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Isolation and analytic characterization of rebaudioside A and GC-MS analysis of methanolic leaves extract of *Stevia rebaudiana* Bert.

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Abstract

During the present research endeavour, a glycoside *viz.*, rebaudioside A was isolated from the *in vivo* leaves of *Stevia rebaudiana* Bert. and identification of the isolated compound was carried out through thin layer chromatography, R_f value, IR spectroscopy and NMR. Furthermore, the methanolic extracts of leaf were also analyzed by gas chromatography and mass spectroscopy in *S. rebaudiana*. It was observed that 71 secondary compounds were isolated in *S. rebaudiana*, respectively.

Key words: *Stevia rebaudiana* Bert., Rebaudioside A, TLC, IR, NMR

Introduction

Our ancestors, whose lives were intimately connected with the plants for their needs and rhythms for living because they provided their food, fiber, shelter and required medicines. Plant genomics has a critical role to play in 21st century agriculture, energy, pharmacology and environmental stewardship (Raskin *et al.*, 2002).

The reach of plant sciences stretches way, beyond direct improvement in crops. Plants can provide scientists with a window to many types of biological phenomena and help to answer fundamental biological questions. Plant genome science has significant cross-disciplinary applications and has already spurred advances in medicine, chemistry, pharmacology and engineering, in addition to basic biology (Ajose, 2007).

With reference to this, plants are known to have a treasure ground of many kinds of biochemicals which have proved to be a boon for the whole of our mankind. These biochemicals are also referred to as phytochemicals (from the Greek word phyto, meaning plant), which are biologically active, naturally occurring chemical compounds found in plants (Verpoorte, 1994).

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These phytochemicals obtained as a result of various metabolic processes, occurring at different cellular levels, involving many kinds and types of organic compounds, which further result in the production of various metabolites. These metabolites are grouped into primary and secondary metabolites. The metabolic pathways that are essential for sustaining life and organization of plant body are synthesis of primary metabolites *viz.*, protein, proline, carbohydrate, amino acid, phenol, ascorbic acid, chlorophyll *etc*. These play recognized role in photosynthesis, respiration, transpiration, nutrient assimilation *etc*. The secondary metabolites are having a restricted distribution in plant kingdom. Nevertheless, secondary metabolites are found specific to a particular plant species or a taxonomically related group of species; whereas the basic primary metabolites are found throughout the plant kingdom (Taiz and Zeiger, 1996).

Plant secondary metabolites bear chemical and pharmaceutical properties concerning with the human health (Raskin *et al.*, 2002; Reddy, 2003). Compounds belonging to the terpenoids, alkaloids, glycosides, stereols and flavonoids are currently used as drugs or as dietary supplements to cure or prevent various diseases (Raskin *et al.*, 2002) and in particular, some of these compounds seem to be efficient in preventing and inhibiting various types of cancer (Watson *et al.*, 2002; Reddy, 2003), asthma (Savithramma *et al.*, 2007), diabeties (Bhuiyan *et al.*, 2009), cataract (Kebapci *et al.*, 1999) and many more diseases. The most powerful technique developed in biophysics, such as IR, NMR, help us in identification of 2-dimensional structure of huge biological molecules (Terskikh *et al.*, 2005).

For the past few decades, various reports are available as well as investigations have been carried out, showing the importance of extracting, isolating, identifying, utilizing and exploring various medicinally bioactive compounds, from many plant species in *in vitro* and *in vivo* conditions such as in *Nerium oleander* (Huq and Hodeges, 1999), *Sericocalyx schomburgkii* (Phuruengrat and Phaisansuthichol, 2006); *Piper nigrum* and *Piper longum* (Hamrapurkar *et al.*, 2011); *Pavetta indica* (Prasad *et al*., 2011); *Momordica charantia* (Ullah *et al*., 2011) *etc*. Hence, this field has opened up new vistas in the field of plant sciences and medicinal world.

During the present research endeavour, rebaudioside A has been extracted from the leaves of *S. rebaudiana*, and then purified and identified by some spectroscopic methods like IR, NMR *etc*. Besides this, gas chromatography and mass spectroscopic studies have been done using crude methanolic extracts of *Stevia rebaudiana*.

