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Phytochemical evaluation of Northern Hungarian horsemint (*Mentha longifolia* (L.) L.) populations

PhD THESIS

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I. INTRODUCTION AND AIMS OF THE STUDY

Introduction

Conservants and antioxidants are used to control oxidative spoilage in the food industry widely. These additives enhance the shelf life of foodstuff together with the preservation of nutritional and organoleptic values.

Since the early 1990s, there is a strong interest towards plant-originated antioxidants. A preparation based on guaconic acid isomers, guaiac gum (nowadays listed under code E314 as antioxidant and E214 as preservative) is in use since a longer time. The recent researches attracted attention to further plant metabolites and their raw materials. After an array of investigations and discussions that had begun in 1996, the European Union accepted the usage of a new food additive, the antioxidant E392/Rosemary exracts (The European Parliament and Council, 2010). Besides rosemary, other species (wild marjoram, garden sage etc.) producing rosmarinic acid and antioxidant flavonoids became subject of research. However, less attention was drawn to *Mentha* species, although they also produce these ingredients, demonstrate considerable *in vitro* antioxidant activity (Damien-Dorman et al. 2003), and in a couple of sporadic investigations some extracts of them are proven to be preservative in selected food matrices (Bandhopadhyay et al. 2008).

Besides the cultivated edible plants, there is widening attention on wild-growing (or once cultivated but nowadays not exploited) edible species. These may be potential raw material and genetical sources for the agriculture if they are more adaptive and their chemical profile is similar or more advantageous than the cultivated relatives are. (Bacchetta et al. 2016). The quality of the wild-collected plant materials is fluctuant from year to year but it can be controlled with selection and cultivation of taxa, chemotaxa bearing advantageous chemical profile.

The investigation and, in long term, cultivation of horsemint (*Mentha longifolia* (L) L.) as a less studied source of antioxidants may be justified by the abundance of the species in Hungary and its well-known genetical diversity. On the one hand, it offers a genetic pool for the selection of types with good polyphenol profile for the food industry (e.g. richness in rosmarinic acid). On the other hand, the species is known as a polymorphic one in terms of its essential oil chemistry. Thus, chemotypes rich in antioxidant or antimicrobial volatiles (Mimica-Dukić et al.) may be raw materials of anti-spoilage preparations.

Aims of the present study

1) Screening of wild-growing horsemint populations living in selected parts of the Northern Hungarian Montainous Region. This is aimed at to elucidate the natural variability of the species in terms of phenolic and volatile composition.

2) Determination of the factors influencing the chemosyndromes of horsemint from the viewpoint of both phenolic and volatile compounds. Furthermore, one goal was to clarify the effect of ***a)*** *biotic factors*: intraspecific (chemo-)taxa, phenophases, age of the plant ***b)*** *local weather and habitat factors* **c)** if possible, *the nexuses of environmental effect and taxon.*

3) Determination of some *in vitro* antioxidant (AO) properties of horsemint populations. Establishment of the correlations between the concentration of the identified phenolics and the antioxidant parameters.

4) Making proposal to a reproducible, efficient solvent extraction method in processing horsemint, which results in polyphenol-rich extracts with high *in vitro* antioxidant activity.

5) Making proposal, if possible, to optimization of the agrotechnology of the species in terms of the yielding of different active ingredients.

6) Designation of the potential area of industrial utilization where selected chemotypes of horsemint may be exploited as safe sources of shelf-life enhancer antioxidants, flavouring and anti-spoilage agents.

II. MATERIALS AND METHODS

Sampling and botanical identification of the wild-growing horsemint populations

Thirty-six populations (Table 1) were sampled in two (2016 and 2017) years, in the stage of full bloom. One sample contained 5-20 randomly chosen shoots, which were cut ca. 10 cm above the ground. Identification of the species was performed via macromorphological determination, according to Simon (1994).

Table 1. Location of the sampled wild-growing horsemint populations.

