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Extraction and characterization of steviol glycosides from *Stevia rebaudiana* bertoni leaves.

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Abstract
Steviol glycosides are highly sweet diterpene glycosides found in the Paragyan shrub, *Stevia rebaudiana* Bertoni. Steviol glycosides, mainly stevioside and rebaudioside, principal components of *Stevia rebaudiana* leaf, has become well known for their sweetness and are used as non-caloric sweeteners in several countries due to their nutritional and pharmacological benefits. Hence, craving for sweetness led man to discover several forms of alternative sweeteners. In this study, different polar and non-polar solvents were used for extracting glycosides from *stevia* leaves and the components isolated through column chromatography were characterized as steviolmonoside, stevioside and rebaudioside B on the basis of spectral (IR and ^{1}H-NMR data), physical and chemical characteristics.

Keywords: *Stevia rebaudiana*, stevioside, extraction, artificial sweetener, rebaudioside.

1. Introduction
Stevia rebaudiana Bertoni is a small, herbaceous, perennial shrub of the Compositae family and native to certain regions of South America. The plant made its way to Pacific Rim countries and it became cultivated domestically, used in its raw leaf form and is now commercially processed into sweetener. Since 1970s, *stevia* extracts have been widely used in many countries as a sugar substitute. *Stevia* is cultivated in many countries: Paraguay, USA, Mexico, Central America, Japan, China, Malaysia, South Korea, Spain, Italy and UK. In Brazil, Korea and Japan, *stevia* leaves and refined extracts are officially used as a low calorie sweetener [1].

The content of steviol glycosides found is *Stevia rebaudiana* varies between 4-20% of the dry weight of leaves depending upon the cultivar and growing conditions [2]. Stevioside is the main sweet component in the leaves of *Stevia rebaudiana* and it tastes about 300 times sweeter than sucrose (0.4% solution). Other components present but in lower concentration are steviolbioside, rebaudioside A, B, C, D, E, F and dulcoside A [3]. Stevioside and stevia extract can be used to treat diabetic condition. Intake of aqueous extract of stevia leaves, 5 g % at 6 h intervals for 3 days resulted in significant decrease in plasma glucose level during glucose tolerance test and after overnight fasting in all healthy subjects [4]. Stevioside slowed down gluconeogenesis in the liver via suppression of PEPCK (Phosphoenol pyruvate carboxy kinase) gene expression leading to decrease in plasma glucose level in diabetic rat [5]. In diabetic rat with very low insulin level PEPCK gene was over-expressed [6] and stevioside decreased PEPCK mRNA and protein concentrations in a dose-dependent manner [5]. Stevioside had direct effect on glucagon secretion as well [7]. In addition to stevioside and steviol, isosteviol, a metabolic compound of stevioside improved lipid profile and upregulated expression of key β-cell genes, including insulin regulatory transcription factors, thereby improved glucose homeostasis, increased insulin sensitivity and lowered plasma triglycerides [8].

The advantages of stevioside as a dietary supplement for human subjects are manifold: it is stable [9], non-calorific, maintains good dental health [10] and opened the possibility for use by diabetic and phenylketonuria patients and obese persons.

Structurally, stevioside is a diterpenoid glycoside, comprising an aglycone and three molecules of glucose. The ent-kaurene skeleton of stevioside and hence also of gibberellins (a plant growth regulator) is formed via 2-C-methyl-D-erythritol-4-phosphate pathway (MEP) [11]. Steviol was synthesized via mevalonate and was confirmed by sequencing 5548 expressed sequence tags (ESTs) from stevia leaf cDNA library [12]. A large fraction of the total plant metabolism is committed to the synthesis of these structurally complex molecules.

Profound changes in the regulation of copalyl phosphate synthase and kaurene synthase expression in stevia leaves have enabled the synthesis and accumulation of high concentrations of sweetness [13]. The present study was planned to extract different glycosides from stevia leaves using polar and non-polar solvents and to characterize them so as to know about their accumulation, yield and other characteristics.

2. Materials and Methods

Stevia plants were raised in open fields and green house of herbal garden of department of Agronomy, Punjab Agricultural University, Ludhiana in Randomized Block Design with three replications.

