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Full Length Research Paper

Characterization of biological activity components of *Stevia rebaudiana Bertoni* Leaf Super Fluid Extraction Fraction

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Abstract
The leaves of Stevia Rebaudiana Bertoni represent a natural source of sugar. They are widespread in the food industry and are known as a natural sweetener. The main sweetness in leaves is caused by diterphenoidal glycosides. We have studied the fractions of Stevia leaf (common in Georgia), obtained through the SFE method, and identified the two preparations of various sweetness. Chlorophyll A and B, common carotenes, common flavonoids, catechins and phenol carbonate acids have been identified by the HPLC, UPLC, VIS / UV methods, while their antioxidant activity has been determined by the DPPH method. Among the studied 31 fractions, the total amount (12000- 7000 ppm) of sweet terpenoide-stevioside and rebaudioside has been determined from fractions 20-29 (29% - 93%). As a final product, we have obtained two preparations that are 100 and 300 times sweeter than sugar.

Keywords: *Stevia, supercritical fluid extraction (SFE), antioxidant activity, glycoside, rebaudioside A.*

Introduction

Stevia (Lat. Stévia) is a honey, sweet plant. In world practice, Stevia is used directly as a leaf, extract, dry extract and sweet diterphenoidal glycosides, as an alternative to a synthetic sweetener - sulphate and saccharin. However, only *Stevia rebaudiana* Bertoni, which motherland is South America, is distinguished by the curative properties. The cultivation of Stevia in different countries began in the previous century. Stevia was introduced in Georgia in the 80s of XX century. (Sivaram & Mukundam, 2003).

A leaf of Stevia is 10-15 times sweeter than sucrose. It should be noted that its caloricity is practically zero (eg, there are 387 k / calories in 100 g of sugar, while there are 18 kcal in 100 g of Stevia's dry grass). Stevia does not cause insulin secretion, as it does not change the level of glucose. The population of Paraguay does not suffer from such diseases as fatty acids and diabetes, because each inhabitant consumes 10 kg of Stevia per year. Unlike the refined sugar, it has a rich chemical composition: its leaves contain 8 diterphenoidal glycosides: Stevioside, steviolbioside, rebaudiosides (A, B, C, D, E) and dulkoside; among them the most sweet and resistant of the detrimental glycosides is rebaudioside A. (https://sptnkne.ws/ewNt) A (Geuns J. M.C. (2003). Stevioside. Phytochemistry)Puri et al., 2011). The goal of our work is to study the chemical composition of extracts and preparations, obtained from the leaves of Stevia, cultivated in Georgia, by using SFE extracting method of superccritical pressure, and to determine its antioxidant activity.

Materials and Methods

Study was conducted in the Department of Chemical Analysis and Food Safety of the Agrarian and Membrane Technology Institute, Batumi Shota Rustaveli State University. Also, at West Georgia Chromatography Center. (Grant AP/96/13 Georgia National Science Foundation).

Biochemical and chemical analysis was carried out by different physico-chemical and instrumental methods. Separation, identification and quantative analyses were carried out using UPLC-PDA (Waters Acquity PDA detector), Spectrometer – Cuvette Changer (Mettler Toledo UV5A); chemicals: radical stability - 2,2-Diphenil-1-picrilhydrazyl (Aldrich-Germany), AlCl3, standads – Gallic acid (Sigma), + Catechin (Teodor Schuchard), Waters Acrodisc LC PVDF Filter 13 mm 0.45 µm.

Antioxidant action was determined using DPPH (2,2-Diphenyl-1-pic rylhydrazil) methods [7,8]. For determination of antioxidant action radical retention to the 1 ml of the sample 3 ml of DPPH extract (0.1 mM DPPH-0.004 g/100mL in ethil alcohol) and after 30 minutes the optical density was evaluated on spectrophotometer. DPPH and 96% ethyl alcohol were used as blanks. For determination of action of free radical inhibition (DPPH) the following formula was used: In % = $A_C - A_S/A_C*100\%$, where A_c indicates absorbtion of DPPH/Alcohol solution and A_s indicates absorption of the extract.

Catechins and Procyanidine contents were determined by Swain and Hill spectral method [10]: 1 ml of samples were added 3 ml of 1 % vanillin reagents (1g vanillin added by 70 % -sulfur acid solution). All the solutions except the samples were used as blank.

