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Original article

Formulation of refreshing non-alcoholic beverage with extracts of medicinal plants

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Summary. The paper presents the necessary analytical and technological procedures for the formulation of a new product, refreshing non-alcoholic beverage, with extracts of medicinal plants. The possibility of using ethanol-aqueous extracts of *Satureja kitaibelii*, *Origanum vulgare*, *Nepeta nuda* and *Hyssopus officinalis* for the preparation of the beverage was analysed. The base of refreshing non-alcoholic beverage was an aqueous solution with 7.0% sucrose and 0.2% citric acid. The volatile fractions of the extracts were analysed using GC-FID/GC-MS. The content of total phenols and flavonoids was determined by the spectrometric methods. Antioxidant activity was assessed by FRAP and DPPH test. Antimicrobial activity of the extracts was analysed by broth microdilution test. The extracts of *O. vulgare* and *S. kitaibelii* had the highest content of total phenols and flavonoids (129.2±12.71 and 90.0±10.56 mg GA/g DE; 110.7±2.47 and 63.3±0.58 mg RU/g DE, respectively), enhancing antioxidant activity (EC_{50}=4.77±0.49 and EC_{50}=18.85±0.31 μg/mL, respectively) and antimicrobial activity. Refreshing non-alcoholic beverage with Oregano extract at a concentration of 1 g/L proved to be the best combination, since it exhibited the highest antioxidant and antimicrobial activity, and sensory was the most acceptable. The formulated product had a pleasant, attractive and harmonious taste and aroma, as well as additional health benefits for consumers.

Keywords: Refreshing non-alcoholic beverage, *Satureja kitaibelii*, *Origanum vulgare*, *Nepeta nuda*, *Hyssopus officinalis*, extracts

Introduction

A close relationship between diet and health has been known since ancient times. The idea that food promotes health is not new, because the principle of „Let food be the medicine and medicine be the food" was accepted by Hippocrates, who is considered to be a father of medicine (1). By developing new scientific knowledge, in the field of biochemistry, physiology, chemistry and related branches, a hypothesis that nutrition plays an important role in maintaining good health status and modulation of various bodily functions was confirmed. Food is no longer viewed only from the aspect of adequate intake in order to meet the general metabolic needs and proper development of the organism. Nowadays, it has one of the leading roles in improving the quality of human life in order to promote health and reduce the risk of chronic, non-infectious diseases (2).

In 1998, the European Union, together with the International Life Science Institute Europe (ILSI Europe), adopted the definition of functional food as "a food product can only be considered functional if together with the basic nutritional impact it has beneficial effects on one or more functions of the human organism, thus either improving the general and physical

conditions and/or decreasing the risk of the evolution of diseases" (3). Components that make functional food can have natural origin: animal or plant, microorganisms or products of their metabolism and synthetic origin. The most common are vitamins, minerals, ω-3 unsaturated fatty acids, probiotics, prebiotics, symbiotics, natural pigments and herbal ingredients (4). Functional food is one of the most interesting areas of research and innovation in the food sector (5). Nowadays, beverages represent the most attractive category of functional food, due to the easy distribution and storage, convenience of consuming, but also as products that can be enriched by bioactive substances in a relatively simple way (6). According to Article 3 of the current Regulation on the quality of refreshing non-alcoholic beverages, these products are obtained by a special technological process, from drinking water, to which sugar and herbal extracts can be added to, as well as other ingredients in accordance with the law, with or without the addition of carbon dioxide (7).

Medicinal plants extracts and isolates, as carriers of certain biological activities, have become very important additives in the production of functional foods. Medicinal plant species of the Lamiaceae family have wide use in official and traditional medicine, and also in food production, thanks to the presence of a wide range of biologically active compounds, which exhibit significant antioxidant, antimicrobial, anti-inflammatory, gastroprotective and other activities (8,9). Medicinal plant species of the Lamiceae family, such as *Satureja kitaibelii*, *Origanum vulgare*, *Nepeta nuda* and *Hyssopus officinalis*, are highly regarded in the traditional, but also find their place in the official medicine.