2.1 Extraction and Isolation

Stevia leaves were air dried followed by drying in hot air oven at 50 ºC for 24 hrs. The leaves were then ground to fine powder and then extracted by the method of Kohda *et al* [14]. The procedure involved four main steps: extraction into polar organic solvent, decolourization, coagulation or concentration, column chromatography and crystallization. Powdered *stevia* leaves were soaked in petroleum ether followed by extraction with methanol for 3 to 4 times. The methanol extract was concentrated under vaccum and the suspension was dissolved in distilled water. Green colour of the extract was removed by washing with diethyl ether. This was repeated 3 to 4 times until the colour was removed. The water extract was partitioned into butanol and the solvent was evaporated under vaccum at 60 ºC using Rotary Vaccum Flash Evaporator. The concentrated crude extract so obtained was dissolved in small amount of methanol and allowed to crystallize at 4 ºC and the mother liquor was separated.

2.2 Column Chromatography

To isolate the various components from the mixture of glycosides in the methanol extract, column chromatography was done. After crystallization the mother liquor was concentrated under vaccum and chromatographed over silica gel (400 g) of mesh size (16-120 mesh). The sample was dissolved in small amount of chloroform. Chloroform and methanol was used as the mobile phase with the increasing polarity. 200 ml volume fractions were collected and monitored by TLC.

2.3 Thin layer chromatography (TLC)

TLC involved the use of silica gel sorbent spread on an inert sheet of glass as a stationary phase. The mobile phase was allowed to travel up the plate carrying the sample that was initially spotted on the sorbent just above the solvent.

2.4 Preparation of chromatoplates

Chromatoplates of 5 cm × 20 cm and 20 cm × 20 cm sizes were prepared by making the silica gel (Silica Gel-G for TLC) slurry of appropriate consistency with distilled water. The slurry was spread over the cleaned and dried chromatoplate with the help of glass rod and by tapping the plates with hand on a smooth surface. The plates were dried at room temperature followed by activation at 120 ºC for 45 minutes. The small amount of glycosidic extract of *Stevia* leaves were spotted with the help of a fine glass capillary tube at a place about 2 cm above the lower edge of chromatoplates.

2.5 Development of chromatoplates

Rectangular glass chamber (24 cm × 12 cm × 24 cm) was used for the development of chromatoplates. TLC plates were developed in suitable solvent system like chloroform: methanol: water (30:20:4). The inner side of chamber were lined with solvent soaked filter paper to ensure solvent saturated atmosphere. The time required for chromatoplates to develop by ascending chromatography upto 16-17 cm from the origin was ≈1 hour. The developed plates were removed and dried at room temperature (≈ 28 ºC) and visualization of spots was done with the following methods:

2.6 Iodine vapours

The spots were visualized by introducing the developed chromatoplates into closed chamber containing iodine vapours. Iodine vapours were generated by warming the vessel containing small amount of iodine. Majority of components showed light brown to dark brown colour in iodine atmosphere.

2.7 Methanol sulphuric acid reagent

In a volumetric flask, 5 ml of concentrated sulphuric acid was added to 95 ml methanol. On spraying the oven dried chromatoplates with methanol sulphuric acid reagent, the black spots appeared after heating at 110 ºC for 10 minutes in an oven.

2.8 α-naphtholss reagent

Dissolved 10 mg of α-naphthol in 10 ml methanol and slowly added 0.4 ml of concentrated sulphuric acid. Violet blue coloured spots visualized after spraying with this reagent and heating in oven at 110 ºC for 10 minutes.

2.9 Characterization of isolated pure compounds

The pure isolated compounds were characterized by different spectroscopic techniques (i.e. IR and PMR), physical constants and using detecting reagents.

The IR spectra of pure isolated components 1, 2, 3 were recorded in KBr pellets on Perkin Elmer RXIFT-IR spectrometer. Proton Magnetic Resonance (PMR) spectra of the pure isolated components were recorded in DMSO d_6 using tetramethylsilane (TMS) as internal standard on Bruker AC 300F NMR (400 MHz) spectrometer. While citing PMR data (δ values) following abbreviations have been used: s (singlet); d (doublet); m (multiplet) and t (triplet).