Identifier	Location name	Location
JOF	**Jósvafő**, Dózsa Gy.st.	N 48.483269; E 20.549968
BÜK	**Bükkszentkereszt**/Kaán Károly springs	N 48.083383; E 20.638888
JÁV	**Jávorkút**	N 48.097635; E 20.528104
KÜH	**Kühne Andor tourist path** (roadside)	N 48.098210; E 20.554323
HOR1	**Hór-völgy**/Tebepuszta meadows	N 48.029817; E 20.552683
HOSSZ3	**Hosszúvölgy**-3 sample	N 48.012667; E 20.505166
HOSSZ2	**Hosszúvölgy**-2 sample	N 48.016984; E 20.501000
HOSSZ11	**Hosszúvölgy**-1 sample	N 48.013367; E 20.493202
TIB	**Tibolddaróc**	N 47.931934; E 20.634509
FET	**Felsőtárkány**/Barát-rét meadows	N 47.992750; E 20.460452
HOR3	**Hór-völgy**/Oszlarét meadows	N 47.979290; E 20.520453
HOR2	**Hór-völgy**/Kisrét meadows	N 47.996033; E 20.513726
NOSZ	**Noszvaj** Víz-völgy meadows	N 47.926044; E 20.469540
EGR1	**Eger**/Leányka street, flower beds	N 47.903308; E 20.382339
EGR2	**Eger**/Zúgó st., isle in Eger stream	N 47.890970; E 20.390122
EGR3	**Eger**/Zúgó st., bridge.	N 47.890970; E 20.390122
EGR4	**Eger**/Zalár st., near bridge	N 47.903198; E 20.375530
DOM	**Szentdomonkos**	N 48.088253; E 20.178323
TLE	**Tarnalelesz**	N 48.048387; E 20.177987
HEA	**Hevesaranyos**	N 48.019062; E 20.215930
VÁR	**Váraszó fish pond**	N 48.085338; E 20.094653
PÉV1	**Pétervására outskirts**-1. sample	N 48.003243; E 20.099971
PÉV2	**Pétervására outskirts** -2. sample	N 48.003243; E 20.099971
MDE1	**Mátraderecske**, pasture at Balla stream	N 47.954067; E 20.072450
MDE2	**Mátraderecske**/Nagyrét pastures	N 47.941857; E 20.075533
MDE3	**Mátraderecske**/Baláta & Kovácsói-stream, bridge	N 47.949622; E 20.072554
DEK	**Dekics-juss**, railway	N 47.974139; E 20.037302
SZU	**'Szurdok'** hill between Mátraderecske-Mátraballa	N 47.964397; E 20.051301
MBA1	**Mátraballa**/between dirt road and railways	N 47.986900; E 20.021050
MBA2	**Mátraballa**/Rákóczi út, abandoned building plot	N 47.989079; E 20.018066
NÁD	**Nádújfalu, aut. ford.** ditch behind bus stop	N 48.010023; E 19.969621
MAC	**Maconka aquifer**	N 47.993504; E 19.858783
KBT	**Bátonyterenye** outskirts, Szarisznyó-stream.	N 47.952612; E 19.815339
HAS	**Pásztó-Hasznos**, Kövicses-stream	N 47.929983; E 19.736200
MÁH1	**Mátraháza**-1. sample	N 47.865483; E 19.980816
MÁH2	**Mátraháza**-2. sample	N 47.864483; E 19.984433

Analytical investigations of the wild-growing horsemint samples

a) Extraction

The shoots were hung and dried naturally for 21 days, then leaves and inflorescences were separated from stem and stored in plastic bags at -18°C until analyses. On the plant material collected in 2016, four different extraction methods were performed to clarify which of them is optimal from the viewpoint of total polyphenol and antioxidant properties. Thus, each item of this sample collection provided four extracts to compare.

The methods applied

- Soxhlet extraction, 3 stages, with methanol (MeOH)
- Extraction in cooled (t<30°C) ultrasonic (US) bath, 3 stages; 45 kHz, 1,5 h/stage; MeOH.
- Soxhlet extraction, 3 stages, with water:ethanol 3:7 (WA)
- Extraction in cooled (t<30°C) ultrasonic (US) bath, 3 stages; 45 kHz, 1,5 h/stage; WA.

b) Determination of total polyphenol content and antioxidant activity

Total polyphenol content (Folin-Ciocalteu assay)

The assay based on the protocol of Waterhouse (2003) but with modifications, we performed it in 60-min reaction time. Spectrophotometric evaluation was made at 765 nm using 0-250 mg/l aqueous gallic acid solutions for calibration. Measurements were performed in duplicate. Results given in mg gallic acid equivalents per kg dry plant material (mg GAE/kg dp).

Determination of radical scavenging activity by DPPH assay

EC_{50} (effective concentration-50) values of horsemint extracts were determined to 0,1 mM DPPH (in 96% ethanol), at ambient temperature, normal lighting, in 30-min reaction. Measurements were performed in duplicate, at 517 nm. Results are given as EC_{50} mg/l.

FRAP assay

Assay was performed ont he base of the study of Benzie és Strain (1996) but with modifications. For calibration, 0-20 mg/l aqueous ascorbic acid solutions were used. Measurements were performed in triplicate. Results are given in mg ascorbic acid equivalents per kg dry plant material (mg AAE/kg dp).

c) Determination of some phenolic compounds of the wild-growing horsemint

Identification and quantification of phenolics was performed by HPLC-DAD (Agilent 1200), on an apolar column (ACE Excel C18, 250×4,6mm, 5µm). Eluent 'A' was 5 V/V% aqueous acetic acid solution and 'B' was acetonitrile. Identification is based on a database of retention time and UV-VIS spectra of standards. These were as follows: apigenin, apigetrin, caffeic acid, chlorogenic acid, cynaroside, diosmetin, diosmin, hesperetin, hesperidin, isoquercitrin, luteolin, naringenin, narirutin, quercetin, rosmarinic acid, rutin, vicenin-2. This set of standards later was necessary to complete with lonicerin and eriocitrin.

As the chromatograms gained from the method described above has shown two intensive unknown peaks in all samples of 2016 Unknown „A" and „B") 10 items of the sample set were investigated with HPLC-DAD-MS (apparatus: Shimadzu LCMS-2010EV).

The MS results elucidated that Unknown „A" is derived from the coelution of two flavonoids, namely lonicerin and eriocitrin. These were necessary to separate and quantify in the following samples (batches from experimental cultivation). Unknown „B" is one individual compound that was not identified during this study.

d) Statistical evaluation

In the case of the wild-growing horsemint plants (collection 2016) statistical evaluation on the one hand covered investigation of significant differences between the total polyphenol and antioxidant indices of the four extract types and, on the other hand, correlation calculations. Testing of significant differences were performed using paired t-tests or Welch's tests. All statistical calculations were performed by the use of IBM SPSS 16.0 és MS Excel 2013.

