Wild mint (*Mentha longifolia*) extracts in the production of non-alcoholic beverages

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Summary. Isolates of medicinal and aromatic herbs are used as additives in non-alcoholic drinks to improve the sensory characteristics and antioxidant potential. The method of drying the plant material, among other factors, has a profound influence on the chemical composition and pharmacological activities of plant extracts. This paper analyzes the effect of the drying technique (I - natural drying, II - in the laboratory oven, III - in low-temperature condensational drier) on the volatile fraction of the wild mint (*Mentha longifolia* (L.) Hudson) extract, in order to select an extract of the best quality for preparation of non-alcoholic drinks. The volatile profile of the extracts was determined by the GC-FID and GC-MS techniques, the antimicrobial activity by the microdilution technique, the antioxidant activity by the DPPH and FRAP assays, and the sensory acceptance according to the *Regulation on quality for refreshing non-alcoholic beverages*. The results showed that piperitone was the major component of the volatile fraction extract of the natural drying herb and low-temperature condensational drier herb (53.9% and 61.1%, respectively), while the extract of laboratory oven herb was rich in menthone (35.5%). At the concentrations in the range of 0.8-3.2 mg/mL the extracts better inhibited the Gram (+) bacteria. The beverage to which the extract of naturally dried wild mint was added, showed the antioxidant activity (9.09±0.17 μ mO Fe²⁺/mL by FRAP and 14.00±3.00 μ L/mL by DPPH method) and good sensorial characteristics (concentration of the extract 0.8 g/L).

Key words: Mentha longifolia, drying, antioxidant activity, piperitone, beverage

Introduction

One of important product categories within the functional food segment is non-alcoholic beverages fortified with vitamins or other functional ingredients (1). For the preparation of non-alcoholic beverages with desired sensory and biological characteristics, medicinal and aromatic herbal raw materials (with different biological activities) are added. Species of the genus Mentha and the family Lamiaceae have enjoyed a rich tradition of use for flavouring, food preservation, and medicinal purposes, due to both their curative and their preventive properties (2).

Drying, as one of the oldest complex processes of food conservation, represents a very important phase in processing of medicinal and aromatic herbs. In conventional hot air-drying, high temperatures and long drying periods can cause thermal degradation or volatilization of important flavour compounds (3). Since there are negative concerns regarding the use of synthetic ingredients for food preservation, natural alternatives such as the addition of plant extracts rich in phenolics are gaining popularity among consumers (4).

The influence of the effects of different drying methods on the yield and chemical composition of the essential oil obtained from the herb Mentha longifolia (L.) Hudson (wild mint, horsemint), was studied showing significant differences mainly concerning the chemical composition. Piperitone was the major compound in the all three essential oil from herb (low temperature drying 71.7%, natural drying 50.8% and laboratory drying oven 43.1%) (5). Besides volatile fraction (6), the non-volatile fraction of the ethanolic extract, in terms of chemical composition and antioxidant activities was assessed by Stanisavljević et al. (7) and the highest antioxidant activity and the greatest content of the total phenolics and flavonoids were found for the extract obtained from the raw material dried naturally.

In this paper the impact of different drying techniques on the composition and activity of ethanolic extracts of the herb *M. longifolia* was analysed aiming to select high quality isolates in order to be applied in the production of non-alcoholic beverages. Functional benefits of herbal extracts may provide value-added products and benefits to consumers.

Materials and Methods

Chemicals

All chemical substances were of analytical purity: Ethanol (Zorka-Pharma, Serbia), DMSO (dimethyl sulfoxide) and DPPH (2,2-diphenyl-1-picrylhydrazyl) (Sigma Chemical Company, USA), TPTZ reagent (2,4,6-tripyridyl-s-triazine) (TCI Europe, Belgium), Iron(II)sulfate-7-hydrate (VWR Prolabo, Belgium), Müller-Hinton and Sabouraud broth, Ampicillin, Amikacin and Nystatin (Torlak, Serbia), Sucrose and Citric acid (from the market).