3. Results and Discussions

The methanolic mother liquor of *stevia* leaf extract exhibited many components but four components could be identified with R_f values 0.93, 0.84, 0.63 and 0.50 on thin layer chromatographic plate (Silica Gel-G, solvent system; chloroform: methanol: water in the ratio 30:20:2(v/v/v). These spots showed brown colouration with the slight variation from each other in the iodine atmosphere. These spots were also visualized by 5% methanol sulphuric acid reagent followed by heating at 110 ºC for 10 minutes and spots showed black coloration. With α-naphthol reagent followed by heating at 80 ºC, component with R_f value 0.93 showed violet blue coloration whereas rest of the components showed blue coloration (Table 1).

Table 1: Characteristics of various components of *stevia* extract (mother liquor) on thin layer chromatographic plate (Silica Gel-G, solvent system; chloroform: methanol: water: 30:20:2 v/v/v)

	R_f	Colour in iodine atmosphere	Colour after spraying with 5% methanol sulphuric acid reagent/heating at 110 °C, 10 min	Colour after spraying with α-napthol reagent/ heating at 80 C,10 min
1.	0.93	Reddish brown	Black	Violet blue
2.	0.84	Light brown	Black	Blue
3.	0.63	Yellowish brown	Black	Blue
4.	0.50	Dark brown	Black	Blue

Shibata *et al* [15] used chloroform: methanol: water (10:6:1, v/v/v) for the development of TLC. After development, chromatograms were dried and sprayed with 50% sulphuric acid methanol reagent and heated at 100 °C for 15 minutes to visualize the glycosides. Metivier and Viana [16] extracted soluble sugars with methanol: chloroform: water (12:5:3 v/v/v). Glycosides were isolated using TLC followed by elution and assay by anthrone reaction. Isolated sugars were detected by spraying developed plates with a freshly prepared solution of 1, 3 naphthalindiol and then heating to 80° C for few minutes. Stevioside, the major component produced deep blue colour. Massoud [17] identified *stevia* sweeteners by TLC. The separation of compounds was obtained by solvent system ethyl acetate: acetic acid: water (8:3:2 v/v/v) and the R_f values for stevioside, rebaudioside A, rebaudioside C, rebaudioside E and steviolbioside were 0.42, 0.30, 0.10, 0.16 and 0.50, respectively.

In order to purify and characterize the components of *stevia* extract, column chromatography was done. Gradient elution was performed by increasing the polarity of the solvent (chloroform: methanol). A total of 94 fractions were collected each of 200 ml volume. Each fraction was concentrated by vaccum evaporation. TLC was used to monitor the qualitative composition of the fractions obtained through column. Various fractions which were found to be similar in composition after TLC analysis, were clubbed and the weight of each fraction was recorded (Table 2). TLC monitoring indicated three components to be in pure state. These components were eluted from 10%, 20% and 30% methanol as eluent. Rest of the fractions exhibited mixture of components. Thus, three fractions were further analysed by spectral studies.

Table 2: Details of Column Chromatography

Sr. No.	Eluent (ml)	Weight (g)	TLC based marks
1.	Chloroform (20 × 200)	Traces	—
2.	Chloroform: Methanol (10%) (14 × 200)	0.186	Crystalline solid (1)
3.	Chloroform: Methanol (15%) (10 × 200)	0.051	Mixture
4.	Chloroform: Methanol (20 %) (10 × 200)	0.427	Crystalline solid (2)
5.	Chloroform: Methanol (30%) (10 × 200)	0.290	Crystalline solid (3)
6.	Chloroform: Methanol (40%) (10 × 200)	0.297	Mixture
7.	Chloroform: Methanol (45%) (10 × 200)	0.192	Mixture
8.	Chloroform: Methanol (80%) (5 × 200)	Traces	Mixture
9.	Methanol (5 × 200)	—	Polar viscous liquid