Involving selected horsemint accessions to experimental cultivation

Accessions were selected primarily according to their antiradical activity and stability of this parameter determined from the samples collected in 2016 and 2017. The geographical origin and habitat characteristics of the selected five populations (Hór-völgy-1: HOR1, Hór-völgy-2: HOR2, Eger-3: EGR3, Szentdomonkos: DOM, Bátonyterenye: KBT) are shown in Table 2.

Table 2. Geographical origin and habitat of the chosen populations. (h = height above sea level)

Identifier	HABITAT			
	Location		h, m	Short characterization
HOR1	N 48.029817	E 20.552683	239	Swamp meadow, in periodic drought conditions. Nat. Park area.
HOR2	N 47.996033	E 20.513726	233	Mesophile meadow, at the edge of oak-hornbeam and beech forests. Nat. Park area.
EGR3	N 47.890970	E 20.390122	173	Streambank, urban ruderal area at a bridge in suburbia
DOM	N 48.088253	E 20.178323	398	Between a cultivated field and a dirt road
KBT	N 47.952612	E 19.815339	221	Mesophile meadow between stream, woods and rural ruderal area. A coenosis of nitrophile weeds.

Experimental field in Eger (Habitat EGR)

The rooted shoots of the five populations were planted in Eger on May 16, 2018. The plot is in the botanical garden of the Esterházy Károly Catholic University (N 47.906834; E 20.388122). Meteorological data (daily minimum, maximum and average temperature, precipitation, hours of sunlight) were provided by the Hungarian Service of Meteorology (OMSZ).

Shoots were planted to pairs placed to 20 cm from each other and arranged in five rows (one row per population) of ca. 5 m length and 1 m distance. No fertilizer was used. In intervals with no precipitation longer than 7 days the plants received watering (equal to ca. 20 mm). Manual weeding was performed weekly and occasional insecticide treatment with acetamipride if needed.

Experimental field in SOR (Habitat SOR)

The propagation material was gained from the 2nd year shoots of the five horsemint populations at the EGR habitat in April 2019. A set of ca. thirty rooted shoots per population (20-30 cm in height) were planted in Soroksár, in the MATE experimental farm. Arrangement, care and sampling is identical as at the EGR habitat. Daily minimum, average and maximal temperatures and precipitation were recorded on spot.

Sampling of the experimental plots

Sampling was performed in years 2018-2020 (EGR) and 2019-2020 (SOR). Five different phenophases were sampled. These are the following:

- **L1** – Spring vegetative
- **VK** – Green flower buds
- **V** – Full bloom
- **T** – In ripening of fruits
- **L2** – 2nd (autumnal) vegetative, emerging from regeneration

After sampling the T (fruit ripening) shoots, the rows were cut ca. 5 cm above the ground. The material of L2 samples were gained from the vegetative shoots emerged from the rows newly.

Analytical investigations of the cultivated horsemint accessions

a) Extraction

The harvested shoots were hung and dried naturally for 21 days, then leaves and inflorescences were separated from stem and stored in plastic bags at -18°C till analyses, except that part of

each V samples which is aimed to essential oil investigations. This amount of plant material was stored in paper bags in a dark closet at room temperature.

b) Determination of total polyphenol content and antioxidant activity

These assays were performed as it was described at the investigations of wild-growing horsemint.

c) Determination of some phenolic compounds

This was performed using the Agilent 1200 HPLC-DAD apparatus, mounted with apolar core-shell column (Poroshell EC-120 C18, 150×4,6 mm (+ 5 mm guard column) 2,7 μm). Eluent 'A' was phosphate buffer (pH 2,4), and 'B' was a mixture of gradient grade acetonitrile and 'A' 70:30. Identification was based on retention time and UV-VIS spectra of standards.

d) Investigations of essential oil yield and composition

These analyses were performed on the blooming-stage (V) samples of the cultivated accessions, at the Department of Medicinal and Aromatic Plants of MATE (Budapest). Hydrodistillation was made using a Clevenger apparatus in accordance with the 7th edition of the Hungarian Pharmacopoeia. It was performed in triplicate on the samples of EGR and in duplicate on the batches of SOR (25-35 g dry plant material per distillation). Determination of essential oil composition was performed using GC-MS (Agilent GC 6890 apparatus mounted with 30 m×0,25 mm, 0,25 μm HP-5MS capillary column, Agilent 5975 inert mass detector (250 °C) viand He (1 ml/min) as carrier gas). Identification was based on public databases (Adams, 2017; Steiner et al. 2011); linear retention indices of (LRI) n alkanes and a library of standard spectra.

e) Statistical evaluation

To clarify the significant differences between the polyphenol composition, Kruskal-Wallis' test was applied. For correlations, Spearman's method was used. A rank-based calculation assembled by the author is applied to find the optimal phenophase to harvest horsemint as a polyphenol crop. This involves datasets of polyphenol, DPPH EC_{50}, FRAP, rosmarinic acid, eriocitrin and lonicerin. Significant differences between EO yields were tested with Wilcoxon's signed ranks test. Chemotaxonomical classification is based on Németh et al. (1993). This study defines chemotypes from major EO components with minor or no fluctuations due to the environment (thus, their percentage may be hereditary). Fluctuations were judged on the base of the coefficient of variation (CV) of their percentages in the samples of the 2 sites×2 year. Statistical calculations were ran using the use of IBM SPSS 16.0 and MS Excel 2013.

