilution method according to the Clinical and Laboratory Standards Institute (CLSI, 2015). The only modification of the method was the use of 0.5 mL closed Eppendorf tubes instead of wells of the titration plate (CLSI, 2015). The EOs were treated as a stock solution and were subjected to two-fold serial dilution. The final range of EO concentrations tested was 0.098% to 25%.

The test tubes were incubated at 35°C for 24 h with shaking at 400 rpm. The value of the minimum inhibitory concentration (MIC) was read after the addition of 1% 2,3,5-triphenyl-tetrazolium chloride (TTC) and additional incubation for 24 h. The MIC value was determined as the lowest concentration of EO at which no bacterial growth was observed in the appropriate tube (it was characterized by the lack of red coloration of the culture).

2.6. Statistics

The data were treated and evaluated by principal component analysis (PCA) comparing different data pre-processing techniques, resulting in the selection of mean-centering, and evaluated by hierarchical k-means clustering. The analyses were performed using R for Windows, version 4.0.0 (R-Cran project, http://cran.r-project.org/) including the "factoextra" library (Kassambara and Mundt, 2017).

3. RESULTS AND DISCUSSION

The factors determining the chemical variability of EOs and their biological properties may include physiological changeability, geographical conditions, genetic factors and cultivation method (Park et al., 2016). Biosynthesis of volatile compounds in the genus *Mentha* is a complex process. The monoterpene biosynthesis pathway in peppermint (*Mentha* x *piperita* L.) is the best known. This process is separated from primary metabolism and is carried out by the conversion of isopentenyl diphosphate and dimethylallyl diphosphate to geranyl diphosphate, which is then cyclized by limonene synthase to (4S)-(-)-limonene. Further details

193 of this process are described by Schalk and Croteau (2000), Croteau et al. (2005) and Masomeh
194 et al. (2017).

195 *3.1. Essential oil content*

196 Quantitative content of the EO in the analyzed samples was determined by the
197 hydrodistillation method and expressed in mL/kg of dry plant. The obtained results are
198 presented in Table 1 and the abundance of major compounds in Figure 1.

199 The content of EO in the analyzed taxa was highly differentiated and ranged from 2.0
200 mL/kg in *M. suaveolens* 'Calixte' to 26.5 mL/kg in *M. piperita* 'Mitcham'. In the *Piperita* group
201 the highest EO yield was found in *M. piperita* 'Mitcham'. In the *Suaveolens* group, the highest
202 EO content was determined in *M. suaveolens* 'Jokka' – 10.0 mL/kg. In the *Spicata* group no
203 large differences in the content of EO within the samples were observed except for *M. spicata*
204 'Nanah' and the yields ranged from 8.7 mL/kg in *M. spicata* 'Nanah' to 18.7 mL/kg in *M.*
205 *spicata* 'Strawberry'. It is worth pointing out that the *M. piperita* sample contained 10.0 mL/kg
206 of EO, which is above the specifications in the European Pharmacopoeia for the *Menthae*
207 *piperitae folium* monograph, according to which it should contain not less than 9 mL/kg of EO
208 for a cut drug (Ph.Eur., 2020). Ludwiczuk et al. (2016) determined a higher content of EO in
209 the *M. piperita* group. In the *Suaveolens* group, the highest content of EO was determined in
210 *M. suaveolens* 'Jokka' – 10.0 mL/kg. In the *Spicata* group no large differences in the content of
211 EO were observed. Its value ranged from 8.7 mL/kg in *M. spicata* 'Nanah' to 18.7 mL/kg in *M.*
212 *spicata* 'Strawberry'. It is worth pointing out that the *M. piperita* sample contained 10.0 mL/kg
213 of EO, which is in accordance with the specifications in the European Pharmacopoeia for the
214 *Menthae piperitae folium* monograph, according to which it should contain not less than 9
215 mL/kg of EO for a cut drug (Ph.Eur., 2020). Ludwiczuk at.al (2016) determined a higher content
216 of EO in *M. spicata* 'Moroccan' (26 mL/kg) and in *M. suaveolens* 'Variegata' (8 mL/kg).