After 15 min, spectral adsorption was determined at 750 nm. Total amount of flavonoid content (TFC) was determined by the aluminum chloride colorimetric method as previously described [11]. Samples (0.5 mL) were mixed with 2 mL of distilled water and 150 µL of 5% $NaNO_2$ solution. After 5 min, it was added by 150 µL of 10% $AlCl_3$ and, after 6 min, by 2 mL of 1 mol/L NaOH solution. The end volume was increased to 5 mL with distilled water. Finally, the absorbance was measured at 510 nm. Results were expressed in mg/l of catechine (or Ruthin).

Determination of Chlorophyll - A, B and Total Carotenoid Contents. The amount of these pigments was calculated according to the formulas of Spectrophotometric Determination of Chlorophyll - A, B and Total Carotenoid Contents of Some Algae Species Using Different Solvents. The calculations have been done by the following formula:
Ca = 11.75 xA662 - 2.350x A645
Acetone Cb = 18.61 xA645 - 3.960 xA662
Cx+c = (1000x A470 - 2.270 xCa - 81.4 xCb)/227
Ca = Chlorophyll a, Cb = Chlorophyll b, Cx+c = Total carotene

Research Methods
The object of the research are the leaves of Stevia (collected during its budding), that were brought to Georgia from South America in the 80s of XX century. The processing of Stevia's pre-dried leaf was carried out by fluid (inert gases-carbon dioxide and co-solvent-ethanol) extraction of supercritical pressure (Waters SFE -100-2-C10).

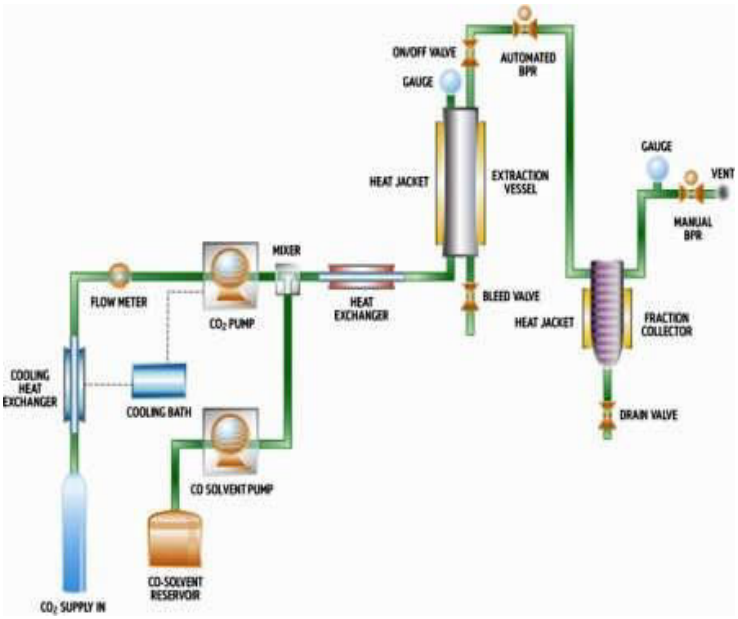

Fig. 1. Equipment diagram**.**

Fig. 2 General view of SFE 100 equipment.

The apparatus consists of the following main components: carbon dioxide reservoir, solvent (pH) pump, co-solvent pump, co-solvent reservoir, mixer, extractor, evaporator-cyclone and other controlling equipment which is managed by a computer.