Satureja kitaibelii Wierzb. ex Heuff, Lamiaceae, is a highly regarded medicinal herb that is most commonly used in the form of a tea beverage. Species of the genus *Satureja* are used to relieve respiratory, urinary disorders and digestive disorders. It is also used in cooking as a spice. Earli studies have shown that *S. kitaibelii* extracts exhibit significant antimicrobial, antitumor and antioxidant activity (10,11). *Origanum vulgare* L., Lamiaceae is a valuable medicinal and aromatic plant, used to strengthen the organism, relieves disorders of the respiratory organs (cough, bronchitis), digestive organs (against diarrhea), problems of urinary tract inflammation (12). It is added to food, to improve flavour and aroma, but also as a natural preservative (13). Oregano extracts exhibit significant antimicrobial and antioxidant activity (14,15). *Nepeta nuda* L., Lamiaceae is also a important medicinal and aromatic plant. It is used in traditional medicine to relieve gastrointestinal and respiratory disorders, such as diarrhea, cough, asthma and bronchitis. Nepeta species also find their application as spices. Early studies have shown that this species, as well as other species of the genus *Nepeta*, exhibit a significant antiviral, antimicrobial, antioxidant, and anti-inflammatory activity (16-18). *Hyssopus officinalis* L., Lamiaceae plays a very important role in traditional and official medicine. In the cuisine, it is appreciated as a spice plant. Extracts are added to alcoholic beverages. It has antimicrobial, antiviral, carminative and mildly spasmolytic activity. It has been confirmed that it exhibits significant antioxidant activity (19,20).

There is a small number of publications dealing with the formulation of refreshing non-alcoholic beverage by adding medicinal plants with antioxidant and antimicrobial properties in order to develop new functional drinks.

So, the aim of this work was the formulation of refreshing non-alcoholic beverage with functional properties, by adding ethanol-aqueous extracts of selected herbs. After examining the chemical composition and biological activities of the extract, we have chosen a product which was the most sensible acceptable and at the same time enriched with the extract with the strongest antioxidant and significant antimicrobial activity. In this way, we have obtained a refreshing product that would have additional benefits for the health of potential consumers.

Materials and Methods

Plant material

The over ground parts of *S. kitaibelii*, *O. vulgare*, *N. nuda* and *H. officinalis* were collected from the region of the Southwestern Serbia. The voucher specimens were deposited at the Herbarium of the Institute of Botany and Botanical Garden „Jevremovac" of the Faculty of Biology, University of Belgrade (numbers 16471, 16472, 16473, 16470, respectively). The plant material in the phenological phase of the blooming was dried in the natural way in the shade.

Chemicals

All chemical substances were of analytical purity: Ethanol (Zorka-Pharma, Serbia), Phosphoric acid, Methanol and Sodium carbonate (Merck, Germany), Folin-Ciocalteu reagent-FC, 2,2-diphenyl-1-picrylhydrazyl-DPPH, Gallic acid, Rutin, Dimethylsulfoxide-DMSO, Ampicillin, Nystatin (Sigma, USA), Aluminum(III) chloride hexahydrate and 2,4,6-Tripyridyl-*s*-triazine-TPTZ reagent (TCI Europe, Belgium), Vitamine C, Iron sulfate and Iron(III) chloride hexahydrate (VWR Prolabo, Belgium), Müller-Hinton broth and Sabouraud dextrose broth (Torlak, Serbia), Sucrose and Citric acid (from the market).

Preparation of extracts

Extraction was carried out by percolation with 70% (v/v) ethanol-water solution as a solvent. Preparation of extracts was according to the procedure described by Stanisavljević et al. (21). 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 and were kept in well closed glass vessels, on the dry, cold and dark place.

Preparation of final products

There were two phases of making refreshing non-alcoholic beverage: I - Product base preparation (dissolving 7% sucrose and 0.2% citric acid in water) and II - addition of chosen plant extract (0.50, 0.75, 1.00, 1.25, 1.50 g/L).

Chromatographic analysis

The volatile fractions of the extracts were analysed using GC-FID/GC-MS, according to procedure described by Đorđevićе et al. (22).

The content of total phenols and flavonoids in extracts and final products

The content of total phenols and flavonoids were determined spectrophotometrically (22). The content of total phenols was obtained with Folin-Ciocalteu reagent (22). The extracts solution in 70% ethanol (0.2 mL, 1 mg/mL) or 0.2 mL of liquid samples of readymade products (conc. 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% water solvent Na_2CO_3. For determination of flavonoids content, in plant extracts (2.0 mL, 1.0 mg/mL) in 70% ethanol or in 2.0 mL of liquid samples of readymade products (conc. of 0.2 mL of beverage/mL of solvent) we added 0.1 mL of aluminium(III) chloride (10%), 0.1 mL of potassium acetate (1 M) and 2.8 mL of distilled water.