3.2. Chemical composition of the essential oils obtained by hydrodistillation after GS-MS analysis

EOs obtained by hydrodistillation were subjected to quantitative and qualitative analysis by GC-MS. The obtained results are presented in Table 2 and Figure 1.

Among the taxa of the *Piperita* group, menthol was the dominant compound, ranging from 29.50% in *M. piperita* 'Mitcham' to 46.52% in *M. piperita*. The second characteristic component in this group is *p*-menthone. It was determined in all samples – with the highest abundance in *M. piperita* 'Kümmel' (27.76%) and with the lowest in *M. piperita* var. *citrata* 'Eau de Cologne' (0.04%). Menthofuran was present with a higher percentage in *M. piperita* 'Mitcham' (17.45%) and in *M. piperita* 'Kümmel' (14.69%). In *M. piperita* var. *citrata* 'Eau de Cologne' menthol was not observed, but the main components of the obtained essential oil were linalool (23.88%) and linalyl acetate (19.93%). Menthol, menthone and menthofuran were also designated as the main constituents in *M. piperita* 'Mitcham' by Heydari et al. (2018).

Among the two analyzed essential oils from the *Arvensis* group, *M. arvensis* ssp. *haplocalyx* var. *piperascens* is characterized by a large variety of compounds. Its main component was menthol (54.70%), and the next dominant compounds were isomenthone (16.41%) and *p*-menthone (9.65%). On the other hand, in the *M. arvensis* 'Thai' taxon carvone is observed as the predominant compound (37.16%). A similar profile of the essential oil from *M. arvensis* ssp. *haplocalyx* var. *piperascens* was observed by Akhtar and co-workers with the main compounds menthol and menthone (Akhtar et al., 2017).

Trans-piperitenone oxide was found to be the dominant component in all essential oils of the *Suaveolens* group. It was determined in *M. suaveolens* 'Jokka' (79.72%), *M. suaveolens* 'Calixte' (85.14%) and *M. suaveolens* 'Variegata' (70.05%). Piperitenone oxide (89.5%) was also the main constituent previously determined in *M. suaveolens* 'Variegata' by Ludwiczuk et

al. (2016). In contrast, Schollenberger et al. (2018) reported caryophyllene oxide (64.32%) as the main component of the essential oil of this taxon. In addition, Bouyahya et al. (2019) determined in the basic species of cultivars of the *Suaveolens* group (*M. suaveolens*) piperitenone oxide (56.28%) as the main compound. Therefore, it can be assumed that this is the dominant component of this group of mints. The authors mentioned above also identified in this taxon piperitenone (11.64%) and pulegone (6.16%). In analyzed EOs of this group pulegone was not identified, but piperitenone was detected with a small percentage only in *M. suaveolens* 'Jokka' (0.25%). A similar result was obtained by Ludwiczuk et al. (2016).

In the essential oils of the *Spicata* group, carvone was the dominant component, and it was determined in four of six oils – *M. spicata* 'Russian' (50.44%), *M. spicata* var. *crispa* 'Persian' (66.55%), *M. spicata* 'Moroccan' (63.09%), and *M. spicata* 'Nanah' (63.89%). Carvone was also identified as the main component of the EO from *M. spicata* 'Moroccan' by other authors (Ludwiczuk et al. 2016; Soilhi et al 2019). In the EO of *M. spicata* 'Dionysos', *p*-menthone (34.64%) and menthol (29.77%) were the most abundant. In *M. spicata* 'Strawberry' the dominant component was piperitenone (85.05%).