Supercritical Fluid Extraction (SFE) of Stevia leaf. There have been selected two methods of Fluid Extraction. The first extraction method was used to produce diterphenoidal glycosides from Stevia leaves, while the purpose of the second method is the removal of the obstructive substances (including colored ones) from the Stevia leaves, what allows us to obtain the total preparation of diterphenoidal glycosides by hot extraction of leaves (ethyl alcohol / water mixture). 31 fractions have been obtained from 10 grams of green dried Stevia leaf, extracted by the SFE method.
The mode of conducting the SFE method consisted of four stages; the following fractions were obtained:
First stage - extraction by carbonate dioxide of supercritical pressure;
Fraction 1 -30 min, 500 bar at 40°C at speed of carbon dioxide 20 g / min;
Fraction 2 - 20 min, 500 bar at 60°C at speed of carbon dioxide 20 g / min;
Fraction 3 - 20 min, 500 bar at 80°C at speed of carbon dioxide 20 g / min;
At the second stage, there was added 5% co-solvent (96% ethanol)
Fractions 4-7 - 350 bar at 60°C at speed of carbon dioxide 20 g / min;
Third stage -10% co-solvent was added (96% ethanol)
Fractions 8-16 - 350 bar at 60°C at speed of carbon dioxide 20 g / min;
Fourth stage - 5% co-solvent was added (50% ethanol/water)
Fractions 16-24 - 350 bar at 60°C at speed of carbon dioxide 20 g / min;
Fifth stage - 5% co-solvent was added (96% ethanol/water);
Fractions 24-31 - 350 bar at 60°C at speed of carbon dioxide 20 g / min.
The equipment is depicted in Fig. 2, while the first stage is graphically illustrated in Fig. 2,3

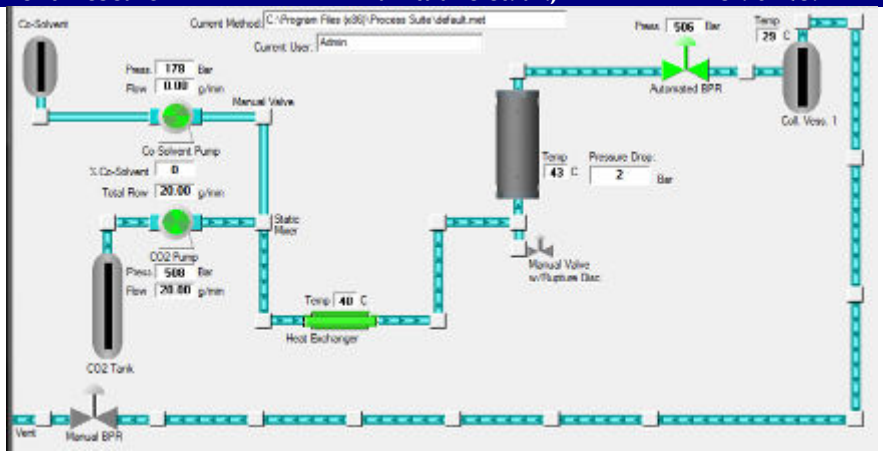

Fig. 3 Processing Scheme

Fig 4. SFE Praparat in the recycler

Results and Discussion

At the first stage of Stevia leaf processing through SFE method, soluble lipophilic compounds were extracted in organic solvents. Therefore, naturally, chlorophyll A and B (37.54-1.96 mg / g, 24.16-0.427 mg / g respectively) and carotene 19.1-1.0 mg / g prevail in fractions 1-8. The extraction of phenol carbonate acids, catechins and flavonoids (1-8 Fractions -150-7.6 mg / c respectively) was carried out at the water flow in the leaf.

Table 1. SFE fraction characterization

SFE №	Fraction, ml	Extract %	Chlorophyll A, mg/g	Chlorophyll B, mg/g	Total carotene, mg/g	Phenolic Acid mg Caffeic Acid/g	Total Catechine mg /g	Total Flavonols mg RUT/g	IC50 mg of sample
1	20	2.81	12.557	3.462	3.811	16.7	0.0	0.0	3.322
2	20	7.75	37.539	24.161	19.155	0.0	0.0	0.0	12.907
4	30	0.95	6.882	4.191	3.495	9.8	0.0	0.0	1.093
5	30	0.62	3.290	0.514	0.996	17.2	0.0	0.0	0.576
6	50	0.61	3.412	0.066	0.563	14.7	0.0	0.0	0.420
7	30	0.85	2.344	0.035	0.359	10.4	0.0	0.0	0.272
8	50	0.27	1.961	0.427	1.021	7.6	0.0	0.0	0.017
9	40	0.08	0.121	0.021	0.063	3.9	5.2	6.9	0.404
10	30	0.01	0.006	0.001	0.003	1.1	1.0	1.8	0.134
11	30	0.07	0.028	0.005	0.015	0.6	0.3	1.1	0.783
12	100	0.12	0.143	0.029	0.089	3.4	2.2	7.2	0.451
13	80	0.09	0.068	0.013	0.045	2.1	0.5	0.0	0.297
14	65	0.09	0.051	0.009	0.036	1.6	0.0	0.8	0.293
15	40	0.07	0.025	0.005	0.018	1.6	0.0	0.0	0.284
16	30	0.20	0.318	0.111	0.253	4.4	0.0	8.4	0.025
17	10	1.82	3.757	1.493	2.289	3.2	1.3	10.6	0.082
18	10	1.13	1.701	0.904	1.238	8.4	2.4	24.2	0.046
19	10	1.58	3.613	2.332	2.008	7.7	3.5	35.5	0.064
20	10	1.88	2.954	1.670	1.973	8.3	4.0	37.7	0.044
21	10	0.91	0.346	0.090	0.258	6.8	2.8	18.2	0.062
22	25	0.65	1.001	0.507	0.730	11.1	8.9	30.0	0.088
23	25	0.74	0.639	0.252	0.584	11.1	11.6	35.8	0.055
24	25	0.64	0.613	0.201	0.460	11.2	8.7	25.0	0.153
25	40	0.50	0.893	0.183	0.447	12.4	19.9	52.6	0.139

