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Research Article

Characterisation of Extracts from *Stevia rebaudiana* Bertoni Leaves

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ABSTRACT

Stevia rebaudiana Bertoni is widely used as a source of natural sweetening agent in human nutition. The aim of present study was to characterise the leaves extracts as evaluate the polyphenol and carbohydrate contents. The effect of different particle size and various solvents on the antioxidant activity of leaves extract were also studied. The content of fructans, polyphenols, radical scavenge activity (DPPH), metal reducing activity (FRAP) in the extracts were established. It was found that the fructans amount did not depend significantly from the size of grinding (2.8 % DW). The type of the solvent had a highest effect only to the yields of the extract (from 254 mg/g DW to 377 mg/g DW). Additionally, the total polyphenols content (from 12.7 mg GAE/g DW to 15.6 mg GAE /g DW), radical scavenge activity - DPPH (from 135.8 mM TE/g DW to 221.4 mM TE/g DW) and metal reducing activity-FRAP DPPH (from 117.7 mM TE/g DW to 149.5 mM TE/g DW) were influenced mainly from the particle size and degree of grinding. The highest values of the presented parameters concerning to antioxidant activity were obtained when the dried leaves of stevia were finely ground and water were used as extracting solvent. Radical scavenge activity and metal–reducing activity correlated very well with total polyphenol content.

Key words: *Stevia rebaudiana*, leaves extract, fructans content, polyphenols, antioxidant, DPPH, FRAP.

INTRODUCTION

There are many aromatic and medicinal plants, which possess antioxidant activity and successfully prevent oxidative stress. *Stevia rebaudiana* Bertoni, the nature's sweetener, is one of the effective sources to combat the damage related to oxidative reactions in the human body[1]. This herb is an annual plant indigenous to northern part of South America (Brazilians and Paraguayans) with a stem reaching 1 m in height and leaves 2 to 3 cm in length. *Stevia rebaudiana* Bertoni is cultivated in many countries in all over the world for their sweet taste. It is known that stevia leaves contain several sweet glycosides (Stevioside and Rebaudioside and their derivates), which successfully replace the sugar and people with diabetes[2]. Stevia has a high content of other bioactive compounds such as phenols, flavonoids, sterols, terpenes, tannins, vitamins and minerals, which have a positive effect on human health and can be used for treatment of many diseases3. It is known that stevia leaves possessed also antialergic, antimicrobial and detoxicative effect and antioxidant effect. The radical scavenging ability of stevia leaves extracts is very important in the control and prevention of the oxidative stress. It has been reported that aqueous leaf extract of S. rebaudiana has total phenolic content 56.73 mg GAE/g 4, catechin 130.76 µg and quercetin 15.64 µg5. Tadhani et al.6 has established total phenolic compounds 25.18 mg/g for stevia leaves and 35.86 mg/g for callus on dry weight basis. Total antioxidant activity ranged from 9.66 to 38.24 mg and 11.03 to 36.40 mg equivalent to different standards in water and methanolic extract of stevia leaves, respectively. The highest percent of inhibition had been observed in methanolic extract of callus. Shukla et al.7 reported that the leaves ethanolic extract also inhibited the hydroxyl radical, nitric oxide, superoxide anions with IC(50) values of 93.46, 132.05 and 81.08 mg/ml, and these values are higher than the ability of ascorbic acid as a standard. The aqueous extract also inhibited the hydroxyl radical, nitric oxide and superoxide anions. The IC(50) values of aqueous extract and ascorbic acid in DPPH radical scavenging assay had been 83.45 and 26.75 µg/ml, respectively4. Tavarini and Angelini (2013) found high level of phenols (78.24 mg GAE g−1 DW) and high antioxidant activity (812.6 µmol Fe2+ g−1 DW by FRAP assay). The inhibition of DPPH free radicals obtained had been IC50 mean value of 250 µg mL−1. They established significant relationships among the total antioxidant capacity and the analysed compounds and the influence of the environmental, technologycal and physiological factor on the concentration of bioactive substances. Ghanta et al.[8] found that methanolic extract of *Stevia rebaudiana* leaves exhibited preventive activity against DNA strand scission by *OH generated in Fenton's