Determination of antioxidant activity of extracts and final products

The antioxidant activity of the extracts and finished product was evaluated by FRAP (Ferricion Reducing Antioxidant Power) and DPPH (Capacity of neutralizing of DPPH radicals, spectrophotometric determination) assays (22). For FRAP test, solutions of dry extracts (0.100 mL) in concentration 0.2 mg/mL or finished products (0.1 mL) were mixed with 3.0 mL of freshly made FRAP reagent (acetate buffer + TPTZ reagent + $FeCl_3 \times 6H_2O$ in proportion 10:1:1). For DPPH test, the series of dry extracts solutions were made (extracted with 70% ethanol) in the concentration range of 10.0; 50.0; 100.0; 500.0; 1000 μg/mL for extracts, or 10.0, 50.0, 100.0, 500.0, 1000 μL of beverage/mL for readymade product and DPPH reagent of the concentration of 0.3 mM in 70% ethanol. 1.0 mL of DPPH reagent solution was mixed with 2.5 mL of prepared solutions of extracts or beverages of different concentrations.

Determination of antimicrobial activity

Antimicrobial activity of extracts was tested against standard strains of microorganisms, according to Clinical and Laboratory Standards Institute guidelines (23). We used nine bacterial strains (Gram positive bacteria: *Micrococcus luteus* ATCC 9341, *Micrococcus flavus* ATCC 10240, *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 12228, *Enterococcus faecalis* ATCC 29212, *Bacillus subtilis* ATCC 6633; Gram negative bacteria: *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* NCIMB-9111, *Pseudomonas aeruginosa* ATCC 27853); and two strains of *Candida*: *C. albicans* ATCC 10259, *C. albicans* ATCC 24433). Minimum inhibitory concentrations (MICs) of tested extracts were determined using broth microdilution method according to the procedure prescribed by the CLSI (23). All of the MIC determinations were performed in duplicate, and two positive growth con-

trols were included. All tests were performed in Müller-Hinton broth for the bacterial strains and in Sabouraud dextrose broth for *C. albicans*. Overnight broth cultures of microorganisms were prepared for each strain, and the final concentration was adjusted to 5×10^5 CFU/mL. The extracts were dissolved in DMSO and tested within the concentration range of 30 mg/mL to 0.81 mg/mL. After incubation at 35 °C in aerobic conditions, minimum inhibitory concentrations (MIC) were determined. Ampicillin and nystatin were used for the control of the sensitivity of tested microorganisms.

Quality assessment of the refreshing non-alcoholic beverages

The quality of the sample was defined through sensory evaluation and physical-chemical analyses (content of dry matter, refractive index, content of ethanol). The evaluation panel comprised of 30 (thirty) members, 15 (fifteen) evaluators, food technology experts, average age from 30 to 65 years old, and 15 panellists (consumers), males and females, 22-50 years old, who were regular users of such products. Evaluators were followed by a precise protocol in order to define the quality of refreshing non-alcoholic beverages using the same instruments and following the same instructions. All samples were adequately labelled during the sensory evaluation, and the results obtained were statistically processed. The samples were offered to the evaluators at 20 °C, in transparent glasses. Four attributes were evaluated: colour intensity (max. 4 point), homogeneity (max. 4 point), odour intensity (max. 5 points) and taste (max. 7 points). Evaluation of the quality of the refreshing non-alcoholic beverages was done in accordance with the Regulation (7).

Results and Conclusions

The yields of dry extracts of *S. kitaibelii*, *O. vulgare*, *N. nuda* and *H. officinalis* were 11.4, 16.4, 14.3, and 14.4 g/100 g herb (respectively), determined according to Ph. Eur. (24).

Chemical profile of volatile fractions of extracts

The results of the analysis of volatile fractions of extracts of examined medicinal plants are shown in Table 1. In volatile fraction of *S. kitaibelii* extract monoterpenes were dominating (69.2%) in comparison to sesquiterpenes (21.0%). Dominant components in the fraction were neral (12.5%), *trans*-linalool oxide (10.0%) and limonene (7.1%). A significant part in volatile fraction of *O. vulgare* extract, belonged to sesquiterpenes, with *trans*-caryophyllene (34.6%) as the most dominant compound. Among monoterpenes the most prominent was linalool (4.5%). In *N. nuda* volatile fraction the most dominant was 1,8-cineole (37.0%). In the volatile fraction of *H. officinalis* extract monoterpenes prevailed with *cis*-pinocamphone (36.1%) as the most prominent constituent.