Stevia rebaudiana Bert. belonging to the family, Asteraceae, is a sweet herb native of South America. The plant has also been cultivated in China and Southeast Asia. Stevia's crude extract from leaves has been used for a few decades to sweeten soft drinks, soju, soya sauce, yogurt and other foods in Japan, Korea and Brazil (Kinghorn and Soejarta, 1985). The dry extract from the leaves of Stevia contains flavonoids, alkaloids, water soluble chlorophylls and xanthophylls, hydroxycynnamic acids (caffeic, chlorogenic, *etc*.), neutral water soluble oligosaccharides, free sugars, amino acids, lipids, essential oils and trace elements (Komissarenko *et al*., 1994). Different extracts of Stevia have suggested that they exert beneficial effects on human health, including antihypertensive (Chan *et al*., 2000), antihyperglycemic (Jeppesen *et al*., 2002) and antihuman rotavirus activities (Das *et al*., 1992).

Material and Methods

Extraction of glycoside

Shade dried leaves and the powder (100gm) of *Stevia rebaudiana* were defatted with petroleum ether and then soxhlet extracted with ethanol for 48 hrs (Kolb *et al.*, 2001). The resultant viscous mass was hydrolysed with 500 ml of 5% H_2SO_4 and filtered. The filtrate was basified with ammonia and then fractionated by extracting sequentially with ether and chloroform. The mother liquor was evaporated to dryness and the dried residual mass was re-extracted with petroleum ether. Subsequently, all the three fractions were dried and examined on TLC along with the reference markers.

Fresh mature leaves of authenticated *S. rebaudiana* were taken. They were air dried under shed at room temperature and finely powdered with the help of grinder. 50 gm dried powdered leaves of *S. rebaudiana* was taken into thimble and 750 ml of methanol was taken into the flask of soxhlet apparatus and cycled 10-15 times. After that it was decanted into the beaker and was left open, so that the methanol gets evaporated while rotary vacuum evaporator was used at 40-45 °C. Afterwards, the proper evaporation analysis of materials was done using GC-MS.

Thin-layer chromatography

TLC Glass plates (20 ´ 10 cm) were coated (0.2-0.3 mm thick) with silica gel (30gm/60 ml. distilled water) dried at room temperature and activated before use at 100ºC for 30 min. in an oven and cooled at room temperature. Each of the crude extracts was applied separately 1.0 cm above from the lower edge of the activated silica gel plates along with the standard reference compound of rebaudioside A. The plates were developed in an air tight chamber containing chloroform: methanol: water (60:25:15). The developed plates were air dried, sprayed with 50% sulphuric acid and subsequently heated at 100ºC for 15 min, it showed spots which coincided with that of the reference rebaudioside A (bluish grey). When plates were placed in a chamber saturated with I2 vapors, it also showed deep brown color of rebaudioside A (Figure B). Rf value coincides with the Rf value 0.37-0.40 (Kedik *et al.*, 2003) of the standard rebaudioside A. The marked spots were scrapped and collected along with the silica gel and eluted with ethanol. Elute was crystallized with chloroform. The purified material was subjected to its IR and NMR spectral analysis.

Results and Discussion

During the present research endeavour, rebaudiaside A ($C_{44}H_{70}O_{23}$) was isolated from the leaves of *S. rebaudiana*. The characteristic IR, 1H NMR and 13C NMR spectral peaks of compound was found to be super imposable with those of their respective standard reference compound of rebaudioside A. Its IR and NMR spectra showed different peaks (Table 1).

Volatile organic materials are products of the secondary metabolism of plants and are generally consisting of complex mixtures of mono-, sesqui-, di-, tri-terpene hydrocarbons and oxygenated materials biogenically derived from them. Some of the compounds identified through GC-MS in *S. rebaudiana* are listed in Table 2. The dominant components in total compounds of the methanolic extract of *S. rebaudiana* were D-Allose (96%), 1-Octadecanol (95%), Phytol (95%), 1,2 benzenedicarboxylic acid (94%), Squalene (94%), 9-Eicosyne (93%) etc (Graph 1).

The steviol glycosides are responsible for the sweet taste of the leaves of the Stevia plant. These compounds range in sweetness from 40 to 300 times sweeter than sucrose. They are heat, pH-stable and do not ferment. They also do not induce a glycemic response when ingested, making them

attractive as natural sweeteners to diabetics and others on carbohydrate-controlled diets.

The diterpene known as Steviol is the aglycone of Stevia's sweet glycosides (JECFA, 2007), which are constructed by replacing steviol's carboxyl hydrogen atom with glucose to form an ester and replacing the hydroxyl hydrogen with combinations of glucose and rhamnose. The two primary compounds, stevioside and rebaudioside A, use only glucose: stevioside has two linked glucose molecules at the hydroxyl site, whereas rebaudioside A has three, with the middle glucose of the triplet connected to the central steviol structure (Starratt *et al.,* 2002).