III. RESULTS AND DISCUSSON

Extractability, antioxidant activity and polyphenols of the wild-growing samples

Results demonstrate that in terms of the antioxidant activity of the extracts, the most efficient method was the hydroalcoholic ultrasonic (US) extraction and the less effective was the methanolic Soxhlet (although this method is widespread). Total polyphenol content calculated from the hydroalcoholic (WA) US extraction was 23390-67910 mg GAE/kg dp, which is in the ca. 10 000-90 000 mg GAE/kg dp interval outlined from literature data.

Regarding antiradical activity, EC_{50}<350 mg/l was gained only from WA extracts. EA US extracts showed EC_{50} 227-806 mg/l (di-*tert*-butyl hydroxytoulene (BHT) as control: 87.5 mg/l). Measurements performed at similar reaction conditions (Damien-Dorman et al. 2003) resulted 150-350 mg/l EC_{50} from 9 samples (hot water extracts) of different *Mentha* taxa (where BHT EC_{50}~85 mg/l), FRAP values outline medium reducing activity; from 36 samples, in 4 were as active as BHT or stronger (12169 mg AAE/kg). The interval of FRAP values calculated from hydroalcoholic US extracts was 5761-12453 mg AAE/kg. Both antioxidant indices and total polyphenol content were heterogenous regarding their coefficient of variation (CV) as CV≥20% in all extract types. This indicates large variability in polyphenol composition of the plant material. Regarding the 36 population sampled in 2016, 4 cases demonstrated high (58278-67910 mg GAE/kg dp) total polyphenol content, and strong AO activity at both of two assays (EC50 227-316 mg/l, FRAP 11115-12543 mg AAE/kg dp). These were DOM (Szentdomonkos, Heves-borsodi Hills), HAS (Pásztó-Hasznos, Western Mátra) HOR2 (Tebepuszta, Bükk Mountains), MBA2 (Mátraballa-2, Eastern Mátra). The weakest antioxidant properties were resulted by accession EGR3 (Eger).

Based on the results of extractability, the wild-growing samples collected in 2017 and later the samples from experimental cultivation were investigated only from hydroalcoholic US samples. The accessions to involve to cultivation (HOR1, HOR2, EGR3, DOM, KBT) has been chosen primarily on the base of their antiradical activity data from 2016 and 2017 (Table 3).

Table 3. Antiradical activity of the chosen horsemint accessions (EC_{50} data)

Identifier	**Hydroalcoholic US extract, EC_{50} mg/l**		**Characterization**
	2016 (average)	***2017 (average)***	
HOR1	359	384	stable, relatively strong AO activity
HOR2	308	389	stable, relatively strong AO activity
EGR3	806	737	stable, weak activity
SZD	245	718	variable activity
KBT	438	429	stable, medium activity

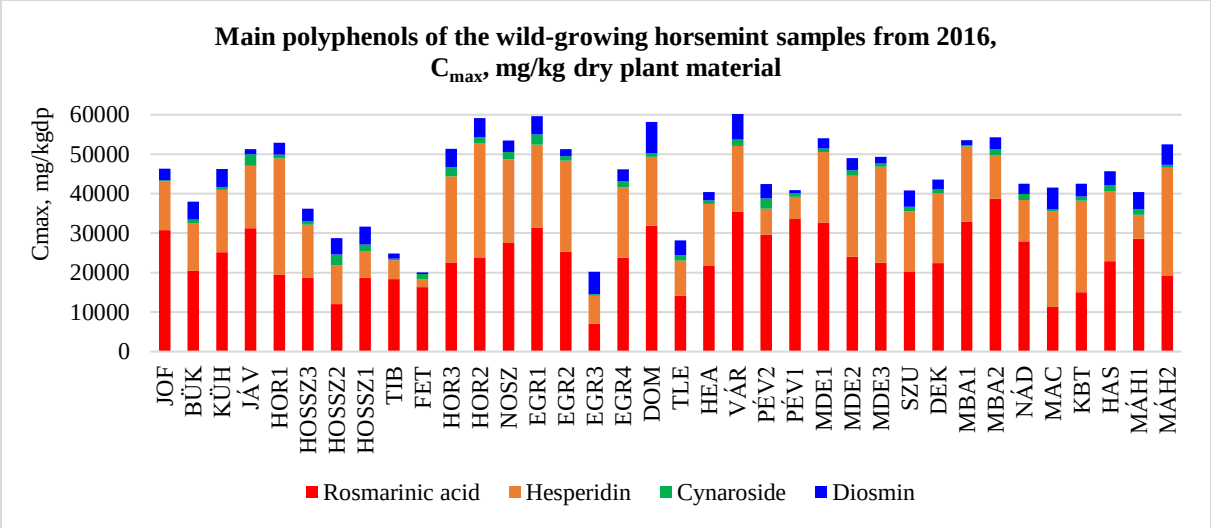
No habitat-based or geographic groups were distinguishable when antioxidant properties of the different populations were compared. For example, the 4 samples demonstrated the highest total polyphenol and strongest AO activity originated from distant populations which belonged to different habitat types.

Four phenolic constituents were identified in the extracts of the wild-growing samples. These are rosmarinic acid, cynaroside (luteolin-7-O-glucoside), hesperidin, diosmin. The four concentrated in the extracts in a manner depending on solvent type. Rosmarinic acid and cynaroside were extracted efficiently in WA extracts while hesperidin and diosmin (methoxyflavonoids) accumulated in MeOH extracts (hesperidin is extracted most efficiently by MeOH US method).