The results of the investigation of essential oils chemical composition of the analysed plant species have been presented in many works. The dominant components of the essential oils of the investigated species were: *S. kitaibelii* - geraniol, *p*-cymene (25,26); *O. vulgare* - carvacrol, thymol, linalyl acetate, (Z)-α-bisabolene (27,28); *N. nuda* - 4aα,7ß,7aα-nepetalactone, germacrene, elemol, β-caryophyllene (29), *H. officinalis* - 1,8-cineole and β-pinene (30).

In our study, we have examined the chemical composition of the volatile fraction of the extract, due to the known biological activities of the expected mono- and sesquiterpenes.

Content of total phenols and flavonoids in extracts

The content of total phenols and total flavonoids in the examined extracts is shown in Table 2.

On the basis of the obtained results, it can be concluded that the highest content of total phenols and flavonoids is present in the extract of *O. vulgare* (129.2±12.71 mg GA/g DE and 110.7±2.47 mg RU/g DE), while the lowest content of these compounds was determined in extract of *N. nuda* (40.9 mg GA/g DE and 20.0±0.6 mg RU/g DE). Dorman and associates (2003) have presented in their studies a high content of phenolic compounds (149 mg GA/g DE) in a deodorized aqueous Oregano extract (31).

Antioxidant activity of extracts

The highest FRAP value (Table 3) showed extract of *O. vulgare* (5.89±0.12 mmol Fe^{2+}/g), while the lowest FRAP value showed extract of *N. nuda* (0.86±0.07 mmol Fe^{2+}/g). The percentage of neutralizing of DPPH radicals was presented with value EC_{50}. The

Table 1. Chemical composition of volatile fractions of extracts of *S. kitaibelii* (S), *O. vulgare* (O), *N. nuda* (N) and *H. officinalis* (H)

Constituents		(%)			
	KIE	S	O	N	H
α-thujene[m]	932	0.7	0.3	0.7	0.3
α-pinene[m]	933	2.2	-	1.0	0.7
camphene[m]	940	1.1	0.2	-	-
sabinene[m]	969	-	1.7	1.8	0.3
β-pinene[m]	975	0.7	-	1.4	0.5
myrcene[m]	996	1.2	-	1.8	1.6
α-phellandrene[m]	1005	2.6	-	-	-
p-cymene[m]	1022	4.8	1.1	-	0.2
limonene[m]	1029	7.1	-	-	-
β-phellandrene[m]	1028	-	-	-	1.8
1,8-cineole[m]	1029	1.6	1.4	37.0	2.1
cis-β-ocimene[m]	1039	1.8	-	-	0.6
trans-β-ocimene[m]	1045	0.6	0.2	-	-
γ-terpinene[m]	1068	-	-	0.5	-
cis-sabinenehydrate[m]	1067	0.6	1.1	-	0.4
trans-linalool oxide[m]	n/a	10.0	-	-	-
artemisiaalcohol[m]	1070	-	0.3	-	-
o-guaiacol	1091	-	3.7	-	-
cis-linalool oxide[m]	n/a	0.5	-	-	-
linalool[m]	1100	4.5	4.5	-	0.3
trans-sabinenehydrate[m]	1105	0.6	-	-	-
trans-pinocarveol[m]	1139	-	-	-	0.3
camphor[m]	1140	1.3	0.7	-	-
3,5-dihydroxy-6-methyl-2,3-dihydro-4H-pyran-4-one	1151	-	2.1	2.0	-
borneol[m]	1163	3.9	2.0	-	-
cis-pinocamphone[m]	1174	-	-	-	36.1
terpinene-4-ol[m]	1175	1.4	0.7	2.1	9.7
α-terpineol[m]	1190	0.4	1.3	1.1	0.6
cis-dihydrocarvone[m]	n/a	2.6	-	-	-
myrtenol[m]	1197	-	-	-	0.5
myrtenal[m]	1199	-	-	-	0.5
trans-dihydrocarvone[m]	1200	1.3	-	-	-
hydroquinone	1207	-	0.3	-	-
1,4:3,6-dianhydro-α-d-glucopyranose*	1215	-	3.4	-	-
cis-carveol[m]	n/a	-	-	-	0.3
neral[m]	1261	12.5	-	-	-
2*Z*-hexenyl isovalerate	1239	-	-	0.7	-
trans-2-hydroxy-pinocamphone[m]	1251	-	-	-	1.9
trans-geraniol[m]	1261	0.8	-	-	-
linalylacetate[m]	n/a	-	-	-	14.2