Component 1 with melting point 185–190 °C exhibited IR bands at 3391 cm^{-1} corresponding to hydroxylic group, 1729 cm^{-1} showed the presence of carbonyl functionality. IR bands at 2928, 1652 and 892cm^{-1} were due to exomethylene group. This data was supported by ^{1}H-NMR signals (400 MHz, DMSO d_6) at δ 1.24 and δ 1.34 as singlets corresponding to two angular methyl groups at C-10 and C-4 position, respectively. The signal at δ 3.6 (J=3.24) appeared as doublet showing the presence of exomethylene group. A doublet at δ 4.4 (J=7.78) confirmed the presence of one anomeric hydrogen. This proved that only one sugar moiety is present in this compound. From the spectral analysis, the compound 1 is shown to have kaurene ring skeleton. Spectroscopic data of all the three compounds revealed that compounds have common aglycone moiety. One glucose that is present in this compound can form either ether linkage with -OH group at C-13 or ester linkage –COOH at C-4. Biogenetically the glucopyranose ring should be present in ether linkage with –OH group at C-13. So, on this basis compound 1 is proved to have the following structure:

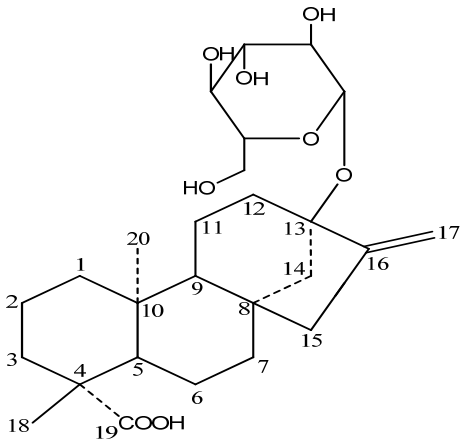

Fig 1: Component 1, Steviolmonoside

Component 2 with melting point ≈199 ºC [18] was proved to be stevioside by its IR and ^{1}H-NMR spectral analysis. IR bands at 3393 cm^{-1} confirmed the presence of –OH group. The presence of ester glycosidic linkage was shown by the band at 1728 cm^{-1}. The IR band at 2929 cm^{-1}, 1653 cm^{-1} and 892 cm^{-1} were attributable to exomethylene group. PMR signals showed the presence of same aglycone moiety as that in compound 1 which revealed that it also had the basic kaurene ring skeleton. The presence of position of two angular –CH_3 groups at C-4 and C-10 position were confirmed by the ^{1}H-NMR signals at δ 0.9 and δ 1.17 respectively. The spectrum showed the signals for three anomeric protons thereby confirming the presence of three glucopyranose moieties in the compound. These appeared as doublets at δ 4.4 (J=7.64), δ 4.6 (J= 7.96), δ 5.3 (J= 8.12) each for one proton. The signals at δ 4.8 and δ 5.1 appeared as singlets which were attributed to the presence of exomethylene protons. The spectral data and their comparison with that of authentic sample available in our laboratory confirmed it to be stevioside having structure 2 as shown below.

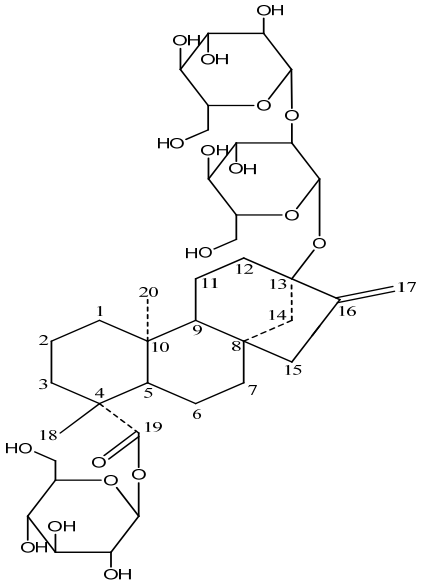

Fig 2: Component 2, Stevioside

Since this compound (2) is already reported in literature and comparison of its spectral data with that of compound (1) indicates that the glucose moiety in (1) can also be present with ester linkage since coupling constant for anomeric proton in (1) is 7.78 Hz and that for (2) is 7.64 Hz. On this basis the following tentative structure can be assigned to compound (1').