Rosmarinic acid content were in significant correlation with both of two antioxidant indices, depending on extract type, $0{,}3550<R<0{,}5555$, $p<0{,}01$. Cynaroside content in most cases is not in correlation with antioxidant properties, though this flavonoid has been demonstrated to be effective antiradical agent (Burda and Ołeszek (2001), our measurements with its standards corroborated this data). Neither hesperidin, nor diosmin was in correlation with AO parameters.

For each plant samples, the four extracts meant four concentration data to every phenolics. The maximum of these 4 values were taken into consideration as highest extractable concentration (C_{max}). C_{max} values of the identified phenolics in the 36 samples are shown on Figure 1. The variability in polyphenol composition of the 36 plant samples indicated by total polyphenol and AO parameters is demonstrated. For rosmarinic acid, interval of C_{max} is 7043 mg/kg dp (*EGR3*)-38667 mg/kg dp (*MBA2*); for cynaroside 319 mg/kg dp *(JOF*)-2944 (*JÁV*); for hesperidin, 7056 mg/kg dp (*EGR3*) - 29518 mg/kg dp (*HOR1*), and for diosmin, 398 mg/kg dp (*FET*)-7987 mg/kg dp (*DOM*). There is heterogeneity among populations in terms of each four components (CV>20%).

Figure 1. Distribution of the four identified phenolics in the sample collection of 2016.



Taking into account the four identified phenolics, the two flavones are dominant in none of the plant samples. Rosmarinic acid dominates 20 of the 36 samples as its C_{max} is 2-8 times larger than of hesperidin. In further eight samples, rosmarinic acid:hesperidin ratio is 1,25-1,5, and in two cases. (HOR3 and EGR3) 1,0-1,1. Four samples were hesperidin-based. (HOR1, KBT, MAC és MÁH2) In these, hesperidin:rosmarinic acid ratio is 1,5-2. The groups of samples with different rosmarinic acid:hesperidin ratios do not build habitat-dependent or geographical groups.

Total polyphenol content and antioxidant properties of the cultivated horsemint

A common feature of the cultivated accessions was observed, as all of them has shown the lowest total polyphenol and antioxidant values in the year of their plantation to Eger (2018). Next year total polyphenol contents increased 1.9-3.5 times in an accession-dependent manner. Antioxidant activity increased simultaneously. Interval of EC_{50} changed from 400-1000 mg/l to ~220-600 mg/l in four accessions of the five. FRAP values increase from ca. to 4500-10 000 mg AAE/kg dp ~6000-12 000, thus approximates BHT (12169 mg AAE/kg) better. Accessions grown in Soroksár show stronger AO parameters and higher total polyphenol contents in their 1st year (2019) as the Eger accessions in 2018. Thus, their results move in less wide intervals. Accession HOR1 had the lowest total polyphenol content. HOR2 showed in 2019-2020 the highest total polyphenol content (in some cases it is ca. 92 000 and 105 000 which is higher than literature data). In addition, HOR2 demonstrated the best antioxidant values. It had 220-350 mg/l EC_{50} and 9000-15 000 mg AAE/kg FRAP values in a major part of the vegetative cycle. EGR3 had in 2019-2020 the second highest total polyphenol content (~50-83 000 mg GAE/kg) and third highest antioxidant activity. (~50-83 000 mg GAE/kg). DOM is the second in these in these terms from the five accessions. In 2019-2020 it is characterized by low EC_{50} (243-395 mg/l) and relatively high 7475-12204 mg AAE/kg FRAP values. Finally, KBT is mediocre in terms of both polyphenol content and antioxidant activity.

Phenolic composition of the cultivated horsemint accessions

In comparison to the wild-growing samples, we could identify a higher number of polyphenols in the samples of the experimental cultivation. These were rosmarinic acid (*caffeic acid derivatives*), eriocitrin and hesperidin (*flavanones*); lonicerin, cynaroside and diosmin (*flavones*). Their concentration is observed to be influenced by the accession (genetical background), the ontogeny of the plants and some environmental factors.

a) Characteristics of the polyphenol profile depending on accession

Kruskal-Wallis test elucidated significant differences in the polyphenol composition (especially in flavonoid profile) of the accessions, which are independent from environmental and ontogenetical factors. Thus, these features may be probably genetically determined. Based on these observations a proposal was made to distinguish polyphenol chemotaxa of *Mentha longifolia* (although *Mentha* spp. are usually classified on the base of their essential oil composition. Figure 2 gives a summary of these polyphenol chemotaxa.

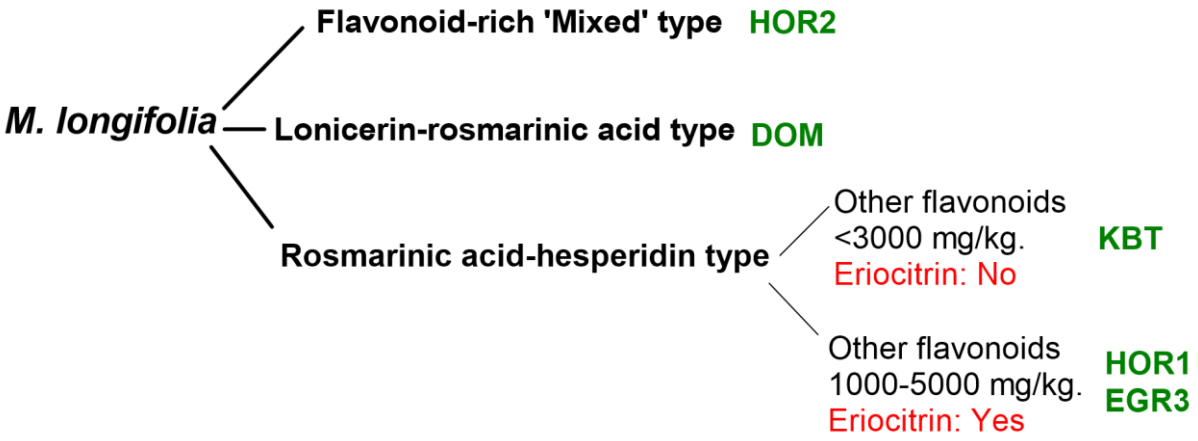


Figure 2. The proposed polyphenol chemotaxa of Mentha longifolia. *The accessions representing each of them are marked with green font.*