geranial[m]	n/a	1.4	-	-	-
bornylacetate[m]	1274	-	1.7	-	-
Indole	1299	-	0.4	-	-
2*E*,4*Z*-decadienal	1289	-	-	2.8	-
perillaalcohol[m]	1303	-	-	-	0.2
carvacrol[m]	1323	3.0	-	-	
p-vinylguaiacol[m]	1318	-	-	-	1.5
myrtenylacetate[m]	1341	-	-	-	0.4
α- terpinylacetate[m]	n/a	-	-	-	0.2
α-copaene[s]	1376	1.6	-	0.4	-
β-bourbonene[s]	1381	2.4	2.1	3.1	0.2
β-elemene[s]	1378	-	0.3	-	-
4a-α,7-ß,7a-α-nepetalactone[m]	1398	-	-	3.7	-
trans-caryophyllene[s]	1416	8.1	34.6	2.3	0.2
α-humulene[s]	1454	-	-	0.7	0.6
trans-ß-farnesene[s]	1459	-	-	4.1	-
γ-muurolene[s]	1482	-	-	5.5	0.3
germacrene D[s]	1474	4.9	0.4	-	-
bicyclogermacrene[s]	1495	1.2	-	2.7	0.2
β-bisabolene[s]	1495	-	0.6	-	-
α-farnesene[s]	n/a	0.3	-	-	-
d-cadinene[s]	1519	-	2.1	0.5	2.4
spathulenol[s]	1572	1.1	3.8	-	1.0
caryophyllene oxide[s]	1576	1.4	2.9	2.3	0.8
viridiflorol[s]	1577	-	1.0	-	-
humulene epoxide II[s]	1593	-	0.4	-	-
α-cadinol[s]	1642	-	0.8	-	0.2
germacra-4(15),5,10(14)-trien-1α-ol[s]	1673	-	0.8	-	-
longiborneol acetate*[s]	1684	-	0.6	-	-
α-bisabolol[s]	n/a	-	-	-	0.9
oplopanone[s]	1725	-	0.4	-	-
hexadecanoic acid[FAD]	1965	-	0.9	1.1	0.8
ethyl hexadecanoate[FAD]	1991	-	8.7	0.4	1.6
phytol[d]	2106	-	0.9	-	1.1
linoleic acid[FAD]	2166	-	-	-	1.0
oleic acid*[FAD]	2173	-	-	-	2.2
ethyl linoleate[FAD]	2098	-	1.2	-	-
ethyl linolenate[FAD]	2151	-	4.4	-	-
ethyl octadecanoate[FAD]	2177	-	6.1	-	0.2
phytol acetate[d]	2117	-	-	1.0	-

m - monoterpenes, s - sesquiterpenes; n/a - not available; * - tentative identification, FAD - fatty acids and fatty acids derivatives; KIE - Kovats (retention) index experimentally determined (AMDIS, Automated Mass Spectral Deconvolution and Identification System software), % - Relative area percentage of the compounds obtained from FID area percent data

Table 2. Content of total phenols and flavonoids in extracts*

Extracts	Total phenols (mg GA/g of DE)	Total flavonoids (mg RU/g of DE)
S. kitaibelii	90.0±10.56 a	63.3±0.58 a
O. vulgare	129.2±12.71 b	110.7±2.47 b
N. nuda	40.9±1.21 d	20.0±0.6 d
H. officinalis	63.3±6.79 c	46.6±0.42 e

Values in the table are the average value ± standard deviation (n=3) GAE-Gallic acid, DE - Dry extract, RU - Rutin, *Different letters in the columns are marking values that differ significantly ($p<0.05$, Scheffé test)

Table 3. Antioxidant activity of examined extracts

Plant extracts	FRAP mmol Fe^{2+}/g	DPPH EC_{50} (μg/mL)
S. kitaibelii	3.22±0.15	18.85±0.31
O. vulgare	5.89±0.12	4.77±0.49
N. nuda	0.86±0.07	86.24±1.85
H. officinalis	1.05±0.02	58.47±3.91

Values in the table are the average value ± standard deviation (n=3)

best DPPH radical scavenging activity was demonstrated for *O. vulgare* extract (EC_{50}=4.77±0.49 μg/mL), whereas extract of *N. nuda* showed the lowest activity (EC_{50}=86.24±1.85 μg/mL).