In terms of weight fraction, the four major steviol glycosides found in the stevia plant tissue are 5-10% stevioside (250-300X of sugar), 2-4% rebaudioside A - most sweet (350-450X of sugar) and least bitter, 1-2% rebaudioside C, ½-1% dulcoside A.

Diterpenoids are a relatively less abundant and less studied group of the secondary metabolism compounds in comparison to other terpenes. Some diterpenoids have unique properties and, therefore, their investigation is of special interest with respect to their fundamental and applied importance. Some diterpenes known as steviols found in *S. rebaudiana*. Leaves of *Stevia rebaudiana* contain high amount of sweet steviol glycosides, that are low caloric, non-toxic and no-mutagenic (Cacciola *et al.,* 2011). Major steviol glycosides are stevioside and rebaudioside A and C (Tamura *et al.,* 1984). Rebaudioside A is usually present 30-40% of total sweetener and has the sweetest taste, assessed as 180-400 times sweeter than sugar (Soejarto *et al.,* 1984).

During the present study, rebaudioside A was isolated and quantified from *in vivo* leaf of *S. rebaudiana* through TLC, Rf value, IR spectroscopy and NMR (H&C). Besides this, rebaudioside A has also been quantified from *in vivo* and *in vitro* plant parts of *S. rebaudiana* by many researcher (Steinmetz and Lin, 2009; Chaturvedula and Prakash, 2011).

Secondary metabolites include a wide variety of compounds with different structures and chemical properties. In order to obtain an overview of the secondary metabolite content of *S. rebaudiana*, a profiling technique with GC-MS method was established. This method allows the qualitative analysis of ionic (charged) compounds like alkaloids, non-ionic (neutral) compounds as terpenoids and phenolic compounds. This technique is also used for the isolation of new compounds.

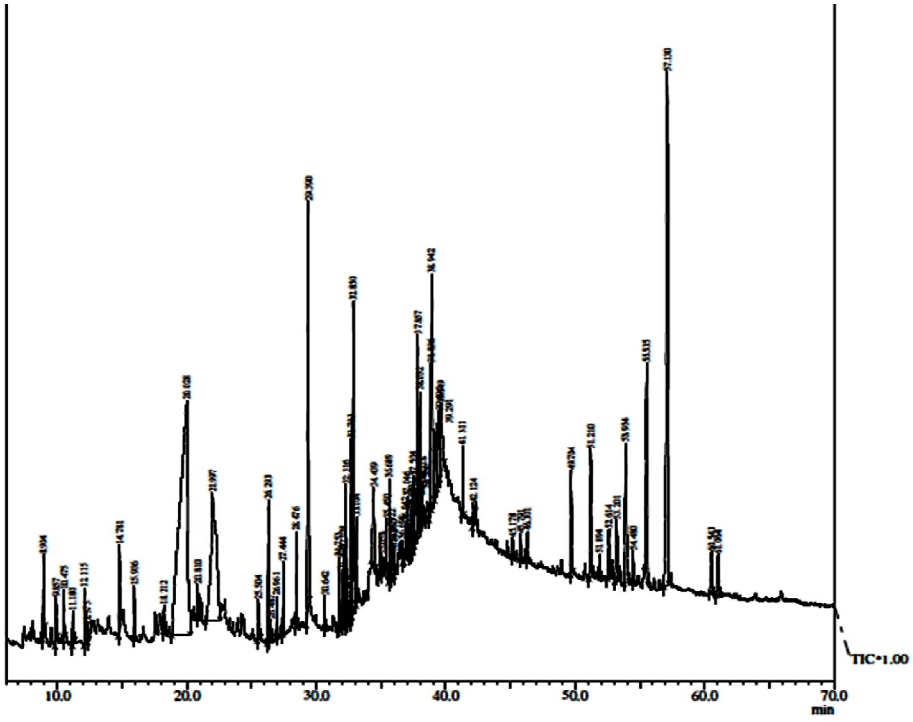
Table 1: Spectral studies of isolated compounds

Name of Compound	UV light absorption band	IR : νcm^{-1}/ max KBr	^{1}H NMR (500 MHz, CD_3OD, δ ppm)	^{13}C NMR(125 MHz, CD_3OD, δ ppm)
Rebaudioside A	210	3348, 1725, 1020,965	0.78 (H_1), 2.22 (H_2), 1.03 (H_3), 44.5 (H_4), 1.05 (H_5), 2.46 (H_6), 1.30 (H_7), 0.88 (H_8), 1.68 (H_9), 2.25 (H_{10}), 86.9 (H_{11}), 2.66 (H_{12}), 2.05 (H_{13}), 154.7 (H_{14}), 5.01 (H_{15}), 1.25 (H_{16}), 1.32 (H_{17}), 6.12 (H_{18}), 4.13 (H_{19}), 6.98 (H_{20})	12.1 (C_1), 15.8 (C_2), 13.9 (C_3), 21.5 (C_4), 28.7 (C_5), 30.5 (C_6), 38.8 (C_7), 40.5 (C_8), 41.6 (C_9), 48.4 (C_{10}), 48.9 (C_{11}), 57.9 (C_{12}), 75.2 (C_{13}), 79.9 (C_{14}), 87.2 (C_{15}), 95.4 (C_{16}), 96.9 (C_{17}), 103.4 (C_{18}), 104.0 (C_{19}), 136.9 (C_{20}).