HOR1, EGR3 and KBT represent a rosmarinic acid-hesperidin type. KBT may be classified as a subgroup of this type as it produces less flavonoids than HOR1 and EGR3. KBT is the only of the five accessions that do not contain eriocitrin.

HOR2 is the richest in flavonoids among the five accessions. It can be described as a 'mixed' type. It was the richest in hesperidin if the sample collection of 2016 is regarded. In cultivation, lonicerin is a dominant component of (ca. 17-30 000 mg/kg dp), together with rosmarinic acid (ca. 10-27 000 mg/kg dp). In the 2nd year of each plantation, eriocitrin became also a dominant component (Eger: up to ca. 46 000, Soroksár: up to ca. 27 000 mg/kg). HOR2 provides an example to that there can be hereditary patterns in the yearly changes of the concentrations of a polyphenol. Concentration of rosmarinic acid in the samples of HOR2 in 2019 and 2020-ban reached maximum in the spring vegetative shoots, then decreased. In in full bloom, it reached a second peak and in fruit ripening decreased again. This pattern with the 'double maxima' was observable at both cultivation sites and is different from the other four accessions; also from the literature data of some mint lines specialized to rosmarinic acid production (Fletcher et al. 2010) because the concentration of rosmarinic acid usually decrease when generative organs begin to develop. Accesion DOM can be classified as a lonicerin-rosmarinic acid type (accumulating both compounds between 10-35 000 mg/kg dp). Additionally, the wild-growing sample collection of 2016 this accession demonstrated the highest C_{max} value of diosmin (7987 mg/kg dp). This trait in the polyphenol profile also refer to a flavone-rich type included by the species. A marked difference of the polyphenol profile of DOM in comparison to the other flavonoid-rich mint HOR2 is that DOM do not produce flavanones in as high concentration as HOR2.

b) Ontogeny-related concentration changes of some phenolics in cultivated horsemints

There were observable changes in the concentration of three major polyphenols, related to the phenophases of the plant and in one case, to the age of the plant.

Concentration of rosmarinic acid usually reached its yearly maximum in the spring vegetative shoots (except the Soroksár plot of accession KBT where the green bud (VK) phenophase brought the maxima). After the yearly maximum, rosmarinic acid concentration decreased following an even pattern (EGR3) or fluctuated (HOR2) in an accession-dependent manner.

Concentration of eriocitrin also shows a specific pattern. We observed that both eriocitrin-producing accessions (HOR1, HOR2, EGR3, DOM) accumulate this flavanone only from the 2nd year of the plots, with no regard to the location. Accumulation of eriocitrin is high primarily in those phenophases when generation and development of shoots is intensive. Thus, its yearly maximum occurred in spring vegetative (L1), in green buds stage or at full bloom.

Concentration of lonicerin culminates in most cases at green bud stage (VK) or at full bloom (V). In a couple of cases it reaches the yearly maximum in spring vegetative (L1) shoots. A common property is at both five accessions is that the lonicerin concentration in fruit ripening (T) shoots are decreased or even means the yearly minimum.

c) Concentration changes of some phenolics related to environmental factors

The accessions which are may be classified to a common rosmarinic acid-hesperidin chemotaxon demonstrated high (KBT, EGR3) or extreme rosmarinic acid concentrations (HOR1). These values (ca. 42 000-56 000 mg/kg dp) were detected only in Eger, and with one exception in the spring vegetative shoots of 2019. Meteorological data revealed that before the collection of these shoots there was extremely rainy and cold weather in Hungary. Since 1901, May 2019 was the third highest precipitation sum in comparison with the same month of the past years. In addition, it was the coldest May since 1991. In Eger, a remarkable decrease in sunlight was also observed in that month in comparison with May 2018 or 2020. An investigation of correlations on the datasets of Eger (75 samples) revealed a weak but significant negative correlation between the rosmarinic acid contents and 7-day sum-of-temperatures: $\mathbf{r_s}$=-0,298, p=0,009 (**). This may refer to that the low temperatures enhance rosmarinic acid production. No correlations were detected between the presence of flavonoids in horsemint and weather parameters. However, location-and accession-related differences in two major flavonoids were observed. Lonicerin production of HOR1, HOR2 and DOM was remarkably higher in Eger. Similarly, eriocitrin accumulation of HOR2 and DOM was higher in this site.

Essential oil yield, composition and chemotypes of the cultivated horsemint accessions

Accession KBT has shown the highest essential oil (EO) yield (1.78-2.08 ml/100 g dp) and EGR3 the lowest (0.87-1.25 ml/100 g dp). Significant difference was between EO yields regarding 2019 and 2020 (Wilcoxon's test value Z = -2,497; p=0,013) despite of the difference of the habitats and the age of the plants at the two plantations. The warm weather and the high irradiance before the 2019 V (full bloom) sampling may be an enhancing factor in the essential oil production of all accessions, on both cultivation habitats.