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reaction on pBluescript II SK (-) DNA. Its efficacy had been better than that of quercetin. The radical scavenging capacity evaluated by the DPPH test had been (IC_{50}=47.66+/-1.04 μg/mL). It is established also additionally inhibition of lipid peroxidation induced with 25 mM $FeSO_4$ on rat liver homogenate as a lipid source - (IC50=2.1+/-1.07 mg/mL). Until now no detailed information about antioxidant activity and fructan contain in stevia plants grown on territory of Bulgaria were found. The data about sugar composition and total phenols were scarcely investigated. The aim of present study was to establish the content of some inulin-type fructans, polyphenols and the effect of particle size and different solvents (water and ethanol) on the antioxidant activity of *Stevia rebaudiana* Bertoni leaves extract.

MATERIAL AND METHODS.

Plant materials and chemicals

Leaves of this plant were provided by the crop cultivated according to the conventional technology in the region of Stara Zagora, Bulgaria.

DPPH and ethanol (95%) were purchased by Sigma Chemical Co, St. Louis, USA. All other chemicals used in this study were analytical grade.

Sample preparation

Dried leaves were ground and sieved in two fractions: 1. Finely ground; 2. Coarsely ground. The prepared samples were analyzed for moisture according to the methods described by the AOAC[9].

Extraction of sugars and inulin-type fructans

Dried leaves were extracted in a Soxhlet apparatus successively with hexane, $CHCl_3$, and ethyl acetate. Then 1 g dried residue from stevia leaves was extracted three times with 95% (v/v) boiling ethanol (20, 20 and 10 ml) under reflux. The duration of each extraction procedure was 60 minutes. The extracts were collected in 50 ml volumetric flask. The low-molecular carbohydrate fraction composed of fructose and FOSs was obtained in the ethanol extracts. For extraction of high-molecular fraction (inulin), the residue in the flask after ethanol extraction was extracted by three following extractions (20, 20, 10 ml) with boiling water as it was described above. The content of low molecules and high molecules fractions of fructans were presented as fructose equivalent was determined by spectrophotometric method at 480 nm [10, 11]. The content of mono-, di-, oligosaccharides and inulin was analyzed by and HPLC analysis.

HPLC analysis of sugars

Chromatographic separations were performed on HPLC Shimadzu, coupled with LC-20AD pump, refractive index detector Shimadzu RID-10A. The analysis of individual sugars in Stevia leaves were performed on a Shodex® Sugar SP0810 with Pb^{2+} a guard column (50 × 9.2 mm i.d.) and an analytical column (300 mm × 8.0 mm i.d.) at 85 °C. The mobile phase used for separation was distilled water with flow rate 1.0 ml/min. The injection volume of the samples was 20 μL. The control of the system, data acquisition, and data analysis were under the control of the software program LC solution version 1.24 SP1 (Shimadzu Corporation, Kyoto, Japan[10].

Total phenolic content

Dry leaf samples (1 g) were ground and exhaustively extracted with 96% (v/v) methanol. The total phenolic content (TPC) was determined using the Folin–Ciocalteu reagent. Basically, 0.2 ml exteracts was mixed with 1 ml Folin–Ciocalteu reagent diluted five times and 0.8 mL 7.5 % Na_2CO_3. The reaction was performed for 20 min at room temperature in darkness. Then the absorbance was measured at 765 nm against blank sample. The results were expressed in mg equivalent of gallic acid (GAE) per g dry weight (dw), according to calibration curve; build in range of 0.02 - 0.10 mg[11].

Determination of DPPH radical scavenging capacity

Radical scavenge activity- DPPH analyse, mM TE/g DW (2,2-diphenyl-1-pycril hydrasil) was established by the methodology: For analyses, 0.15 ml of analyzed extracts were mixed with 2.85 ml freshly prepared 0.1 mM solution of 1,1-diphenyl-2-picrylhydrazyl radical (DPPH, Sigma) in methanol (Merck). The reaction was performed at 37 ºC in darkness and the absorptions at 517 nm were recorded after exactly 15 min against methanol. The antioxidant activity was expressed as mM Trolox equivalents (TE) per g dry weight (DW) by using calibration curve, build by 0.05, 0.1, 0.2, 0.3, 0.4 and 0.5 mM 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, Fluka) dissolved in methanol (Sigma). Determination of EC50: 0.15 ml of each analyzed extracts was mixed with 2.85 ml freshly prepared 0.1 mM DPPH solution in methanol (Merck). Blank sample was developed by the same way, but using methanol instead of plant extract. The reaction was conducted for 15 minutes at 37 ºC in darkness. The absorbance of both blank and sample were recorded at 517 nm and used for calculations of % inhibition and EC_{50} as described elsewhere[12].