Plant material

The *M. longifolia* herb in the phenological phase of blooming was gathered from the region of the municipality of Prokuplje (Rastovnica, 400 m.a.s.l., Serbia). The voucher specimen (N°16469) was deposited

at the Herbarium of the Institute of Botany and Botanical Garden "Jevremovac" (Faculty of Biology, University of Belgrade).

Drying

The herb material was dried under three different types of drying techniques: I - natural drying (ND) in the shade on a draughty place for 15 days, II - in the laboratory oven (LOD) (Stockli, Switzerland) at 45°C for 2 days, III - in low-temperature condensational drier (LTCD) (LT-CD/60S, Freon Eko Kragujevac, Serbia) at 35°C for 2 days.

Extraction

Dried plant material was grinded in the electric coffee mill and extracted by the modified pharmacopoeia procedure (8) of the single percolation by ethanol 70% V/V as a solvent. Chopped herb (50 g) was soaked in Erlenmeyer with solvent (25 mL) and left two hours so that the herb could absorb the solvent and swell. Wet herbal material was moved to percolator, basted by a certain quantity of the same solvent and left at the room temperature for 24 hours. The quantity of solvent was determined by the preliminary extraction. The extract from the percolator was released in the Erlenmeyer flask at the regulated speed, 1-3 mL per minute. By the process of the one way percolation, the obtained quantity of the extract compared to the beginning quantity of the herb raw material was 2:1. Liquid extract was used for GC analysis. For all other analyzes dry extract was used. The obtained percolate was evaporated in the rotary vacuum evaporator (Ika-Werke, D-79219 Staufen, Germany), at 50°C, till dryness. Dried extract was milled into fine powder using the mortar with a pestle, dried into the vacuum-drier at 50°C until the constant mass and was kept in well closed glass vessels, on the dry, cold and dark place.

GC-FID and GC-MS analysis

Chromatographic analyses of the extract volatile fractions were performed by GC-FID and GC-MS techniques.

GC-FID analysis was carried out on a Hewlett-Packard system (HP-5890 Series II gas chromatograph), equipped with split-splitless injector and automatic liquid sampler (ALS), attached to HP-5 column (25 m × 0.32 mm, 0.32 µm film thickness) and fitted to flame ionization detector (FID). Carrier gas flow rate (H₂) was 1 mL/min, injector temperature was 250°C, detector temperature 280°C, while column temperature was linearly programmed from 40-260°C (at rate of 4°C/min), and held isothermally at 260°C next 10 minutes. Undiluted extract were consecutively injected by ALS (1 µL, split mode, 1:30). Area percent reports, obtained as result of standard processing of chromatograms, were used as base for the quantification purposes. All measurements were performed in triplicate, and the results were presented as the mean values. Statistics has been covered by FID specification (results with a range of deviation for the level 1%).

The same analytical conditions as those mentioned for GC-FID were employed for GC-MS analysis, along with column HP-5MS (30 m × 0.25 mm, 0.25 μ m film thickness), using HP G 1800C Series II GCD system (Hewlett-Packard, Palo Alto, CA, USA). Helium was used as carrier gas. Transfer line was heated at 260°C. Mass spectra were acquired in EI mode (70 eV), in m/z range 40-450. Sample solutions were injected by ALS (1 μ L, split mode, 1:30).

The constituents were identified by comparison of their mass spectra to those from Wiley275 and NIST/ NBS libraries, using different search engines. The experimental values for retention indices were determined by the use of calibrated Automated Mass Spectral Deconvolution and Identification System software (AMDIS), compared to those from available literature (9) and used as additional tool to approve MS findings.

Antimicrobial activity

The minimum inhibitory concentration (MIC) was determined by the broth microdilution method (10). Tests were performed in Müller Hinton broth for the bacterial strains, and in Sabouraud dextrose broth for *Candida albicans*. Further, the sensitivity of examined microorganisms to standard antibiotics ampicillin, amikacin and nystatin was evaluated.

The study was conducted against nine different bacterial strains: *Micrococcus luteus* ATCC 9341, *Micrococcus flavus* ATCC 10240, *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 12228, *Enterococcus faecalis* ATCC 29212 and *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 25922, *Klebsiella* pneumoniae NCIMB 9111, Pseudomonas aeruginosa ATCC 27853; and two strains of yeast Candida albicans (ATCC 10259 and ATCC 24433).