The essential oil polychemism of the species manifested in the results of these study markedly. Accession HOR1 do not contain limonene metabolites. Carvacrol (19.28-20.56%), 1,8-cineole (14,87–17.45%), thymol (13.36–13,90%), carvacryl acetate (8.81–10.40%) and para-cymene (7.24–8.01%) are present. Regarding the coefficient of variation (CV) calculated to the concentration of each compounds, these are homogeneous (CV<20%) or extremely homogenous (<10%). Thus HOR1 may represent a novel EO chemotype of horsemint. Till May 2022 there are four records are available about horsemint EO batches rich in γ-terpinene-metabolites (Mimica-Dukić et al. 1993, Serbia; Hassanzadeh et al. 2011, Iran; Akşit et al. 2013, Turkey; Ćavar Zeljkovic et al. 2021, Slovakia). None of them is uniform with HOR1.

Accession HOR2 demands further investigation in terms of EO chemotaxonomy. Three of its four samples is based of cis-dihydrocarvone (47.57–57.06%) accompanied by the trans isomer (9.93–12.28%). In contrast, the HOR2 EO sample from 2020, Soroksár was based on thymol (19.79%) 1,8-cineole (14.93%), γ-terpinene (9,36%), para-cymene (7.22%). Besides horsemint, a couple of data on its hybrid native spearmint (M. x spicata L.) is found to produce γ-terpinene type, cineole/γ-terpinene or cineole/γ-terpinene/limonene-2-oxo essential EOs (Stoeva & Ilyev 1997; Kizil & Tonçer, 2006). But there were no previous data to a similar rearrangement like in HOR2, neither from horsemint nor from native spearmint.

Accession EGR3 is based on cis-piperitone epoxide (44,2-55,34%), 4-5% 1,8-cineole and among the accessions it is the richest in sesquiterpenes (up to 328% in total). Therefore, it is a cis-piperiton epoxide/β-caryophyllene/germacrene D/1,8-cineole chemotype. No similar combination was found in the literature till now.

Accession DOM is rich in menthone (47-64%), isomenthone (9.57-15.14%) β-caryophyllene and germacrene D (5-10%).

Similarly, EO composition of accession KBT is typical to horsemint. Stable percentages of menthone, isomenthone and β-caryophyllene were detected, accompanied by fluctuating proportions (5-16%) of pulegone.

IV. CONCLUSIONS

Polyphenol composition of the wild-growing horsemint populations

No geographical or habitat-based groups were observable in the polyphenol profile and antioxidant indices of the samples collected in 2016 from the 36 wild-growing populations. Even populations in close proximity (e.g. EGR2-EGR3, MÁH1-MÁH2) can give plant material with highly different composition while their morphological characters are similar. This finding demonstrates that the wild-collected horsemint does not give constant quality, thus selection and cultivation is needed for the industrial utilization of the species. Cultivation also may assure the maintenance of the lines bearing utilizable chemical composition and the control of the environmental contamination of the plant material.

Our results also show that the chemosyndromes of the species can be judged properly only if the studied populations are grown and analyzed at the same habitat.

The investigation of different extraction methods concluded to that if the high antioxidant (AO) activity and polyphenol content of the extracts is required, hydroalcoholic ultrasound extraction is the most efficient. Methoxyflavonoids (hesperidin and diosmin) were not extracted totally by this method. However, as the correlation between the concentration of these compounds and in vitro AO properties were weak and not significant, their low yield may not be a major disadvantage if horsemint is processed as a source of industrial antioxidants.

Polyphenol profile of the horsemint accessions involved to experimental cultivation

The total polyphenol content of the investigated five accessions (in decreasing order) can be ranked as follows: **1**. HOR2, **2**. EGR3 and DOM, **3**. KBT, **4**. HOR1. If antioxidant properties are regarded, the order of rank is similar: **1.** HOR2, **2**. DOM, **3**. EGR3, **4**. KBT, **5**. HOR1.

In the five accessions, predominatly the same flavonoid pathways may be present, indicated by their products:

a) generation of eriocitrin (in 4 accessions in the total 5)

b) *the hesperetin pathway pere* in all accessions, indicated by hesperidin

c) *the luteolin pathway* as presence of lonicerin, cynaroside and diosmin demonstrate it

Despite the qualitative polyphenol profile of the five accessions is built by rosmarinic acid and the same flavonoids, their polyphenol composition is significantly different (in quantitative terms), especially if flavonoids are regarded. These (e. g., the total lack of eriocitrin in accession KBT) are independent from ontogeny, year and habitat, thus may be hereditary. Therefore, polyphenol chemotaxa may be distinguished in *Mentha longifolia* (Figure 2).

No previous literature dealing with cinnamate-based chemotaxonomic classification of *Mentha* spp. were found.

Three accessions (KBT, HOR1, EGR3) were dominated by rosmarinic acid and hesperidin. These gave the batches bearing the highest rosmarinic acid content (42 000-56 000 mg/kg dp). However, their results also demontstrated that these high concentrations are present only in the spring vegetative shoots; when generative organs develop, the rosmarinic acid concentration fluctuates. If flavonoid composition is regarded, this rosmarinic acid/hesperidin chemotaxonomic group may be divided to two subgroups. HOR1 and EGR3 build one of these, as they produce all other identified flavonoids as hesperidin, but only in <5000 mg/kg dp amounts. KBT means the other subgroup as it does not accumulate eriocitrin and is poor in flavones (usually <3000 mg/kg dp).