Metal reducing activity, FRAP

Metal reducing activity, FRAP analyse, mMTE/g DW was established by the method of: The assay was performed according to method, described by Benzie & Strain[13] slightly modified as follow: the FRAP reagent was freshly prepared before analyzes by mixing 10 parts 0.3 M acetate buffer (pH 3.6), 1 part 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ, Fluka) in 40 mM HCl (Merck) and 1 part 20 mM $FeCl_3x6H_2O$ (Merck) in dd H2O. The reaction was started by mixing 3.0 ml FRAP reagent with 0.1 ml of investigated extract. Blank sample, prepared with methanol instead of extract was developed as well. The reaction time was 10 min at 37 °C in darkness and the absorbance at 593 nm of sample against blank was recorded. Antioxidant activity was expressed as mM Trolox equivalents (TE) per g dry weight (DW) by using calibration curve, build in range of 0.05-0.5 mM Trolox (Fluka) dissolved in methanol (Merck).

Statistical analysis

Statistical analysis were performed with Statistical 10, StaSoft, Inc. and results were expressed as means ± standard error (SE). Statistical significance was determined by the LSD-Duncan test. Statistical significance was presented for $P<0.05$; $P<0.01$; $P<0.001$.

RESULTS AND DISCUSSION

Table 1. Content of inulin-type fructans presented as fructose units in the leaves of *Stevia rebaudina* g/100 g (DW)

Samples	Low molecule fraction (Fru, Suc and FOS	High molecule fraction (inulin)	Total fructans,
Finely ground sample	2.3 ± 0.1	0.5 ± 0.1	2.8 ± 0.2
Coarsely ground sample	2.1 ± 0.2	0.4 ± 0.1	2.5 ± 0.3
Average	2.2 ± 0.15	0.45 ± 0.1	2.65 ± 0.25

Table 2. Carbohydrate composition in extracts of *Stevia rebaudina* leaves, g/100 g dw

Samples	Fru	Glc	Suc	1-Kes	Nys	Inulin
Finely ground leaves	0.4	0.2	1.3	0.4	0.2	0.5
Coarsely ground leaves	0.3	0.2	1.4	0.3	0.1	0.4

