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ISOLATION AND ANALYTICAL CHARACTERIZATION OF STEVIOSIDE FROM LEAVES OF *STEVIA REBAUDIANA* BERT; (ASTERACEAE)

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ABSTRACT
Stevioside a natural non caloric sweetener isolated from the leaves of the plant *Stevia rebaudiana Bertoni* (Asteraceae or compositae). It has been widely used in many countries, including Japan, Korea, China, Brazil and Paraguay, either as a substitute for sucrose in beverages and foods or as a household sweetening agent isolated from the dried leaves of Stevia. In the present work attempt was made to isolate stevioside from the dried leaves of Stevia in its purest form. Isolated stevioside was purified, analyzed & characterized by using various chromatographic & analytical methods including TLC, UV, FTIR, NMR and HPLC methods. The Rf value for TLC was 0.32, λmax of UV spectra was obtained at 333 nm and HPLC showed the sharp peak with 1.958 min retention time. The isolated stevioside was also compared with standard stevioside with all analytical methods.

KEYWORDS: Stevia, Stevioside, Extraction, TLC, UV, FTIR, NMR and HPLC.

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INTRODUCTION

The subject of phytochemistry or plant chemistry has undergone the significant development in recent year as distinct discipline is concerned with the enormous verity of substances that synthesize and accumulated by plant & structural elucidation of substance. The technology involves extraction, isolation, purification & characterization of phytoconstituent. *Stevia rebaudiana* Bertoni is a perennial herbaceous plant and is member of the Asteraceae (compositae) family which is indigenous to the northern regions of Paraguay, South America [1].It is commonly known as Stevia, honey leaf. It has been reported to have antibacterial [2], anti-microbial [3], anti-retroviral [4], antioxidant [5], and hypoglycemic activity [6].

Although a number of natural products have been isolated from *S. rebaudiana,* more than 100 compounds have been identified from this species, the best known are the diterpenoids, specifically the sweet-tasting *ent*-kaurene glycosides comprising stevioside, rebaudiosides A and C–E, and dulcoside A**.** Stevioside is the major sweet-tasting glycoside about 5–22% of the weight of dry leaves in *S. rebaudiana,* and has been reported to be 250–300 times sweeter than sucrose. The yield of stevioside from dried leaves of *S. rebaudiana* can vary greatly, as it is depending upon the cultivar and growing conditions [7].

MATERIALS AND METHODS

Procurement and Authentication of Plant Material

The plant material was procured from Kamdhenu Agro, Pune, Maharashtra, India.

Preliminary Phytochemical screening

In order to determine the presence of alkaloid, glycosides, flavonoids, tannins, terpenes, sterol, saponins, fats & sugars, a preliminary Phytochemical study (color reaction) with aqueous and methanolic extract was performed which were then further confirmed by thin layer chromatography[8].

Extraction and isolation of stevioside from Stevia plant

The leaves of s. rebaudiana dried in the shade and then pulverized in a grinder. The powdered leaves were utilized for extraction of stevioside using water as a solvent. The powdered material was pretreated with non polar solvent and then the marc was air dried. The marc was extracted using water as a solvent. The aqueous extract was then treated with the tri carboxylic acid to lower the pH at 4. Subsequently calcium containing compound was added. The aqueous extract was neutralized with acid then the extract was treated with water immiscible solvent. The isolated stevioside was purified by recrystalization with methanol[9].

TLC of isolated stevioside

The TLC was performed using mobile phase Methanol: Chloroform: Water (25:65:4) on precoated silica gel –G plates as stationary phase. Visualization of spot was done by spraying with Libermann- Burchard reagent[10].

U.V spectroscopy

10µg/ml solutions were prepared of isolated stevioside and standard stevioside by dissolving in the distilled water and then the solution was filled in one cuvvet and other cuvvet with distilled water kept as blank in UV/VIS spectrophotometer- JASCO – V530 and the λmax was determined.

Fourier Transform Infra Red Spectrophotometer (FTIR)

The isolated stevioside and standard compound was mixed with potassium bromide in the ratio of (1:10) in agate mortar and pestle and solid dispersion was prepared. This solid dispersion was then loaded in FTIR (JASCO– FT/IR- 4100) to get an IR spectrum of isolated product and standard compound.

NMR Spectroscopy

H1 NMR of stevioside was recorded in a Varian mercury -NMR-mercury300, NMR instrument. The solvent used was D_2O. The scanning was carried out using T.M.S. (reference) as a marker compound.

HPLC

The isolated stevioside was analyzed by using Acetonitrile: water (80:20) as mobile phase on Jasco HPLC system which consisting of Jasco PU-2080 plus HPLC pump and UV-2075 plus UV/VIS detector and JASCO Borwin 1.50 was used for analysis. Version software was used for analysis. Separation was carried out on ODS 295 C_{18} column (150 x 4.6 mm internal diameter.) Samples were injected using Rheodyne injector with 20 µL loop[11].