3.3. HS-SPME/GC-MS analysis

The headspace profiles of volatile compounds of analyzed mints were obtained by headspace solid-phase microextraction (HS-SPME) combined with GC-MS analysis. The following mints were characterized with menthol as the main constituents: *M. piperita* (43.06%), *M. piperita* 'Mitcham' (49.30%), and *M. arvensis* ssp. *haplocalyx* var. *piperascens* (42.24%). Carvone was present in higher abundance in *M. arvensis* 'Thai' (75.57%), *M. spicata* 'Russian' (65.95%), *M. spicata* var. *crispa* 'Persian' (77.48%), *M. spicata* 'Moroccan' (73.50%), and *M. spicata* 'Nanah' (72.15%). *Trans*-piperitenone was detected as the main compound in the headspace profiles of *M. suaveolens* 'Jokka' (86.16%), *M. suaveolens*

265 'Calixte' (81.07%), *M. suaveolens* 'Variegata' (67.31%), and *M. spicata* 'Strawberry' (47.09%).
266 In *M. piperita* 'Kümmel' headspace menthofuran dominated (25.98%), in *M. piperita* var.
267 *citrata* 'Eau de Cologne' linalyl acetate was the major compound (51.55%), and *p*-menthone
268 was the major compound in *M. spicata* 'Dionysos' (29.75%). The results of HS-SPME/GC-MS
269 analysis are shown in Table 3.

270 Comparing GS-MS analyses of EOs obtained by hydrodistillation and volatile fractions
271 obtained by HS-SPME, several differences in the profiles of the main compounds can be
272 observed. In the *Piperita* group, only in *M. piperita* var. citrata 'Eau de Cologne' the main
273 component was changed from linalool in hydrodistillation (23.88%) to linalyl acetate in HS-
274 SPME (51.55%), which could be the consequence of linalyl acetate hydrolysis during
275 hydrodistillation, and in the *Spicata* group in *M. spicata* 'Strawberry' from piperitenone in
276 hydrodistillation (85.05%) to *trans*-piperitenone oxide in HS-SPME (47.09%). In the other
277 taxa, the main compounds were the same, but with varying abundance.

278 *3.4. Presence of potential toxic compounds in analyzed mint essential oils*

279 Several compounds of mint essential oil such as menthone, pulegone and menthofuran
280 can show toxic effects when used both externally and internally (Zárybnický et al., 2018;
281 Mancianti and Ebani, 2020). That is why it is so important to know the chemical composition
282 of EOs, in particular newly introduced mint species hybrids. High doses of pulegone, menthone
283 or menthofuran may show hepatotoxicity and mutagenicity, genotoxicity or carcinogenicity in
284 animals tests (Prinsloo et al., 2018; Malekmohammad et al., 2019). Humans are exposed to
285 pulegone and menthofuran in medicinal products and in food, and as part of the EO in
286 flavorings, confectionery, and cosmetics. The highest known recommended daily dose in the
287 EU is 1.2 mL of peppermint oil, which contains a maximum of 32.97 mg of pulegone and 87.92
288 mg of menthofuran (EMA, 2016). The European Pharmacopoeia defines in its monograph for

289 *M. piperita aetheroleum* minimum and maximum percentages for selected compounds. For
290 menthone it is 14.0% to 32.0%, for menthofuran 1.0% to 8.0%, and for pulegone a maximum
291 of 3.0% (Ph.Eur., 2019). Among all analyzed samples, only in EO for *M. spicata* 'Dionysos'
292 was the level of menthone exceeded (34.64%). In two EOs the content of menthofuran was
293 higher in comparison to that recommended by the pharmacopoeia (in *M. piperita* 'Mitcham'
294 17.45% and in *M. piperita* 'Kümmel' 14.69%). The level of pulegone in all analyzed samples
295 was within the limits allowed by the pharmacopoeia. These minor exceedances of substances
296 with potential toxic effects in three out of fifteen tested samples of essential oils may indicate
297 the safety of their use. This is of particular importance because the purpose of creating mint
298 hybrids is, among other things, to obtain such plant material that would match the specific taste
299 or olfactory requirements of the food or cosmetic markets, while being safe.