The moisture content in leaves were established to be 8 - 10 %. The content of low molecule fraction of carbohydrates (fructose, sucrose) presented as percent in dry weight is average 2,2 % with variation from 2,1 to 2,3 % (Table 1). The amount of fructooligosacharides and inulin was higher in the finely ground fraction. Total amount of polysaccharides presented as fructans as percent in the dry weight was average 2,65 % with variation from 2,5 to 2,8 %. Finely ground sample has a higher amount of fructans which is due to the better extraction of the polysaccharides from the substrate with the small and fine ground particle size. The differences in the content of low molecule fractions, inulin and fructans in DW among the finely ground and coarsely ground leaves are not statistically significant. From HPLC analysis of stevia leaves extracts it was found the presence of sugars fructose, glucose and inulin-type fructans. The amount of monosaccharides were lower than reported in literature, while sucrose content was typical for wild type stevia[14]. The level of fructooligosacharides 1-kestose and nystose which is inulin type fructan were below 0.6%. The inulin content in stevia leaves reached to 0.5 % which was similar to the results were reported by[3] (Table 2). Therefore, *Stevia rebaudiana* leaves can be considered as a source of inulin-type FOS and its presence in the leaves indicates a possible application of extracts as a dietary supplement. During the process of extraction the total volume of the extract obtained from the Stevia leaves was average 315,7 ± 5.58 mg/g dry weight with variation in the range from 224.6 ± 2,1 to 387,0 ± 8,6 (Table 3). The amount of the obtained extract varied significantly depending on the type of the solvent and the particle size of grinding. Water is a better solvent for the extraction of chemical compounds from stevia. Average with the water was obtained 377.4 **± 7.55** mg/g DW extract with comparison to Ethanol 95 % - **254 ± 3.6** mg/g DW. The differences among the values for the two solvents give the advantage of the water as a better solvent extracting with 48.6 % more extract compared to the 95 % ethanol. The amount of the extract was influenced also from the particle size and grounding process. Average from the two solvent the higher amount of the extract was obtained when finely grounding of the leaves was applied – 335.2 ± 6.85 compared to 296.2 ± 4.3 coarsely ground. The difference is 13.2 % increasing of the extractable ability when grinding of the stevia dried leaves are finely ground. The differences of extract content are statistically significant at P<0.001 among ethanol and water solvents and at P<0,05 among the finely and coarsely ground samples for the water. Total polyphenol content varied in the range from 10,7 ± 0,8 to 20,1 ± 0,4 mg GAE/g. Water was a better solvent and the amount of the polyphenols 15.6 ± 0.4 was higher compared to 95 % ethanol extract 12.75 ± 0.55. Total phenolic content in investigation by Jahan et al.[15] ranged between 2.53 – 6.52% gallic acid equivalents in ethanol extracts of stevia leaf. Sunanda S et al[16] explorated the content of phenols in different stevia organs found 11.04±3.16 mg GAE phenols from the stevia methanolic leaves extract. The difference is with 22.4 % higher extracted from the water compared to ethanol. Grinding of the leaves and particle size have the higher influence on the extraction of the polyphenols than the solvent. The amount of polyphenol from the finely ground leaves was average 17,45 ± 0.35 compared to 10.9 ± 0.6 for coarsely ground one. The advantage is with 60.1% more extracted polyphenols when finely grounding of samples is performed. Differences in the total phenols content, mg GAE/g DW among ethanol and water extracts from coarsely ground samples are not statistically significant, while for finely ground are statistically significant at P<0.001. Zayova et al.[17] has obtained 10.51-14.65 Phenols, mg g DW by water extract from stevia leaves with different origin – USA and Paraguay.

Radical scavenge activity

DPPH analyse, mM TE/g DW was average 178.575 ± 7.45 with the scope from 81,7±1,2 to 361,0 ± 11,9. The antioxidant ability was the higher when water was used as solvent – average 221.35 ± 6.55 compared to 221.35 ± 6.55 for 95 % ethanol. Finely ground sample extracted with water has given the highest oxidant activity - 361,0 ± 11,9 which confirm the advantage of the water as better solvent for extraction of solutions from stevia with high antioxidant activity. Higher Radical scavenging activity was obtained from finely ground samples - 272 ± 9.5 which exceeded more than three times coarsely ground sample - 85.15 ± 5.4. Differences in the Radical scavenge activity-DPPH, mM TE/g DW, among ethanol and water extracts from coarsely ground samples are not statistically significant, while for finely ground are statistically significant at P<0.001.

The ferric reducing properties are generally associated with the presence of reductions by breaking the free radical chain by donating a hydrogen atom[18]. The ferric reducing power is widely used in the evaluation of the antioxidant component in dietary polyphenols[19].

Table 3. Antioxidant activity and total polyphenols in the extracts of *Stevia rebaudiana* in the dry weight(DW)

Sample	Solvent	Extract mg/g DW	Total polyphenol content, mg GAE/g DW	Radical scavenge activity- DPPH analysis, mM TE/g DW	Metal reducing activity, FRAP analysis, mM TE/g DW
Finely ground sample	95% Ethanol	283,4 ± 5,1***	14,8 ± 0,3***	183,0±7,1***	145,7 ± 2,9***
Coarsely ground sample	95% Ethanol	224,6 ± 2,1***	10,7 ± 0,8a	88,6 ± 9,6a	89,8 ± 4,6*
Finely ground sample	Water	387,0 ± 8,6*	20,1 ± 0,4***	361,0 ± 11,9***	224,1 ± 7,1***
Coarsely ground sample	Water	367,8 ± 6,5**	11,1 ± 0,4a	81,7±1,2a	74,8±1,3*
Average for solvent	95 % Ethanol	254 ± 3.6	12.75 ± 0.55	135.8 ± 8.35	117.75 ± 3.75
	Water	377.4 ± 7.55	15.6 ± 0.4	221.35 ± 6.55	149.45 ± 4.2
Average for the size of grounding	Finely ground sample	335.2 ± 6.85	17.45 ± 0.35	272 ± 9.5	184.9 ± 5.0
	Coarsely ground sample	296.2 ± 4.3	10.9 ± 0.6	85.15 ± 5.4	82.3 ± 2.95
Average for the size of grounding and solvent		315.7 ± 5.58	14.175 ± 0.475	178.575 ± 7.45	133.6 ± 3.98
Standard Deviation		68.56	3.96	117.90	61.27