The further two accessions HOR2 and DOM are characterized by high concentrations of flavonoids, both flavanones and flavones. HOR2 was the 2nd highest in hesperidin when the wild-growing sample collection of 2016 is regarded. In cultivation, HOR2 is dominated by lonicerin (up to 30 000 mg/kg dp) and after the 2nd year after planting, eriocitrin up to46 000 mg/kg dp. Further characteristic of HOR2 is the highest cynaroside content among the five experimental accessions (9116 mg/kg dp). Another unique trait in the chemism of HOR2 is that its rosmarinic acid concentrations showed double maxima. After the yearly maximum (usually in L1 phase) the concentration decreased but in full bloom, it reached a second peak. DOM is as rich in lonicerin as HOR2 but it can be distinguished by the lesser production of flavanones (eriocitrin contents not higher than 8-10 000 mg/kg dp).

Our results also may refer to that the function of the different phenolics in the horsemint plant's physiology is not the same (and if the literature is surveyed it is not clear). An example is the common property of the eriocitrin-producing accessions: they do not accumulate this flavanone in their first year after plantation. Thus, the physiological role of eriocitrin may differ from the function of hesperidin as the latter accumulated continuously in the plants.

Based on our results, the optimal harvesting time of the horsemint as a polyphenol (antioxidant) crop may be in the spring vegetative phase in the 2nd year after plantation. This differs from the optima of harvesting mints as essential oil crops.

Essential oil composition of the horsemint accessions in the cultivation experiment

According to the literature of species, the typical essential oil constituents are limonene-3-oxo ketones and epoxides (but menthol is rare) and limonene-2-oxo metabolites. Three of the investigated five accessions corroborated it, although EGR3 shows an unique combination of typical compounds. However, HOR1 represents a previously unknown chemotype of horsemint. It is based on cymyl compounds and 1,8-cineole thus it is atypical in the species and also in the *Mentha* genus. Chemotype of HOR2 could not be defined because of highly variable concentrations of dihydrocarvone, thymol and 1,8-cineole regarding the different years and cultivation sites. The essential oil chemotypes and polyphenol chemotaxa of the accessions observed to be not uniform. For example, the accessions classified as a 'rosmarinic acid/hesperidin type' (HOR1, EGR3, KBT) represent three different essential oil chemotypes (Table 4).

Table 4 Essential oil chemotypes and polyphenol chemotaxa of the five accession

Accession	Essential oil chemotype	Polyphenol chemotaxon
HOR1	Carvacrole/carvacryl-acetate/Thymol/1,8-Cineole	Rosmarinic acid/hesperidin based, eriocitrin and flavones in low concentrations
HOR2	Not defined. (Dihydrocarvones vs. thymol)	'Mixed' type, rich in flavanones, luteolin glycosides and rosmarinic acid.
EGR3	*cis*-Piperiton epoxide/β-Caryophyllene/Germacrene D	Rosmarinic acid/hesperidin based, eriocitrin and flavones in low concentrations
DOM	Menthone/Isomenton	Flavone-rich. Lonicerin- and rosmarinic acid-dominated.
KBT	Menton/Izomenton/Pulegon	Rosmarinic acid/hesperidin based. No eriocitrin. Poor in flavones.

V. NOVEL SCIENTIFIC RESULTS

Novel scientific results

1) Four flavonoids of the species were quantified in a reliable manner for the first time. Hesperidin established to be present up to 30 000 mg/kg dp, dioismin up to ca. 8000 mg/kg, eriocitrin up to 46 000 and lonicerin up to 33 000 mg/kg dp, high enough for practical utilization of horsemint.

2) Regarding the polyphenol composition of the five accessions, significant differences were detected, independently both from environmental and ontogenetical factors. Based on these, polyphenol chemotaxa were distinguishable in *Mentha longifolia*. Three accessions (KBT, HOR-1, EGR3) may be included by a 'rosmarinic acid/hesperidin' chemotaxon. One (HOR-2) is rich both in flavones (lonicerin, cynaroside) flavanones (eriocitrin, hesperidin) and rosmarinic acid, thus it was described as 'mixed type'. Accession DOM is classified as a rosmarinic acid-lonicerin type.

3) Two novel essential oil chemotypes of the species were determined. From accession HOR1, carvacrol acetate was detected as a major essential oil constituent for the first time. It was accompanied in the essential oil by high percentages of carvacrol, thymol, 1,8-cineole, and para-cymene which are atypical not only in the species but in the entire *Mentha* genus. Similarly, accession EGR3 combining high percentages of cis-piperiton epoxide and sesquiterpenes shows a new chemotype. Our results refer to that polyphenol and essential oil chemotaxa in the species are independent.

Novel results from practical viewpoint

1) A proposal was made to the optimal harvest time of the horsemint as a polyphenol crop. Simultaneous evaluation of total polyphenol, DPPH EC_{50}, FRAP, rosmarinic acid, eriocitrin and lonicerin concentration datasets using a rank-based calculation assembled by the author, the optimum may be reached in spring vegetative shoots. This finding differs from the well-known optima of harvesting mints as essential oil crops.

2) Regarding utilization, further investigation of three horsemint accessions is proposed. These are HOR1 as a new source of cymyl compounds, HOR2 because of its rich polyphenol production and adaptivity and EGR3 as the base of a potential cultivar specialized to rosmarinic acid production.

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