RESULTS AND DISCUSSION

Preliminary phytochemical screening

The extract obtained after extraction was characterized by preliminary phytochemical test for rough ideas of constituents present in extract. Extracts showed the presence of steroids, proteins, carbohydrates, amino acids, glycosides, alkaloids, flavonoids, tannins and phenolic compounds. (**Table 1**)

Thin layer chromatography profiles (Table 2)

IR spectrum of isolated product (Table 3)

IR spectrum of standard stevioside (Table 4)

FTIR was performed on isolated product, the spectra was compared with that of the standard and was found to be in accordance with the standard.

H1 NMR data for isolated stevioside (Table 5)

HPLC

The sharp and highest sharp peak with 1.958 retention time is of isolated stevioside.

CONCLUSION

Pure stevioside isolated from leaves of *Stevia rebaudiana* Bertoni & further structure was established on the basis of chromatographic & spectroscopic evidence. Isolated stevioside showed similar results of chromatographic & spectroscopic to those of standard stevioside. The data obtained from the TLC, UV, FTIR, H_1NMR and HPLC confirmed that the isolated was stevioside and its structure is as follows.

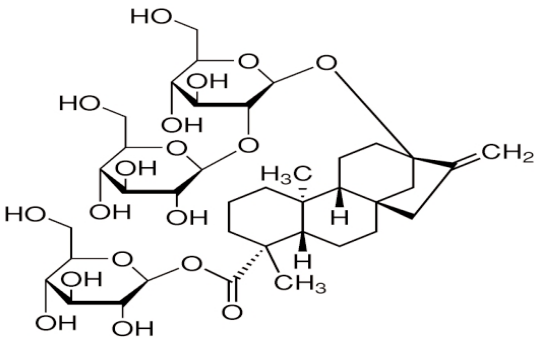

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Table: 1.Preliminary phytochemical screening of extracts of the *S. rebaudiana*
(+ indicates presence of constituents) (- indicates absence of constituents).

Sr. No.	Chemical test	Aqueous Extract	Methanolic Extract
1.	**Carbohydrate:** a) Molisch Test b) Fehilings Test c) Benedicts Test d) Barfoed's Test	 + + + -	 + + + -
2.	**Protein:** a) Biuret Test b) Millions Test c) Xanthoprotien Test	 + + -	 + + -
3.	**Steroids:** a) Salkowski Test b) Liebermann-Burchard test c) Liebermann's reaction.	 - + -	 + + -
4.	**Glycoside:** a) Deoxysugares (Killer-Killani) b) Legal's Test c) Borntrager's Test d) Modified Borntrager's Test	 + + + +	 + + + +
5.	**Alkaloids:** a) Drogendroff's Test b) Mayers Test c) Hagers Test d) Wagners Test	 + + + +	 + + + +
6.	**Test for Flavonoids:** a) Lead Acetate b) Sodium Hydroxide c) Shinoda test	 + + +	 + + +
7.	**Test for Saponins:** a) Foam formation Test	 +	 +
8.	**Tannins & Phenolic Compounds:** a) 5% Ferric Chloride Test b) Lead Acetate Test c) Dilute Iodine Test. d) Dilute Nitric acid Test. e) Potassium Permanganate Solution	 + + + + +	 + + + + +

Table 2: Thin layer chromatography profiles

Sr. no.	Compounds	Mobile Phase	Visualizing Agent	Color of Spot	Rf Value
1.	Glycoside	MeOH:chloroform:Water (25:65:4)	Sodium nitroprusside	Yellow	0.74
2.	Alkaloids	Chloroform: MeOH: Diethylamine (80:20:10)	10%Ethanolic sulphuric acid	Light brown	0.32
3.	Terpenoides	Benzene: Ethyl acetate (5:95)	Vanillin-sulphuric acid	i) Pink ii)Light pink iii)Violet	i)0.66 ii)0.77 iii)0.88
4.	Carbohydrates	Benzene: GAA: MeOH (20:20:60)	Anisaldehyde-sulphuric acid	Yellow	0.55
5.	Flavonoids	Ethyl acetate:GAA:water (90:10:10)	5%$FeCl_3$ solution	Grey	0.82

Table 3: Interpretation of IR spectrum of isolated product

Frequency (cm^{-1})	Functional Group
1031.73	Carboxylic acid ,esters
1365.35	O-H bending
1681.37	> C=O
1859.04	Lactone ring
2833.88	C-H stretching
2921.63	-CH3
2978.52	C=C-H, some unsaturation
3558.02	O-H stretching

Table 4: Interpretation of IR spectrum of standard stevioside

Wave number (cm^{-1})	Vibrations
1011.48	Carboxylic acid ,esters
1380.78	O-H bending
1658.48	> C=O
1859.04	Lactone ring
2852.1	C-H stretching
2916.81	C=C-H, some unsaturation
3556.2	O-H stretching

Table 5: Interpretation of ^{1}H NMR of isolated product

Sr.No.	Signals ppm	Types of Proton
1	0.767-0.908	R-CH3
2	1.018-2.813	R-CH2 in the ring
3	3.214-5.424	Sugar moiety
4	4.820	D2O
5	6.287-6.411	=CH2