The dry extract was dissolved in DMSO and it was tested in the concentration range 0.81-30 mg/mL. Positive control of bacterial growth was set in the test as well as the sterility control of extract. Plates were incubated in aerobic atmosphere for 24 h at 37°C for bacteria and 48 h at 26°C for *Candida albicans*. Subsequently, the growth of bacteria and yeast was recorded semiquantitatively as turbidity of the medium and pellet at the bottom of the wells.

The preparation of non-alcoholic beverages

The base for beverages was made as the water solution of sucrose and citric acid (14% and 0.4%, respectively). The beverages were prepared with different percent of dry extract (0.6, 0.7, 0.8, 1.0, 1.2 g/L) obtained from naturally dried herbs ND (7), and kept in well closed glass dishes at dry, cold and dark place.

Antioxidant activity of non-alcoholic beverages

The antioxidant activity of the prepared nonalcoholic beverage as finished product was evaluated by FRAP and DPPH assays. For the Ferric Reducing Antioxidant Power (FRAP) assay, an aliquot of 0.1 mL of the sample was added to 3.0 mL of freshly prepared FRAP reagent. The measures were taken at 593 nm and the calibration curve was prepared using ferrous sulphate as the standard (11).

For determining antioxidant activity of samples the series of solutions in the range of concentrations 10.0; 50.0; 100.0; 500.0; 1000.0 μ Lof sample/mL were made for finshed product and DPPH radicals of 0.3 mM in 70% ethanol. 1.0 mL of DPPH radical solution and 2.5 mL of already prepared samples of finished product of different concentrations were mixed (12, 13):

The capacity for neutralizing DPPH radicals (%) = = $100 - [(A_s - A_b) \times 100/A_c]$ (1)

where: A_s - absorbance in the presence of the product in the DPPH solution, A_c - absorbance of the control solution (containing only DPPH) and A_b - absorbance of the sample product solution without

DPPH. The EC_{50} value was calculated according to the experimental data by the use of the sigmoidal noncurve method and SigmaPlot 2000 Trial software.

The content of total phenolics in non-alcoholic beverages

The content of total phenolics was determined by Folin-Ciocalteu reagent. 0.2 mL of non-alcoholic beverage (concentration of 0.2 mL of beverage/mL of solution) was mixed with 1 mL of Folin-Ciocalteu reagent and 0.8 mL of 7.5% of water solution of Na₂CO₃. After thirty-minute incubation at room temperature and dark place, the absorbance of reactive compound was measured at 765 nm at spectrometer "VARIAN Cary-100". The content of total phenolic compounds was obtained by the equation of curve (R=0.9919) with gallic acid as a standard shown as mg of gallic acid/mL of beverage:

Absorbance=7.2328
$$c_{gallic acid}(\mu g/mL) - 0.2286$$
 (2)

Calibration was carried out with standard solutions of gallic acid. Regarding calibration curve and absorbance of the examined samples the content of total phenolics in samples was obtained.

The content of flavonoids in non-alcoholic beverages

The content of total flavonoids was determined by the spectrometric method. 0.1 mL of the solution of aluminum(III) - chloride (10%), 0.1 mL of the solution of potassium acetate (1M) and 2.8 mL of distillated water were added into 2.0 mL of non-alcoholic beverage (conc. of 0.2 mL of beverage/mL of solution). After thirty-minute incubation at room temperature the absorbance of reactive compound was measured at 415nm in relation to distilled water. Rutin was used as a standard, and the total content of flavonoids was shown as mg of rutin/mL of beverage and was determined by the equation of curve (R=0.9994):

Absorbance=
$$12.722 c_{rutin} (\mu g/mL) + 0.0034$$
 (3)

Sensory evaluation of non-alcoholic beverages

The quality of readymade products was defined by sensory evaluation. The evaluation committee was composed of 15 members, the experts from the field of food technology, age between 30 and 65. The samples were offered to the evaluators at 20°C, in transparent glasses. Four attributes were evaluated: color intensity (max. 4 points), homogeneity (max. 4 points), fragrance intensity (max. 5 points) and taste (max. 7 points). Evaluation of the quality of the non-alcoholic beverages was carried out according to the *Regulation* on quality for refreshing non-alcoholic beverages (Serbian national regulative) (14).