26	80	0.36	1.265	0.260	0.568	19.8	32.2	75.7	0.151
27	35	0.32	0.465	0.122	0.213	7.6	11.6	20.5	0.158
28	50	0.23	0.484	0.050	0.089	8.5	8.9	29.1	0.132
29	50	0.51	1.112	0.201	0.404	15.0	15.5	59.1	0.214
30	50	0.37	0.620	0.108	0.265	7.6	22.7	28.0	0.212
31	50	0.61	0.541	0.381	1.291	9.5	68.0	23.2	0.393

There are almost no pigments in fraction 8. The amount of pigments after the addition of co-solvent to the fractions (31- 9) is in the form of a trace, while in fractions 17- 19 it exceeds 3 mg / g. The number of all phenolic compounds increases from fraction 17. The exception is phenol carbonate acid, which content varies without any regularity. This can be explained by various compounds of organic solvents in different solutions of phenol carbonate acid. The extract is rich in dry substances in fractions 1-8. It varies from 7.75% to 0.27%. Their content reduces in fractions 9 - 15 and, therefore, the number of analyzed substances also decreases. The content of extracted substances, as well as catechins, phenol carbonate acids and common flavonoids increases in fractions 16-20. The composition of extracted compounds gradually decreases in fractions 21-31, while the total number of individual components of phenolic natural compounds is preserved (Table 1). The sweet terpenoidal glycoside was obtained from the analyzed fractions 20-29 (total amount of steviosides and rebaudiosides 12000- 7000 ppm respectively)(Fig. 5). However, its amount is 500 ppm in fraction 31. As a final product, two preparations (sweeter than sugar in 100 times and 300 times respectively) were obtained. The total amount of steviosides and rebaudiosides in them was 29% and 93 % respectively. (Table 2)