Table-2: Organic compounds found in *Stevia rebaudiana* Bert

No.	Compound	Retention Time(Min.)	Area	Area%
1	Stevioside	6.625	1782472	0.57
2	Proceroside	8.900	1267297	0.73
3	2,3-dihydro-5-hydroxy-6-methyl-4h-pyran-4-one	9.858	990040	0.57
4	Levoglucosenone	10.475	796672	0.46
5	1,5-anhydro-6-deoxyhexo-2,3-diulose	11.183	580471	0.33
6	4-methyl-2-oxopentanenitrile	12.117	1652230	0.95
7	Benzoic acid, 2-hydroxy-, methyl ester	12.375	211258	0.12
8	2-heptanol, acetate	14.783	2317693	1.33
9	Phenol, 2-methoxy-4-(2-propenyl)-	15.933	650292	0.37
10	1-dodecanol	18.208	459715	0.26
11	D-Allose	20.025	40677116	23.31
12	Spathulenol	20.808	405731	0.23
13	Viridiflorol	20.967	15359605	8.80
14	Methyl-.alpha.-d-ribofuranoside	22.000	1095716	0.63
15	Tetradecanoic acid	24.175	1850561	1.06
16	2,3-Bis(1-methylallyl)pyrrolidine	25.500	1095716	0.63
17	2,6,10-trimethyl,14-ethylene-14-pentadecne	26.292	453991	0.26
18	2-pentadecanone, 6,10,14-trimethyl-	26.483	251726	0.14
19	3,7,11,15-tetramethyl-2-hexadecen-1-ol	26.958	1049470	0.60
20	9-Eicosyne	27.442	924623	5.30
21	Palmitic acid, methyl ester	28.475	349776	0.20
22	Pentadecanoic acid	29.392	789836	0.45
23	Cyclopropanebutanoic acid, 2-[[2-[[2-[(2-pentylcyclopropyl) methyl]cyclopropyl]methyl]cyclopropyl]methyl]-, methyl ester	30.642	628556	0.36
24	1-Octadecanol	31.750	1014529	0.58
25	Linoleic acid, methyl ester	31.975	1660976	0.95
26	9,12,15-octadecatrienoic acid, methyl ester, (z,z,z)-	32.108	242172	0.14
27	Phytol	32.317	3320582	1.90
28	Tetradecanoic acid, methyl ester	32.475	6980810	4.00
29	10,12-hexadecadien-1-ol	32.700	1142094	0.65
30	9,12,15-octadecatrien-1-ol, (z,z,z)-	32.850	3103306	1.78
31	Octadecanoic acid	33.100	887224	0.51
32	flavone 4'-oh,5-oh,7-di-o-glucoside	34.442	204395	0.12
33	13,15-octacosadiyne	34.925	759786	0.44
34	Humulane-1,6-dien-3-ol	35.058	1640255	0.94
35	9-octadecenal, (z)-	35.450	940607	0.54
36	4,8,13-cyclotetradecatriene-1,3-diol, 1,5,9-trimethyl-12- (1-methylethyl)-	35.692	314761	0.18
37	Flavone 4'-oh,5-oh,7-di-o-glucoside	35.950	355910	0.20
38	3-methyl-5-(2,6,6-trimethyl-1-cyclohexen-1-yl)-1-pentyn-3-ol	36.042	581858	0.33
39	Globulol	36.700	841357	0.48
40	Palustrol	36.942	335480	0.19
41	4,8,13-cyclotetradecatriene-1,3-diol, 1,5,9-trimethyl-12- (1-methylethyl)-	37.050	732491	0.42
42	2,4a,8,8-tetramethyldecahydrocyclopropa[d]naphthalene	37.208	1110371	0.64
43	2-Beta.-hydroxy-9-oxoverrucosane	37.342	2327237	1.33
44	Uvidin A	37.500	2971507	1.70
45	Lactaropallidin	37.642	1818907	1.04
46	Lanosterol	37.858	352074	0.20