The obtained results show that antioxidant activity of tested extracts was decreasing in the following way: *O. vulgare*>*S. kitaibelii*>*H. officinalis*>*N. nuda*. Generally, there is a relatively good matching of the results obtained for antioxidant activity using different techniques for determination (correlation coefficient, R^2 =0.7501), but using Scheffe test at the level of reliance of 95% it was shown that results obtained by DPPH assay for all extracts were significantly different, which was not the case with results obtained applying FRAP method. Commercial antioxidant vitamin C showed the greatest capability of neutralizing DPPH radicals in comparison to other tested samples, with value EC_{50} of 11.12±0.62 μg/mL. By comparing DPPH values of vitamin C with DPPH values for Oregano extract, a higher antioxidant potential of the extract is noticed.

Antimicrobial activity of extracts

Antimicrobial activity of extracts on nine bacterial strains and two strains of *Candida albicans* is shown in Table 4.

The examined extracts have shown moderate to low antimicrobial activity in comparison with standard antibiotics. The results of MIC assay have showed that the extract of *S. kitaibelii* exhibits the best antimicrobial activity against *M. luteus,* and moderate to *M. flavus*, *St. epidermidis*, *St. aureus*. Thus, MIC value was

Table 4. Antimicrobial activity of extracts of *S. kitaibelii*, *O. vulgare*, *N. nuda* and *H. officinali*

Extracts	*S. kitaibelii*	*O. vulgare*	*N. nuda*	*H. officinalis*	Ampicillin[a]	Nystatin[a]
Microorganism	**MIC (mg/mL)**				**MIC (μg/mL)**	
Gram positive bacteria						
M. luteus ATCC 9341	0.81	3.25	1.62	3.25	2.5	n.t.
M. flavus ATCC 10240	1.62	1.62	0.81	1.62	3.0	n.t.
St. aureus ATCC 25923	1.62	1.62	1.62	3.25	0.5	n.t.
St. epidermidis ATCC12228	1.62	3.25	3.25	1.62	0.2	n.t.
E. faecalis ATCC 29212	7.5	7.5	7.5	3.25	5.0	n.t.
B. subtilis ATCC 6633	3.25	7.5	3.25	1. 62	n.t.	n.t.
Gram negative bacteria						
E. coli ATCC 25922	7.5	7.5	7.5	3.25	2.0	n.t.
K. pneumoniae NCIMB 9111	7.5	7.5	7.5	3.25	4.0	n.t.
P. aeruginosa ATCC 27853	7.5	7.5	7.5	3.25	12.8	n.t.
Fungi						
C. albicans ATCC 10259	7.5	7.5	7.5	1.62	n.t.	3.0
C. albicans ATCC 24433	7.5	7.5	7.5	1.62	n.t.	5.0

a - average values, n.t. - not tested

in the range 0.81-7.5 mg/mL. The antifungal activity of the extract was also moderate. Methanolic (10) and petroleum ether, chloroform and ethyl acetate extracts (11) expressed a wide range of inhibiting activity against both Gram positive and Gram negative bacteria. Extract of *O. vulgare* herb had the value of MIC in range 0.81-7.5 mg/mL, against the tested microorganisms. The extract exhibited the best activity against *M. flavus* and *St. aureus.* Moderate activity was observed to all Gram negative bacteria. Also, extract showed moderate antifungal activity. Previoulsy it was shown that the extracts of *O. vulgare* were more active against bacteria, especially against Gram positive bacteria (15). Commercial extract *O. vulgare* had the ability of reducing the growth of *Fusarium* and *Penicillium* species (13). Extract of *N. nuda* herb showed the best activity against Gram positive bacteria *M. flavus, St. aureus*, and *M. luteus*, and mild against *St. epidermidis* and *B. subtilis.* Extract also showed moderate antifungal activity. Methanol extracts of some *Nepeta* species showed significant antibacterial and antifungal activity (17). Minimum inhibitory concentrations of *H. officinalis* herb extract were in the concentration interval between 1.62 mg/mL and 3.25 mg/mL. Extract showed the best activity against Gram positive bacteria *M. flavus, St. epidermidis and B. subtilis.* Also, unlike the other tested extracts, it exhibited better activity against *C. albincans.* In earlier studies it has been shown that the extracts of Hissop have significant antimicrobial activity (19).