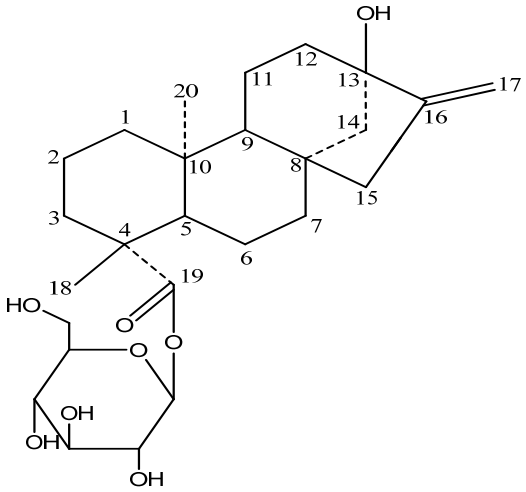

Fig 3: Component (1')

Component (3) with melting point 205-210 ºC showed IR bands at 3384 cm^{-1} corresponds to the presence of –OH group. The bands at 2929 cm^{-1}, 1653 cm^{-1}, 1074 cm^{-1}, 892 cm^{-1} were attributed to the exomethylene group. The band at 1720 cm^{-1} confirmed the presence of carbonyl group. The signals due to the protons of the aglycone moiety were at almost the same positions as that of other two components. ^{1}H-NMR signals at δ 0.9 and δ 1.19 as singlets confirmed the presence of methyl groups at C-4 and C-10 positions respectively. Signals appearing in ^{1}H-NMR spectrum at δ 5.02 and δ 5.1 were attributed to the two protons of exomethylene group at C-17. This indicated that all these components 1, 2 and 3 have the same aglycone moiety. The three glycopyranose units were indicated by the three doublets in the region δ 4.5 (J= 7.72), δ 4.7 (J=7.76) and δ 5.3 (J= 8.24) which were attributed to three anomeric protons. So, on this basis structure (3) can be assigned to this compound and it is the known compound, Rebaudioside B.

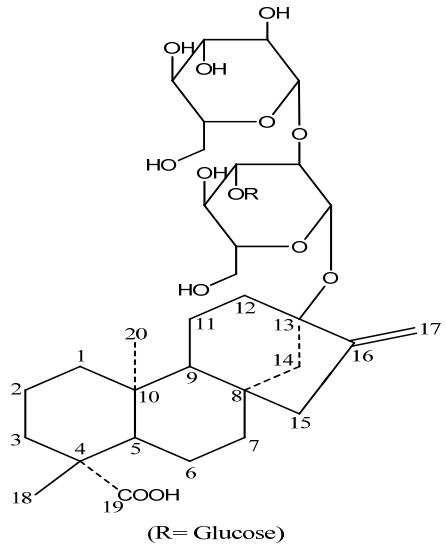

Fig 4: Component 3, Rebaudioside B

The characteristics of all the three components are shown in Table 3 and R_f values of these three components were observed in TLC plate shown as Fig. 5.

Table 3: Characteristics of pure isolated compounds

No. of components	R_f values	Nature of compound	Name of component	Melting point
1.	0.93	Solid	Steviolmonoside	185 -190 °C
2.	0.63	Solid	Stevioside	≈ 199 °C
3.	0.50	Solid	Rebaudioside B	205-210 °C

Fig 5: Thin layer chromatographic plate of isolated components from the mother liquor of glycosidic extract.

The percentage recovery through column chromatography were found to be 4.65, 10.67 and 7.25 percent for component 1, 2 and 3 respectively. Kohda *et al* [14] reported the isolation of stevioside (0.33%) and rebaudioside B (0.04%) in *S. rebaudiana* leaves. Methanol with 10, 20 and 30 percent combination with chloroform was found to be suitable for the high yield of three extracted steviol glycosides in this study. This could further be employed at large scale and could be used as an effective method for extracting higher yield of glycosides for industrial applications.