Results and Conclusions

Chemical composition of volatile fraction of *M. longifolia* extracts are shown in Table 1. The greatest differences in the volatile profile of the extracts can be noticed in the content of piperitone, menthone and *iso*-menthone.

It is noticeable that monoterpenoids were found to be the most abundant class of compounds identified (ND 76.7%, LOD 77.7%, LTCD 81.4%), followed by sesquiterpenes. Chromatographic analysis showed that in ND and LTCD extracts the monoterpene piperitone was the major compound (53.9% and 61.1%, respectively). In the LOD extract was observed the lowest content of piperitone (18.5%), while the presence of menthone was recorded in the LOD extract as the dominant component (35.5%). Because of that, plant species from this locality can be inserted in the pipetitone chemotype. In the previous study of the essential oil of the same plant species, the authors found similar results (5). Piperitone dominated in all three oils (LTD 71.7%, ND 50.8%, LOD 43.1%), carvone in ND oil (20.0%), and menthone and iso-menthone in LOD oil (17.5% and 8.3%, respectively). It is obvious that there is an agreement in the content of menthone, isomenthone and piperitone in extracts and essential oils, and way, temperature and drying time have an impact on the chemical composition of the studied isolates. Analyzing the essential oil M. pulegium originated from Iran (15), piperitone as the main component was obtained (38.0%).

According to the *Regulation on quality for refreshing non-alcoholic beverages* (16), the use of pulegone in food and beverages has limits of: 100 mg/kg for mint/ peppermint containing alcoholic beverages; 20 mg/kg for mint/peppermint containing non-alcoholic bever-

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Constituents (%)	KIE	KIL	ND	LOD	LTCD
α-thujene ^m	933	924	0.6	-	0.4
α-pinene ^m	n/a	932	0.9	0.9	0.8
sabinene ^m	973	969	-	-	0.4
β-pinene ^m	976	974	0.7	0.7	0.8
myrcene ^m	994	988	1.0	1.6	0.5
3-octanol ^m	1005	988	1.5	0.9	0.9
limonene ^m	1029	1024	1.9	0.2	2.0
1,8-cineole ^m	1032	1026	2.9	3.5	4.6
<i>cis</i> -β-ocimene ^m	1041	1032	1.1	0.6	0.7
benzene acetaldehyde	1051	1036	0.4	-	0.3
menthone ^m	1154	1148	-	35.5	-
<i>iso</i> -menthone ^m	1165	1158	0.7	9.5	-
α -terpineol ^m	1195	1186	0.4	-	0.4
<i>cis</i> -dihydrocarvone ^m	n/a	1191	0.7	0.6	1.3
<i>trans</i> -dihydrocarvone ^m	1200	1200	1.3	0.6	0.4
(3 <i>Z</i>)-hexenyl 3-methyl butanoate	1239	1232	0.5	0.3	0.3
pulegone™	1243	1233	0.3	0.2	-
carvone ^m	1247	1239	3.0	1.7	4.1
piperitone™	1257	1249	53.9	18.5	61.1
<i>cis</i> -piperitone epoxide [™]	1258	1250	0.7	0.2	-
<i>trans</i> -piperitone epoxide ^m	1258	1252	0.5	-	0.2
carvacrol ^m	1303	1298	3.3	2.3	2.1
6-hydroxy-carvotanacetone ^m	1308	1309	1.0	-	0.5
<i>para</i> -vinyl guaiacol ^m	1322	1309	0.3	0.2	0.2
9-decenoic acid*FAD	1359	1359	0.8	0.2	-
β-bourbonene ^s	1386	1387	1.2	0.6	0.9
<i>trans</i> -caryophyllene ^s	1420	1417	4.7	2.4	5.6
α-humulene ^s	n/a	1452	0.7	0.6	-
<i>cis</i> -muurola-4(14),5-diene ^s	1459	1465	0.6	0.7	0.5
γ-muurolene ^s	1483	1478	3.4	2.0	3.6
bicyclogermacrenes	1498	1500	0.5	-	0.6
<i>n</i> -hexadecanoic acid ^{FAD}	1970	1951	0.4	0.6	-
ethyl hexadecanoate ^{FAD}	1999	1992	0.6	1.6	0.6
phytol ^d	2118	2114	1.7	1.4	2.0
cis-9, cis -12-octadecadienoic acid (linoleic acid)*FAD	2173	2132	0.9	2.3	0.8
Sum of contents (%)			92.9	90.5	96.4