In general, it is well documented that a large number of plant species of the Lamiaceae family have antimicrobial activity, and have a diverse application in pharmacy, medicine and the food industry. Extracts are often used as preservatives to prevent contamination of food from oxidizing process and the microorganisms (9).

Sensory evaluation of the quality of readymade products

The combination of refreshing non-alcoholic beverage with extracts of the tested plant species gave good results in relation to the sensory characteristics. Samples of refreshing non-alcoholic beverages in which medicinal herbs extracts were added at a concentration of 0.5 to 1.5 g/l have been sensitively evaluated. Results of sensory mark of the sample quality of refreshing non-alcoholic beverage with different concentrations of extracts of *S. kitaibelii*, *O. vulgare*, *N. nuda* and *H. officinalis* were shown in Table 5. The best sensory characteristics were shown by samples with an extract concentration of 1 g/L.

The samples of refreshing non-alcoholic beverage enriched with extract of *S. kitaibelii* in concentration of 1.00 g/L, was light green and clear, with pleasant fragrance and harmonious taste. It gained 16.80 points (84.0% of maximum possible quality). The refreshing non-alcoholic beverage sample with extract of *O. vulgare* in concentration of 1.00 g/L was light brown, with extremely nice fragrance and harmonious taste. It was evaluated as the best with 17.15 points (85.8% of maximum value).

Extracts of *N. nuda* and *H. officinalis* were sensory less acceptable, and thus omitted from further evaluation.

Average sensory value of refreshing non-alcoholic beverage samples enriched with extract of *O. vulgare* was significantly better than average values of all other samples.

Refreshing non-alcoholic beverage with extract of *S. kitaibelii* for fragrance got $\bar{X}$=3.96 out of maximum 5 (79.2% of maximum). The highest individual mark ($\bar{X}$=3.45) the sample got for color (86.3%). For homogeneity (clearness) obtained average mark $\bar{X}$=3.43 (85.8%). For taste, out of 7, it got $\bar{X}$=5.96 points (85.1%). It was obvious that good evaluated refreshing non-alcoholic beverage sample satisfied taste, color and general appearance, but was not completely accepted from the point of fragrance. The evaluators have expressed their impressions and evaluations after consuming the refreshing non-alcoholic beverage with the extract of *S. kitaibelii*, stating that the beverage has had a characteristic refreshingly mild sour taste.

In samples with of extract of *O. vulgare*, which was collectively sensory best rated, fragrance was better evaluated, while the lowest individual mark ($\bar{X}$=3.00) it got for homogeneity (75.0%). The mark for color was 85.3%. The average value for fragrance was 96.4% of maximum possible. Out of maximum 7, it obtained $\bar{X}$=5.92 (84.6%) for taste. The evaluators have expressed their impressions and evaluations after consuming the refreshing non-alcoholic beverage with Oregano extract, stating that the beverage has had a characteristic spicy note and rich taste. Fragrant Oregano spice which makes the base of Mediterranean cuisine along with all its beneficial properties, intense aroma and smell can

Table 5. Sensory evaluation of the quality of refreshing non-alcoholic beverage with extracts of *S. kitaibelii*, *O. vulgare*, *N. nuda* and *H. officinalis*

Sensory parameters	Extracts of plant species	Extract concentration (g/L)				
		0.5	**0.75**	**1.0**	**1.25**	**1.5**
Colour max. 4	*S. kitaibelii*	3.00	3.30	3.45	3.40	3.25
	O. vulgare	3.00	3.25	3.41	3.35	3.20
	N. nuda	2.90	3.20	3.40	3.30	3.20
	H. officinalis	2.90	3.15	3.35	3.25	3.15
Homogeneity max. 4	*S. kitaibelii*	3.21	3.36	3.43	3.38	3.30
	O. vulgare	2.65	2.80	3.00	2.90	2.75
	N. nuda	3.20	3.30	3.40	3.35	3.20
	H. officinalis	2.60	2.75	2.90	2.80	2.70
Fragrance max. 5	*S. kitaibelii*	3.25	3.60	3.96	3.75	3.55
	O. vulgare	4.45	4.65	4.82	4.80	4.60
	N. nuda	3.20	3.50	3.90	3.70	3.50
	H. officinalis	4.40	4.60	4.75	4.70	4.50
Taste max. 7	*S. kitaibelii*	5.20	5.70	5.96	5.85	5.55
	O. vulgare	5.25	5.60	5.92	5.70	5.55
	N. nuda	5.10	5.60	5.90	5.80	5.50
	H. officinalis	5.15	5.50	5.85	5.60	5.45
Total max. 20	*S. kitaibelii*	14.66	15.96	16.80	16.38	15.65
	O. vulgare	15.35	16.30	17.15	16.75	16.10
	N. nuda	14.40	15.60	16.60	16.15	15.40
	H. officinalis	15.05	16.00	16.85	16.35	15.80

Values in the table are the average value (n=3)

serve not only to enrich the meals, but also to prepare a refreshing soft drink of strong and refined taste.