300 *3.5. Principal component analysis (PCA) and hierarchical k-means tree clustering*

301 The datasets containing volatile composition determined by GC-MS analysis of EOs
302 and HS-SPME demonstrated very clear natural clustering and good separation of three groups
303 (Figures 2 and 3). In the case of the data obtained for the EO, the data were mean-centered and
304 the first two PCA factors explained 73.0% of variance among the samples. In the case of the
305 data obtained for the headspace, mean-centering was applied prior to the analysis, and the main
306 2 factors explained 84.6% of variance in the dataset. The variables providing the greatest
307 contribution to the first two principal components were: *trans*-piperitenone oxide (accounting
308 for more than 70% of factor 1), carvone (accounting for nearly 25% and 50% of factors 1 and
309 2, respectively), menthol (accounting for nearly 30% of factor 2), *p*-menthone and piperitenone
310 (EO); *trans*-piperitenone oxide (accounting for about 40% of both factors 1 and 2), carvone
311 (accounting for about 20% and 60% of factors 1 and 2, respectively), menthol (accounting for
312 about 25% of factor 1), *p*-menthone and menthofuran (HS-SPME). The samples naturally
313 formed 3 groups – chemotypes characterized by the abundance of *trans*-piperitenone oxide,

carvone or menthol and related compounds. The first group characterized by *trans*-piperitenone oxide was composed of the samples from *M. suaveolens* cultivars, but also *M. spicata* 'Strawberry' (the results from HS-SPME), the second group characterized by carvone was composed of the majority of *M. spicata* cultivars (*M. spicata* 'Dionysos', *M. spicata* 'Moroccan', *M. spicata* 'Strawberry', *M. spicata* 'Nanah'), excluding *M. spicata* 'Dionysos' and *M. spicata* 'Strawberry', along with one cultivar of *M. arvensis* - *Mentha arvensis* 'Thai'. The third group characterized by the abundance of menthol and related compounds included all *M. piperita* cultivars along with some *M. spicata* (*M. spicata* 'Russian' and *M. spicata* 'Moroccan') and *M. arvensis* ssp. *haplocalyx* var. *piperascens* cultivars.

The results obtained by the hierarchical k-means clustering algorithm are shown in Figure 4. The pre-defined number of clusters was four, according to the number of the analyzed species. The data, however, tended to form three groups both in the case of the headspace and the essential oil dataset. One sample, visibly most closely related to the last group, was left apart due to pre-defined settings. The clusters based on headspace or EO datasets did not overlap with each other, but they were perfectly in accordance with natural clustering previously observed on PCA.

3.6. Antimicrobial screening

Not only certain types of food, such as fruits and vegetables or livestock, but also some products used in the cosmetic or pharmaceutical industry, are particularly at risk of contamination by bacterial and fungal pathogens due to their high moisture content. This can lead to their microbiological contamination, which is an important safety factor. Particularly dangerous pathogens for humans and animals are *Staphylococcus aureus* found in the nasopharyngeal cavity and on human and animal skin, *Escherichia coli*, which is a part of the physiological bacterial flora of the human colon and other warm-blooded animals, and *Pseudomonas aeruginosa*, living mainly in soil and water, on the surface of plants and on the skin of animals.

339 Infections with these pathogens can lead to severe diseases. EOs, due to their content of
340 biologically active compounds, can help to prevent contamination of products by pathogens
341 and thus protect against the illnesses they may cause. Therefore, they can also have a preventive
342 function (Antunes and Cavaco 2010; Kalemba and Synowiec 2020) . There are studies being
343 carried out in order to explain not only the mechanisms of such action and compounds
344 responsible for them, but also their potential therapeutic or protective use in both health care
345 and cosmetic or food products (Singh et al., 2015). The antimicrobial properties of peppermint
346 EO are quite well described, while those of mint hybrids are not (Uribe et al., 2016; Chouhan
347 and Sharma, 2017; Pľuchtová et al., 2018; Desam et al., 2019; Kang et al., 2019; Peer et al.,
348 2019; Gishen et al., 2020).Therefore, it is appropriate to study the microbiological activity of
349 new mint cultivar EOs. The investigation of microbiological activity of EOs from the mints
350 obtained by hydrodistillation was carried out against three pathogens *Staphylococcus aureus*,
351 *Escherichia coli* and *Pseudomonas aeruginosa*. The obtained results are presented in Table 4.