Table 4. Total phenols and antioxidant activity in *Stevia rebaudiana* extract

Sample	Solvent	Total polyphenol content mg GAE/g extract	Radical scavenge activity- DPPH analyse, mM TE/g extract	ECso, DPPH analyse, mg extract/mL	Metal reducing activity, FRAP analyse, mMTE/g extract
Finely grouned sample	95% Ethanol	53,2 ± 0,5a	663,2 ± 2,2***	0,7	518,8 ± 8,4***
Coarsely ground sample	95% Ethanol	50,4 ± 2,8a	431,6 ± 5,6***	1,3	433,7 ± 9,6***
Finely ground sample	Water	52,2 ± 0,9a	962,4±51,7***	2,2	576,7 ± 3,1***
Coarsely ground sample	Water	32,6 ± 0,2***	220,2 ± 2,7***	0,6	200,9 ± 2,9***
Average for solvent	95 % Ethanol	51.8 ± 1.65	547.4 ± 3.9	1	476.25 ± 9.0
	Water	42.4 ± 0.55	591.3 ± 27.2	1.4	388.8 ± 3.0
Average for the size of grounding	Finely grouned sample	52.7 ± 0.7	812.8 ± 1.95	1.45	547.75 ± 5.75
	Coarsely ground sample	41.5 ± 1.5	325.9 ± 4.15	0.95	317.3 ± 6.25
Average for the size of grounding and solvent		47.1 ± 1.1	569.35 ± 3.0	1.2	432.525 ± 6.0
Standard Deviation		8.89	288.87		149.54

Metal reducing activity, FRAP analyse, mMTE/g DW was average 133.6 ± 3.98 with range from 74,8±1,3 to 224,1 ± 7,1. Higher metal reducing activity was obtained from the water extract and from the finely ground samples. Comparison among the Radical scavenge activity DPPH and Metal reducing activity, FRAP demonstrates lower Metal reducing activity, FRAP for the adequate samples and factors – solvent and size of ground of samples. The differences of Metal reducing activity, FRAP are statistically significant at $P<0.001$ among ethanol and water solvents for finely ground samples and at $P<0,05$ among the finely and coarsely ground samples for the two solvents. Total polyphenol content, mg GAE/g extract derived from the dry leaves of Stevia rebaudiana Bartoni was average 47.1 ± 1.1 (Table 4). The range was in a scope from 32,6 ± 0,2 to 53,2 ± 0,5. With average 51.8 ± 1.65 polyphenols the ethanol 95 % confirms that is better solvent for extracting of polyphenols than the water - 42.4 ± 0.55. The higher content of polyphenols in the extract has obtained Ismet Ara Jahan et al. 15 after ethanol extraction- 65.21±0.97 and lower by water extraction - 41.49± 0.86 .

Total polyphenols are higher in finely ground samples. The amount of polyphenols in the extract is approximately 4

Table 5. Correlation among the parameters.

Sample	Extract, mg/g DW	Total polyphenols, mg GAE/g DW	Radical scavenge activity- DPPH, mM TE/g DW	Metal reducing activity, FRAP, mM TE/g DW	Total polyphenols, mg GAE/g extract	Radical scavenge activity- DPPH, mM TE/g extract	Metal reducing activity, FRAP, mM TE/g extract
Extract, mg/g DW	1.000						
Total polyphenols, mg GAE/g DW	0.566	1.000					
Radical scavenge activity- DPPH , mM TE/g DW	0.544	0.994***	1.000				
Metal reducing activity, FRAP, mMTE/g DW	0.458	0.989***	0.993***	1.000			
Total polyphenols, mg GAE/g extract	-0.392	0.533	0.544	0.629*	1.000		
Radical scavenge activity- DPPH, mM TE/g extract	0.271	0.942***	0.951***	0.979***	0.767**	1.000	
Metal reducing activity, FRAP, mMTE/g extract	-0.123	0.747**	0.760**	0.825***	0.952***	0.919***	1.000