Taking into account the content of total phenol and flavonoids in the extracts, the antioxidative and antimicrobial activity of the extract, the parameters of the sensory quality of the product, refreshing non-alcoholic beverage with extracts of *O. vulgare* and *S. kitaibelii*, proved to be the most acceptable products. Furthermore, these samples were tested on the general quality parameter for non-alcoholic drinks.

General quality of readymade products

Refreshing non-alcoholic beverage with extract of *S. kitaibelii* and refreshing non-alcoholic beverage with extract of *O. vulgare* had values for dry matter (6.5 to 7.0% w/w, respectively), the refractive index (1.3440 and 1.3450, respectively) and the content of ethanol (0.13 and 0.12% v/v, respectively). The alcohol content of the products was below 0.5%, which is the maximum value according to the Regulation on non-alcoholic beverages (7).

The content of total phenols and flavonoids and antioxidant activity of readymade products

The antioxidant activity of final product determined by FRAP and DPPH methods, as well as total content of phenols and flavonoids of readymade products were given in Table 6.

The capacity of neutralizing DPPH radicals reached maximum 33.6% for base of the beverage, 94.46% for the beverage with the addition of the extract of *S. kitaibelii*, 93.67% for the beverage with the addition of the Oregano extract, while the calculated value of the EC_{50} in the final product data in Table 6. Obtained results pointed out that the base itself had certain antioxidant activity which was rather lower in comparison to the readymade product, confirming the contribution of *S. kitaibelii* and *O. vulgare* extracts to the antioxidant potential of prepared products.

Table 6. Antioxidant activity of refreshing non-alcoholic beverage

Product	Total phenols, mg of GA/mL of beverage	Total flavonoids, mg of RU/ mL of beverage	FRAP μmol Fe^{2+}/ mL of beverage	DPPH μL/mL
Refreshing non-alcoholic beverage with extract of *S. kitaibelii*	0.640±0.016	0.235±0.002	6.97±0.15	22.50±1.16
Refreshing non-alcoholic beverage with extract of *O. vulgare*	1.010±0.020	0.497±0.005	15.79±0.32	12.59±0.73

Values in the table are the average value ± standard deviation (n=3)

Refreshing non-alcoholic beverage with Oregano extract had higher content of total phenols and flavonoids and manifested stronger antioxidant activity. These results were expected and are in accordance with results of analysis of extracts, where extract of *O. vulgare* had highest values for phenols and flavonoids contents as well as for antioxidant activity. Results of analyzing readymade product showed that the difference in the content of total flavonoids and antioxidant activity determined by FRAP assay was statistically significant ($p<0.05$; Scheffe test).

Good correlation of total content of phenols and antioxidant activity was shown in different cocoa liquors (32) and some commercial herbal liqueurs (33), while these results were opposite to results published by Heinonen and associates (34). They claimed that there was no good correlation between phenol and antioxidant activity in fruit liquors and wines. Over more than 20 types of alcoholic and non alcoholic beverages were tested by Tabart et al. (35) for phenols, flavonoids and antioxidant activity, where it was determined that red wine and orange juice had the highest content of bioactive compounds and manifested highest antioxidant activity.

By selecting the best extract of four extracts obtained from plants of the family Lamiaceae, a new non-alcoholic product, was obtained. Refreshing non-alcoholic beverage with ethanolic-aqueous extract of *O. vulgare* at a concentration of 1.00 g/L had the best sensory characteristics combined with a preferred chemical profile and good antioxidant activity.

Therefore, designing or formulating a new product, in this case, refreshing non-alcoholic beverage was preceded by the necessary and logically connected, analytical and technological procedures. They ensured made the product of high biological and sensory quality possible. Our future research will focus on defining the shelf life of refreshing non-alcoholic beverage, which contains a natural preservative extract of oregano at the selected concentration. We will also consider the challenge and possibility of combining plant extracts, or plant extracts and fruit juices for making refreshing non-alcoholic beverage, to targeted health problems.

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