47	5,6-Dihydrostigmasterol, acetate	38.033	331721	0.19
48	1,2-Dioxaspiro[5.5]undec-4-en-3-ol, 3,5,7,7-tetramethyl-11-methylene-	38.125	500065	0.29
49	1,2-benzenedicarboxylic acid	38.217	3482833	2.00
50	5,6-Dihydrostigmasterol, acetate	38.550	5289398	3.03
51	1H-Benzocyclohepten-7-ol, 2,3,4,4A,5,6,7,8-octahydro-1,1, 4A,7-tetramethyl-, Cis-	38.833	2888378	1.66
52	Widdrol	38.942	2442435	1.40
53	Steviol	39.292	2948017	1.40
54	Campesterol	39.500	738713	0.42
55	Beta-sitosterol	39.650	391511	0.22
56	Squalene	41.308	289397	0.17
57	1-triacontanol	42.125	401098	0.23
58	1-hexacosanol	45.175	437830	0.25
59	Cholesta-4,6-dien-3-ol, (3.beta.)-	45.792	2197681	1.26
60	Alpha.-Tocopherol-.beta.-D-mannoside	46.300	3133521	1.80
61	Stigmasterol	49.700	383877	0.22
62	Stigmast-5-en-3-ol, (3.beta.)-	51.208	1197792	0.69
63	Methyl commate c	51.892	146943	0.84
64	Methyl commate a	52.617	4140656	2.37
65	Lup-20(29)-en-3-yl acetate	53.200	77925	0.45
66	Betulin	53.933	5612955	3.22
67	Lanosta-8,24-dien-3-ol, acetate, (3.beta.)-	54.483	17041910	9.77
68	Alpha-amyrin	55.533	130.3934	0.75
69	Methyl commate b	57.133	1441656	0.83
70	2-isopropenyl-4a,8-dimethyl-1,2,3,4,4a,5,6,8°-octahydro -naphthalene	60.542	1303934	0.75
71	Alpha-selinene	61.067	1441656	0.83



Graph 1: GC-MS chromatogram of methanolic extract of *Stevia rebaudiana* Bert.

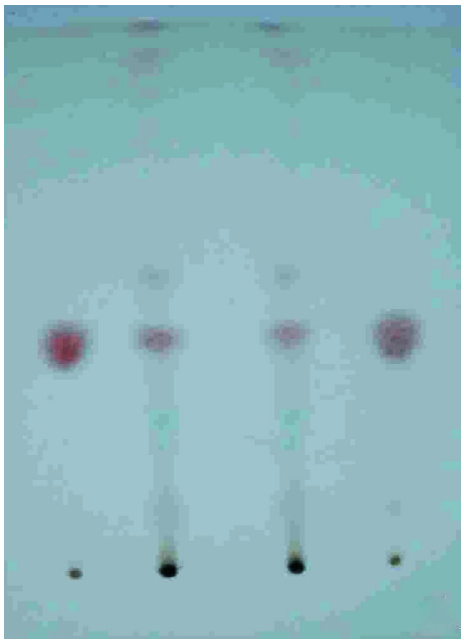


Figure : Developed TLC plate of rebaudioside A

In the present study, the methanolic extracts of leaf were analyzed by GC-MS in *S. rebaudiana*. However, this method has already been adopted for many species including *Melilotus officinalis* (Kovaleva *et al.*, 2009*); Melissa officinalis, Urtica dioica, Lamium album, Matricaria chamomilla* (Iordache *et al.*, 2009); *Polycarpaea corymbosa* (Karuppasamy *et al.*, 2012); *Calotropis gigantean* (Shirsat *et al.*, 2013).

Gas chromatography mass spectroscopy is designed to separate volatile compounds from a complex mixture. This technique is based on the temperature of vaporisation specific to each compound to separate them from a solution by passing the sample through a heated column, where it is partitioned between an inert gas under pressure and a thin layer of non-volatile liquid coated on an inert support inside the column. However, many compounds are difficult to vaporise, like the polyhydroxylated alkaloids. However, their capacities to vaporise could be improved by replacing the hydroxyl groups by other chemical groups like trimethylsilyl groups prior to the injection onto the GC-MS (Rispail *et al.*, 2004).

Conclusion

This particular plant is full of important medicinal compounds. Biochemical estimation of rebaudioside A has been done on the leaves extracts of *Stevia rebaudiana* during the studies. However, each part of the plant possesses important secondary metabolites. More research work could be performed to accelerate these invaluable compounds for their further use in pharma industry.

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Conflict of interest

The authors declare no conflict of interest.

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