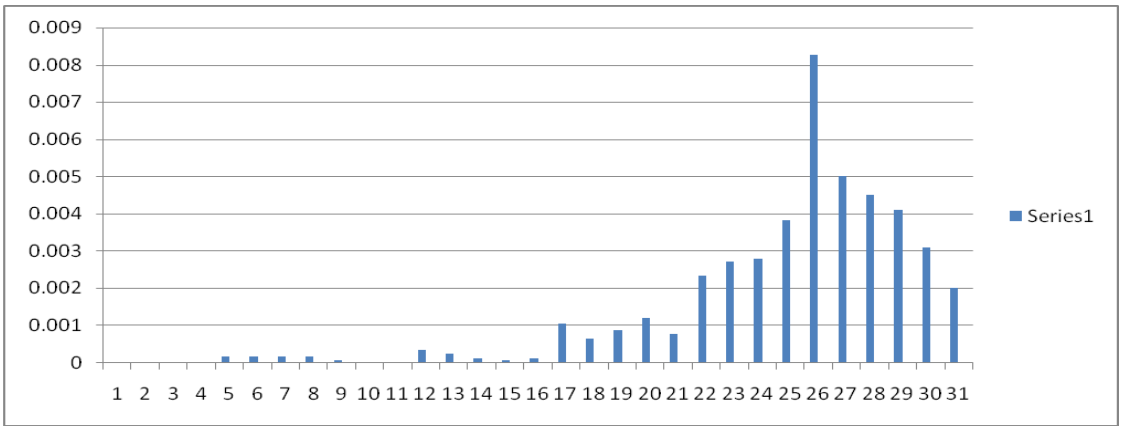

Fig. 5 Amount of sweet terpenoid glycoside by fraction

The total glycosides have been identified and quantified in the same fractions (20,29) and the both preparations by UPLC-PDA method. The antioxidant activity of the obtained fractions and preparations was determined, which increases with the total growth of phenolic compounds of different types (fractions 8-23) (Table 1).

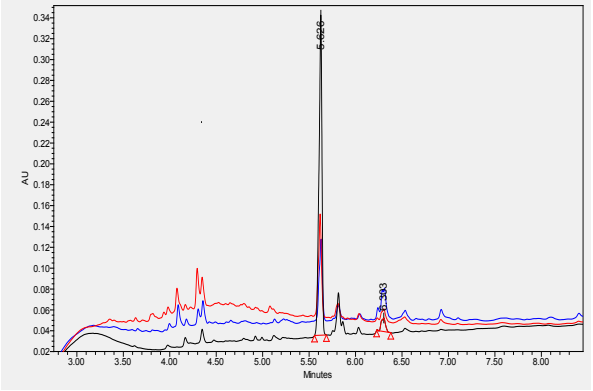

Fig.6 stevia total preparation UPLC-PDA-214 nm

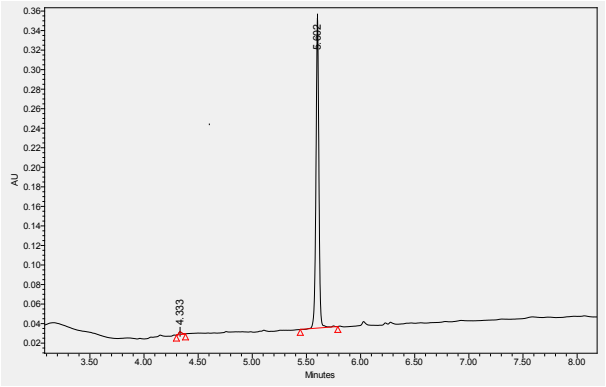

Fig.7 Rebaudioside A UPLC-PDA-214 nm

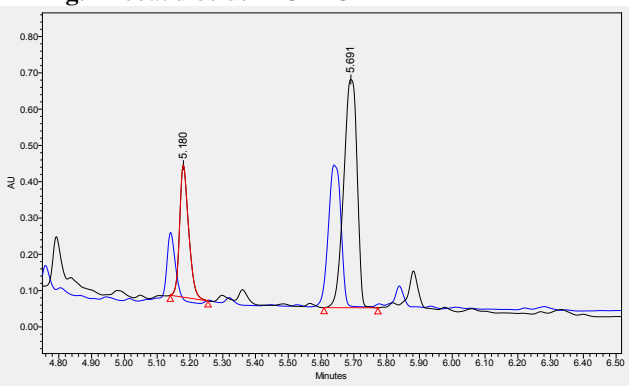

Fig.8 Stevia SFE fraction №20, 29 UPLC-PDA -214 nm

Table 2. Characterization stevia leaf sweet glycosides of SFE fraction and dray preparation

	Name	Retention Time	Area	% Area	Height	Amount	Units
1	Rebaudioside A +stevioside	5.602	594316	99.04	317392	2000	ppm
2	Fraction 29	5.640	1052679	76.29	388070	7084,98	ppm
3	Fraction 20	5.640	1789435	71.92	388070	12043,66	ppm
4	Stevia 100	5.619	225691	77.26	98311	29,00	%
5	Stevia 300	5.626	688461	94.38	306916	93,64	%

Conclusion

Using the SFE method, we have fractionated the components of Stevia leaf and identified chlorophyll A and B, common carotenes, common flavonoids, catechins and phenol carbonate acids for each fraction. Two preparations, containing different quality of sweetness, have been obtained; the quantitative content of glycosides in them was determined as well. The antioxidant activity has been established both for fractions and preparations.

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