Statistical significance at *P<0.05; **P<0.01; P<0.001

times higher than those in the dry matter. The difference in the total phenol content GAE/g extract is statistically significant only for the water extract from coarsely ground sample. Radical scavenge activity- DPPH, mM TE/g extract is average 569.35 ± 3.0 with the range in margin 220,2 ± 2,7 to 962,4±51,7**.** The highest radical scavenge activity is obtained from water extract and from fine grounding of the leaves. The difference among average values of radical scavenge activity of the water and the ethanol as solvents is a small 591.3 ± 27.2 - 547.4 ± 3.9 respectively. High antioxidant activity has been established for *Stevia rebaudiana* aqueous extract when compared to α-tocopherol, BHA and green tea extract in sardine oil and linoleic acid systems[20]. Esmat A et al[21] had also found higher radical scavenge activity from water extract than methanolic extract, respectively -37.36b ± 1.47 31.61c ± 0.90. Oppositely the difference in antioxidant activity is 2.5 times higher from the finely ground sample than coarsely ground sample - 812.8 ± 1.95 to 325.9 ± 4.15 respectively. The difference in the radical scavenge activity- DPPH, mM TE/g extract is statistically significant among all examined factors.ECso, DPPH, mg extract/mL was average 1.2 with the range from 0,6 to 2,2. The highest value was obtained from finely ground sample with water extract and the lowest from the coarsely ground water also from water extract. Metal reducing activity, FRAP assay, mMTE/g extract obtained in the present survey was average 432.525 ± 6.0 with scope from 200,9 ± 2,9 to 576,7 ± 3,1**.** Although the highest values of metal reducing activity is obtained from water, ethanol 95 % has higher average values than those of water due to the very low metal reducing activity obtained from water extraction from the coarsely grounding. The same tendency was obtained for total polyphenol content, mg GAE/g extract.

Kim, et al.[22] investigated influence of the method of extraction (hot water extraction (HWE) at $120^{\circ}C$ for 4 hr, vacuum extraction (VE) at $65^{\circ}C$ for 4 hr under 0.08 MPa, and fermentation of hot water extract (FHWE) using Lactobacillus buchneri) on the antioxidant activity of stevia leaves extract found that the antioxidant activities measured by radical scavenging activity, ferric-reducing antioxidant potential ability, and thiobarbituric acid reactive substance showed the highest values in vacuum extract. The antioxidant activities of all extracts have been higher than those of stevioside and rebaudioside at the same concentrations, known as the major active components in stevia. Good correlation among the total polyphenol content and metal reducing activity was established (Table. 5). The difference in the metal reducing activity is statistically significant among all examined factors. For better extraction and obtaining of higher radical scavenge and metal reducing activity is necessary very fine grounding of the leaves. Factor analyze for establishing the relations among the factors and the controlled parameters demonstrates that solvent and particle size of ground leaves have different effect on the expression of the parameters. The amount of the leaves extract depends mainly from the solvent – 88.34 %, while the particle size of grounding of the sample effect is only 8.82 % (Table 6). Oppositely the concentration of the extracted total polyphenols, depend mainly from the particle size of leaves grounding- 43.21 – 74.32 % for g/extract and g/DW, respectively. Radical scavenge activity- DPPH, was influenced mainly from the particle size of grounding of the sample – 68.50% for mM TE/g DW and 77.48% for mM TE/g extract. On the Metal reducing activity-FRAP, bigger effect has also particle size of the samples – 64.76% mM TE/g extract and 76.46 mM

Table 6. Influence of the factors solvent and particle size of the sample on parameters