352 The MIC values for *S. aureus* were in the range of 0.098–3.125% of the stock solution of EOs.
353 The EOs from *M. suaveolens* 'Jokka' and *M. arvensis* ssp. *haplocalyx* var. *piperascens* exhibited
354 the strongest effects (MIC ≤ 0.098%) while EO from *M. suaveolens* 'Calixte' (MIC = 3.125%)
355 showed the weakest effects. Compared with the mint EO inhibition for *S. aureus*, eight EOs
356 exhibited MIC = 0.39% and two showed MIC = 0.78%. The MIC value obtained for *E. coli* was
357 in the range of 0.098–1.56 % of the EOs stock solutions. The strongest effect (MIC ≤ 0.098%)
358 was found for EO form *M. spicata* 'Nanah'. Four EOs showed the same MIC value of 0.19%
359 or 1.56% and seven 0.39%. Of three microorganisms tested,
360 *P. aeruginosa* showed the lowest susceptibility to the aetheroils tested. The range of the
361 antimicrobial concentrations of EOs for this microorganism was from 0.39% of the initial
362 concentration of *M. spicata* 'Strawberry' to ≥ 25% (MIC of *M. spicata* 'Russian', *M. suaveolens*
363 'Calixte', *M. arvensis* ssp. *haplocalyx* var. *piperascens*). Five EOs inhibited growth only at

concentrations ≥ 12.5%.

The present research showed that the majority of isolated EOs inhibited the growth of all 3 species of bacteria. However, the effectiveness of their antibacterial activity varied. The oil from *M. spicata* 'Strawberry' exhibited similar effectiveness in inhibiting bacterial growth after at least 256-fold dilution. Most of the EOs showed a stronger effect on *S. aureus* and *E. coli* than the peppermint oil. *P. aeruginosa* was found to be the least susceptible to the EOs. In the case of the oils from *M. spicata* 'Russian', *M. suaveolens* 'Calixte', and *M. arvensis* ssp. *haplocalyx* var. *piperascens*, due to their weak inhibitory action, the exact MIC value for this strain could not be determined.

The EOs from *M. spicata* 'Russian' and *M. suaveolens* 'Calixte' showed the weakest antibacterial activity against all bacteria. In turn, the EO from from *M. arvensis* ssp. *haplocalyx* var. *piperascens* inhibited *S. aureus* and *E. coli* more strongly than *P. aeruginosa*. A noteworthy observation is that the mint EOs differ in the activity towards particular Gram-negative bacilli, and both *E. coli* and *P. aeruginosa* have a similar type of cell wall structure. Usually, the presence of an outer membrane, which limits the transport of various substances into the cell, leads to increased resistance of this group of microorganisms to antimicrobials such as antibiotics. In the present research, however, it was observed that *E. coli* susceptibility to the EOs was similar to, or even greater than, that of *S. aureus*, and significantly different from that of *P. aeruginosa*. The EOs extracted from various mint cultivars showed antibacterial activities especially concerning *S. aureus* and *E. coli*, and it will be the subject of further research to check whether the antimicrobial activity results from the action of the individual compounds or it is the synergistic effect of the substances.

The dependence of the antibacterial action of mint EOs on their main chemical components is inconclusive. Menthol, *trans*-piperitenone oxide, piperitenone and carvone are