Table 1. The chemical composition of volatile fraction of *M. longifolia* extracts

% (w/w) – mass percent defined by peak area percent determined by integration (GC-FID); m – monoterpenoids, s – sesquiterpenoids, d – diterpenoids, FAD – fatty acids and fatty acid derivatives; KIE – Kovats (retention) index experimentally determined (AMDIS), KIL – Kovats (retention) index, literature data, n/a – not available, * – tentative identification ages. As a pure ingredient, pulegone may not be added to foodstuff. The low content of pulegone in the studied isolates could be considered as a premium criterion due to extremely toxic properties, especially a high abortive potential and possible carcinogenic effect for humans (17). On the basis of the content of certain components it can be concluded that the quality of the extract depends on the drying process. This is specifically valid with the use of the laboratory oven at 45°C. At higher temperature the decrease in the content of piperitone was noticed, as well as of limonene, *cis*- β ocimene, α -terpineol, carvone, β -bourbonene, *trans*caryophyllene and γ -muurolene.

The results of antimicrobial activity of *M. longifolia* extracts and antibiotics against microorganisms are presented in Table 2.

Hand-made food products, which did not go under a thermal process, are at risk of contamination by Staphylococcus spp., which proves the significance of examining the antimicrobial activity of extracts. It is well known that the antimicrobial activity is the result of the presence of terpenes and their common influences (18). The examined extracts have shown moderate to low antimicrobial activity. The most sensitive were Gram (+) bacteria: M. luteus, M. flavus, S. aureus and S. epidermidis. Mahboubi and Haghi (15) stress the significance of piperitone as the oil component which has shown the germicide and antimicrobial effect especially against the Gram (+) bacteria S. aureus, while the least sensitive were Gram (-) bacteria, especially Escherichia coli. The present study confirmed the antifungal activity of Serbian M. longifolia extracts, as well.

Table 2. Antimicrobial activity of M. longifolia extracts

	MIC									
Microorganism	Amp. (μg/mL)	Amk. (µg/mL)	Nys. (µg/mL)	ND (mg/mL)	LOD (mg/mL)	LTCD (mg/mL)				
Micrococcus luteus ATCC 9341	3.6	n.t.	n.t.	0.8	0.8	0.8				
Micrococcus flavus ATCC 10240	n.t.	n.t.	n.t.	1.6	1.6	1.6				
Staphylococcus aureus ATCC 25923	4.8	n.t.	n.t.	1.6	3.2	1.6				
Staphylococcus epidermidis ATCC12228	2.6	n.t.	n.t.	1.6	1.6	1.6				
Enterococcus faecalis ATCC 29212	4.0	n.t.	n.t.	15.0	7.5	15.0				
Bacillus subtilis ATCC 6633	3.2	n.t.	n.t.	7.5	7.5	7.5				
Escherichia coli ATCC 25922	6.4	n.t.	n.t.	7.5	7.5	7.5				
Klebsiella pneumoniae NCIMB-9111	8.6	5.2	n.t.	7.5	7.5	7.5				
Pseudomonas aeruginosa ATCC 27853	n.t.	12.5	n.t.	7.5	7.5	7.5				
Candida albicans ATCC 10259	n.t.	n.t.	3.8	7.5	7.5	7.5				
Candida albicans ATCC 24433	n.t.	n.t.	6.2	15.0	7.5	7.5				

n.t. - not tested, Amp. - Ampicillin, Amk. - Amikacin, Nys. - Nystatin

The results obtained for the antimicrobial activity of the extracts of M. *longifolia* provide a foothold to the possibility of its use as a natural preservative in non-alcoholic drinks. It could help in addressing specific consumer needs as healthy diet is a part of the lifestyle that maintains or improves overall health.