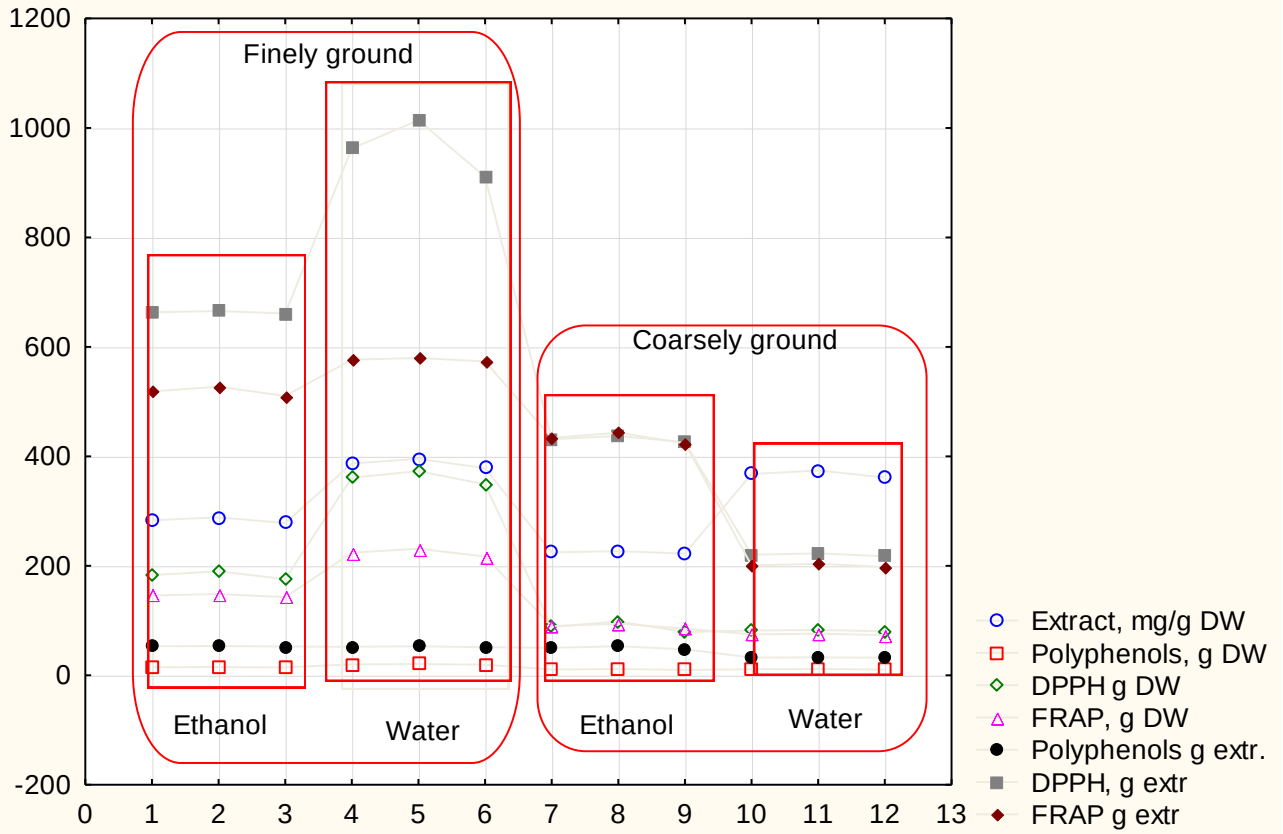
Parameters/ Factors	SS	DF	MS	F	P	%
Extract, mg/g DW						
Particle size	4563	1	4563	124.48	0.000004	8.82
Solvent	45683	1	45683	1246.20	0.000000	88.34
Particle size*Solvent	1176	1	1176	32.08	0.000474	2.27
Error	293	8	37			0.57
Total polyphenols, mg GAE/g DW						
Particle size	128.708	1	128.708	490.314	0.000000	74.32
Solvent	24.368	1	24.368	92.829	0.000011	14.07
Particle size*Solvent	18.008	1	18.008	68.600	0.000034	10.40
Error	2.100	8	0.263			1.21
Radical scavenge activity- DPPH, mM TE/g DW						
Particle size	104738.8	1	104738.8	1466.827	0.000000	68.50
Solvent	21956.4	1	21956.4	307.491	0.000000	14.36
Particle size*Solvent	25641.0	1	25641.0	359.093	0.000000	16.77
Error	571.2	8	71.4			0.37
Metal reducing activity-FRAP, mM TE/g DW						
Particle size	31580.3	1	31580.3	1546.73	0.000000	76.46
Solvent	3014.7	1	3014.7	147.65	0.000002	7.30
Particle size*Solvent	6542.7	1	6542.7	320.44	0.000000	15.84
Error	163.3	8	20.4			0.40
Total polyphenols, mg GAE/g extract						
Particle size	376.32	1	376.32	168.38	0.000001	43.21
Solvent	265.08	1	265.08	118.60	0.000004	30.44
Particle size*Solvent	211.68	1	211.68	94.71	0.000010	24.30
Error	17.88	8	2.24			2.05
Radical scavenge activity- DPPH, mM TE/g extract						
Particle size	711215	1	711215	1047.298	0.000000	77.48
Solvent	5782	1	5782	8.514	0.019356	0.63
Particle size*Solvent	195534	1	195534	287.934	0.000000	21.30
Error	5433	8	679			0.59
Metal reducing activity-FRAP, mM TE/g extract						
Particle size	159322	1	159322	3525.98	0.000000	64.76
Solvent	22943	1	22943	507.75	0.000000	9.33
Particle size*Solvent	63380	1	63380	1402.67	0.000000	25.76
Error	361	8	45			0.15