388 the dominant components of the most active EOs. *S. aureus* is most strongly affected by *M.*
389 *arvensis* ssp. *haplocalyx* var. *piperascens* EO, whose main component is menthol (54.7%) and
390 by *M. suaveolens* 'Jokka' EO with dominant *trans*-piperitenone oxide (79.72%). Other EOs,
391 which also contain menthol or *trans*-piperitenone oxide as the main components, did not show
392 such activity. An example is the *M. suaveolens* 'Calixte' sample containing 85.14% *trans*-
393 piperitenone oxide and exhibiting the lowest activity against *S. aureus*. In relation to *E. coli* the
394 highest inhibitory activity was shown by the *M. spicata* 'Nanah' sample, whose main
395 component was carvone (63.89%). The remaining EOs, in which this compound was also the
396 dominant component – *M. arvensis* 'Thai', *M. spicata* 'Russian', *M. spicata* var. *crispa*
397 'Persian', and *M. spicata* 'Moroccan' – did not have such a strong effect. *P. aeruginosa* was
398 most strongly inhibited by the *M. spicata* 'Strawberry' sample with piperitenone as the main
399 constituent (85.05%). Only in two samples, *M. suaveolens* 'Jokka' and *M. spicata* 'Russian',
400 was this compound determined in small amounts, while the remaining samples did not contain
401 it at all. The dependence of the antimicrobial activity of mint cultivars' EOs on their main
402 component suggest synergy or additivity of their action against these pathogens. Similar
403 relationships were observed by other authors (Bassolé and Juliani 2012; Chouhan and Sharma
404 2017; Requena et al. 2019). A stronger antibacterial effect against *S. aureus* than that of
405 ciprofloxacin was observed for EOs from *M. arvensis* ssp. *haplocalyx* var. *piperascens* and *M.*
406 *suaveolens* 'Jokka' (MIC 0.098% of the EO baseline concentration) and for *M. piperita*
407 'Mitcham' and *M. spicata* 'Strawberry' (MIC 0.19% of the EO baseline concentration). None
408 of the tested EOs showed a stronger inhibitory effect against *E. coli* than the reference
409 substance. Against the third tested pathogen, *P. aeruginosa*, only the EO from *M. spicata*
410 'Strawberry' proved to be stronger than ciprofloxacin.

411 **4. CONCLUSIONS**

412 The results of the conducted research clearly indicate that there is great diversity in both
413 the chemical composition and antimicrobial activity among the investigated EOs of *Mentha*
414 cultivars. It can therefore be concluded that the chemical profile of volatile compounds and
415 their antibacterial effect are not directly associated with the relationship between the analyzed
416 plant materials. In the following cultivars, the analysis of volatile compounds from leaves and
417 the antibacterial effect of the obtained EOs was carried out for the first time: *M. piperita* var.
418 *citrata* 'Eau de Cologne', *M. piperita* 'Kümmel' , *M. arvensis* 'Thai', *M. suaveolens* 'Jokka' ,
419 *M. suaveolens* 'Calixte', *M. spicata* 'Russian', *M. spicata* 'Dionysos', *M. spicata* var. *crispa*
420 'Persian', *M. spicata* 'Strawberry' and *M. spicata* 'Nanah'. The quantitative content of EO
421 obtained by hydrodistillation in the leaves of analyzed mints ranged from 2 mL/kg in *M.*
422 *suaveolens* 'Calixte' to 26.5 mL/kg in *M. piperita* 'Mitcham'. The GC-MS analysis of these
423 EOs showed a great diversity even within the same taxa. Using PCA analysis, the taxa were
424 naturally grouped into three chemotypes. The first group, characterized by *trans*-piperitenone
425 oxide, contained mainly the samples from *M. suaveolens*, while the second group, characterized
426 by carvone, contained *M. spicata* cultivars. The third group, characterized by an abundance of
427 menthol and related compounds, included all *M. piperita* cultivars along with *M. spicata*
428 'Russian', *M. spicata* 'Moroccan' and *M. arvensis* ssp. *haplocalyx* var. *piperascens*. Comparing
429 GS-MS analyses of EOs obtained by hydrodistillation and volatile fractions obtained by HS-
430 SPME, several differences in the profile of main compounds can be observed. The analysis of
431 potentially toxic compounds in tested mints' EOs such as menthone, pulegone and menthofuran
432 showed that these compounds are present in the majority of analyzed mints at a level ensuring
433 their safe use. Antimicrobial tests carried out on mint oils against the three pathogens
434 *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* showed that the
435 majority of tested EOs inhibited the growth of all these species of bacteria, but the effectiveness
436 of their antibacterial activity varied.

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