Results of antioxidant activity, content of total phenolics and flavonoids of the non-alcoholic drink prepared with dry extract obtained from naturally dried herbs, are given in table 3.

Total antioxidant potential was $9.09\pm0.17 \mu mol$ Fe²⁺/mL (FRAP method). The capacity of neutralising of DPPH radical shighly reaches 36.69% for the base of beverages, and 93.58% for non-alcoholic beverages, and the obtained value for EC₅₀, in the final product is (EC₅₀=14.00±3.00 µL/mL). The product obtained by adding the extract of wild mint dried naturally generally presents the best characteristics in comparison to the other two drying processes. The highest antioxidant activity was found for the ND extract (21.00±2.00 µg/mL) while LOD and LTCD showed significantly weaker activity (36.00±4.00 and 33.00±1.00 µg/mL, respectively) (7).

In the test of sensory evaluation, the sample of a beverage with the extract of wild mint in concentration of 0.8 g/L was significantly better than the other samples (Table 4). Well accepted by every evaluator it won 17.25 points, which is 86.25% out of maximum ideal

value of 100%. The formulated product is light yellow and homogenous. It is of harmonic taste, typical, pleasant fragrance. The results of dry matter content 13.9% and ethanol content 0.13% (V/V) are in accordance to quality demands prescribed by the *Regulation on quality for refreshing non-alcoholic beverages* (14). Appreciating the results of FRAP and DPPH assays we can conclude that the addition of the extract to the finished product increases its antioxidant activity in relation to the prepared base.

In the category of the evaluated, the juice obtained from the addition of the extracts of wild mint dried naturally is the best of all others presented. However, it also has some failures, which should be removed. The highest individual mark (\overline{X} = 3.61) it got for the sensory characteristic of quality (homogeneity) which is 90.25% of the maximum possible quality. Colour as the analyzed parameter of sensory quality was marked \overline{X} = 3.49 out of maximum 4, which is 87.25% of the maximum possible quality. Sensory characteristics (fragrance and flavor), with the analysis of the product obtained \overline{X} =4.21 and \overline{X} =5.94 points of maximum 5 and 7, that is, 84.20% for fragrance and 84.85% for flavor of maximum quality. The experts showed disagreement when they evaluated fragrance when the coefficient of variation was 13.73, whereas they were in agreement when evaluated flavor of the juice.

Table 3. Antioxidant activity, content of total phenolics and flavonoids of the juice with the extract of M. longifolia

FRAP µmol Fe²+/ mL of beverage	DPPH µL/mL	Total phenolics, mg of gallic acid/mL of beverage	Total flavonoids, mg of rutin/ mL of beverage
9.09±0.17	14.00±3.00	0.536±0.007	0.398±0.007

Tab	le 4	I. S	Sensory	eva	luation	of	the	juice	with	the	extract	of	М.	long	ŗif	Ъl	ia

Samples	Concentration of the extracts of Wild mint (g/L)	Colour max. 4	Homogeneity max. 4	Fragrance max. 5	Flavour max. 7	Total max. 20
1.	0.6	2.90	3.25	3.20	5.35	14.70
2.	0.7	3.20	3.70	3.70	5.45	16.05
3.	0.8	3.49	3.61	4.21	5.94	17.25
4.	1.0	3.35	3.80	3.85	5.50	16.50
5.	1.2	3.10	3.55	3.40	5.40	15.45

Preliminary results of the author, as well as the results presented in this work, indicate that drying technology of wild mint herb has a significant impact on the content of some compounds in the volatile fraction (essential oils or extracts). It is further reflected on the content of phenolics and flavonoids, the biological activity of the isolates and the product (antioxidant and antimicrobial activity), as well as on the sensory properties of the product. Non-alcoholic beverage based on wild mint extract may have a preventive effect on human health.

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