TE/g DW. Comparison the effect of the factors on the controlled parameters it is evident that the main influencing factor on the Radical scavenge activity and Metal reducing activity is a particle size of grounding of the leaves. The solvent as factor have a bigger effect only for the amount of the extract obtained during the process of derivation. The influence of the factors on the all investigated parameters was statistically significant at $P<0.001$. It can be concluded that the particle sizes and preparation of the substrate for extraction has a predominant effect on the composition of the extract and the concentration of polyphenols and antioxidant activity of stevia. Finely grounding of leaves is an essential condition and provide higher amount of polyphenols and antioxidant activity (Fig.1). If grounding is fine, water is better and more easy applicable extragent for extracting of polyphenols than ethanol. When the leaves are coarsely ground it is better to use ethanol as extragent. Among the investigated parameters a good correlations were found to exist. Radical scavenge activity and metal reducing activity correlate well with total polyphenols content. Coefficient of correlation $r=0.747 – 0.989$ is very high and statistically significant at $P<0.001$ (Table 5). Sunanda et al[16] considers that higher phenol content directly correlated with high radical scavenging activity. Kim et al.[21] also considers that the antioxidant activity of stevia extract is mainly due to the phenolic compound components.It was found that no correlation exited between total polyphenols and antioxidant actity and the volume of the extract.

CONCLUSION

The amount of the extracted from *Stevia rebaudiana* leaves low molecule and high molecule (inulin) fructans do not depend significantly from the size of grinding. Total polyphenol content extracted from *Stevia rebaudiana* leaves is higher when water is used as solvent and from finely ground sample. Radical scavenge activity- DPPH, average 178.575 ± 7.45 mM TE/g DW and average 569.35 ± 3.0 mM TE/g extract was higher from water as solvent and from finely ground substrate. Metal reducing activity, FRAP, average 133.6 ± 3.98 mMTE/g DW and average 432.525 ± 6.0 mMTE/g extract is also higher from water as solvent and from finely ground substrate. ECso,

Fig. 1. Disposition effect of the solvent and size of grounding on the parameters
Stevia-DPPH-FRAP 14v*16c



DPPH, mg extract/mL was average 1.2 with the scope 0.6-2.2. The highest value was obtained from finely ground sample with water extract and the lowest from the coarsely ground leaves also from water extract. The type of the solvent (water or ethanol- 95 %) has a highest effect only to the amount of Extract, mg/g DW. The content of Total polyphenols, mg GAE/g DW and mg GAE/g extract Radical scavenge activity- DPPH mM TE/g DW and DPPH mM TE/g extract, Metal reducing activity- FRAP, mM TE/g DW and mM TE/g extract were influenced mainly from the particle size and grounding as factor. The highest values of the presented parameters concerning to antioxidant activity - Radical scavenge activity and Metal scavenge activity were obtained when the dried leaves of stevia are finely ground and water is used as solvent for extraction. Radical scavenge activity DPPH and metal scavenge activity FRAP correlate very well with total polyphenol content. Correlation dependence is higher for Radical scavenge activity- DPPH, mM TE and lower for Metal reducing activity, FRAP, mM TE.

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