4. References

1. Mizutani K, Tanaka O. Use of Stevia rebaudiana sweeteners in Japan. In: Kinghorn A D (Ed.), Stevia, the Genus Stevia. Medicinal and Aromatic Plants-Industrial Profiles. 2002; Taylor and Francis, London and NY. 19:178-195.
2. Geuns JMC. Stevioside. Phytochem 2003; 64:913-921.
3. Kennelly EJ. Sweet and non-sweet constituents of Stevia rebaudiana (Bertoni) Bertoni. In: Kinghorn A D (Ed.), Stevia, the genus Stevia. Medicinal and Aromatic Plants-Industrial profiles Vol 19, Taylor and Francis, London and NY, 2002, 68-85.
4. Curi R, Alvarez M, Bazotte RB, Botion LM, Godoy JL, Bracht A. Effect of *Stevia rebaudiana* on glucose tolerance in normal adult humans. Brazilian J Med Biol Res 1986; 19:771-774.
5. Chen TH, Chen SC, Chan P, Chu YL, Yang HY, Cheng JT *et al*. Mechanism of the hypoglycemic effect of stevioside, a glycoside of Stevia rebaudiana. Planta Med 2005; 71:108-113.
6. Chan P, Wong KL, Liu IM, Tzeng TF, Yang TL, Cheng JT *et al*. Antihyperglycemic action of angiotensin II receptor antagonist, valsartan, in Streptozotocin-induced diabetic rats. J Hypertens 2003; 21:761-769.
7. Hong J, Chen l, Jeppesen PB, Nordentoft I, Hermansen K. Stevioside counteracts the α-cel hypersecretion caused by long-term palmitate exposure. Am J Physiol Endocrinol Metab 2006; 290:416-422.
8. Nordentoft I, Jeppesen PB, Hong J, Abdula R, Hermansen K. Isosteviol increases insulin sensitivity and changes gene expression of key insulin regulatory genes and transcription factors in islets of the diabetic KKAy mouse. Diabetes Obes Metab 2008; 10:939-949
9. Chang SS, Cook JM. Stability studies of stevioside and rebaudioside A in carbonated beverages. J Agric Food Chem 1983; 31:509-512.
10. Das S, Das AK, Murphy RA, Punwani IC, Nasution MP, Kinghorn AD *et al*. Evaluation of the Cariogenic Potential of the Intense Natural Sweeteners Stevioside and Rebaudioside A. Caries Res 1992; 26:363-366.
11. Totte N, Charon L, Rohmer M, Compernolle F, Baboeuf I, Geuns JMC *et al*. Biosynthesis of the diterpenoid steviol, an ent-kaurene derivative from *Stevia rebaudiana* Bertoni, via the methylerythritol phosphate pathway. Tetrahedron Letters 2000; 41:6407-6410.
12. Brandle JE, Richman A, Swanson AK, Chapman BP. Leaf ESTs from *Stevia rebaudiana*: a resource for gene discovery in diterpene synthesis. Plant Mol Biol 2002; 50:613-622.
13. Richman AS, Gijzen M, Starrat AN, Yang Z, Brandle JE. Diterpene synthesis in stevia rebaudiana: recruitment and upregulation of key enzymes from the gibberellin biosynthetic pathway. Plant J 1999; 19:411-421.
14. Kohda H, Kasai R, Yamasaki K, Murakami K, Tanaka O. New sweet diterpene glucosides from *Stevia rebaudiana*. Phytochem 1976; 15:981-983.
15. Shibata H, Sonoke S, Ochiai H, Nishihashi H, Yamada M. Glycosylation of steviol and steviol glycosides in extracts from *Stevia rebaudiana* Bertoni. Plant Physiol 1991; 95:152-156.
16. Metivier J, Viana AM. Determinaion of microgram quantities of steviosides from leaves of *Stevia rebaudiana* Bertoni by two dimensional thin layer chromatography. J Exp Bot 1979; 30:805-810.
17. Massoud MI. Chemical and Technological Studies on Natural Sweeteners from *Stevia* Plant. Ph.D. Thesis in Food Technology, Food Science and Technology Department, Faculty of Agriculture, Alexandria University, 2001.
18. Kinghorn AD, Soejarto DD. Current status of stevioside as a sweetening agent for human use. In: Wagner H, Hikino H, Farnsworth NR. (Eds.). In: Economic and Medicinal Plant Research 1985; 1:152.