Table 6: HPLC chromatogram analysis for isolated stevioside

Sr. No.	RT	Area	Height
1	0.125	699.197	98
2	1.958	2338765.291	173021
3	2.275	572652.011	45097
4	2.900	1816781.381	53721
5	4.250	30728.701	1794
6	4.525	75262.187	4383
7	6.275	1092.500	138

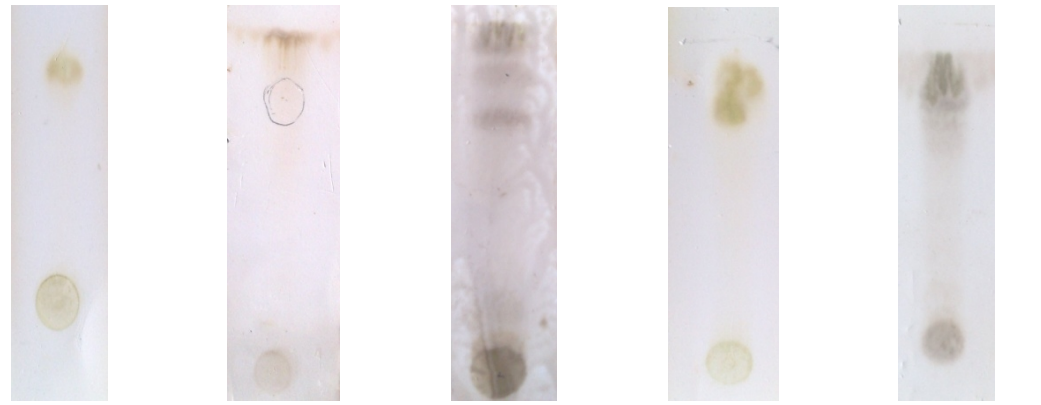

Fig 1: Thin Layer Chromatography of extract of *Stevia rebaudiana*

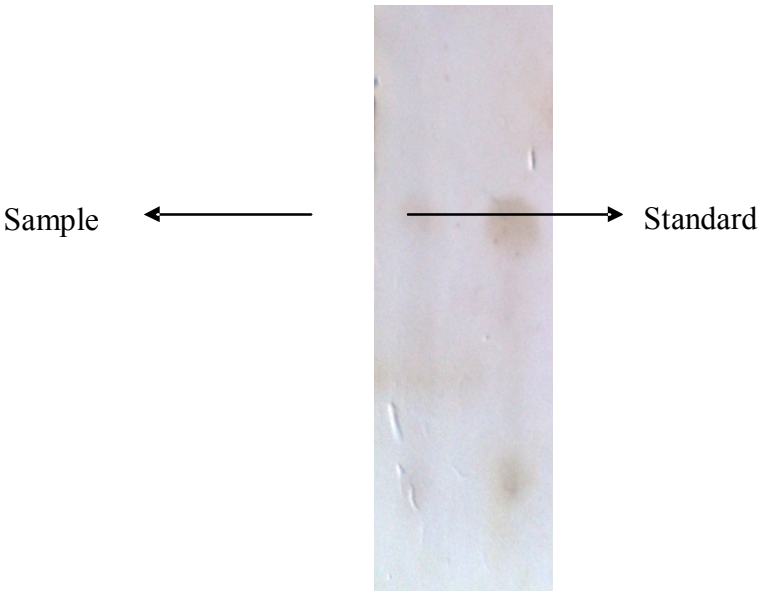

Fig 2: TLC of isolated stevioside and standard

Rf value of isolated product was found to be 0.32 and is within the range specified in literature 0.30-34

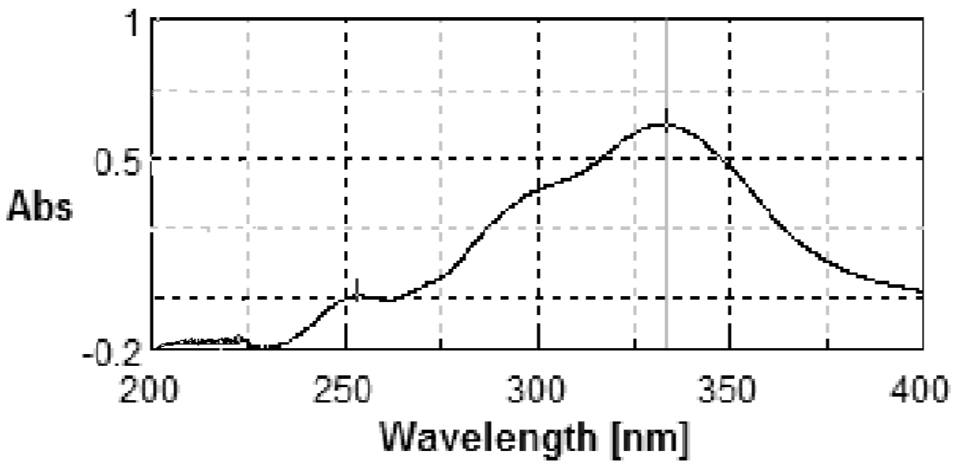

Fig 3: U.V spectra of isolated stevioside

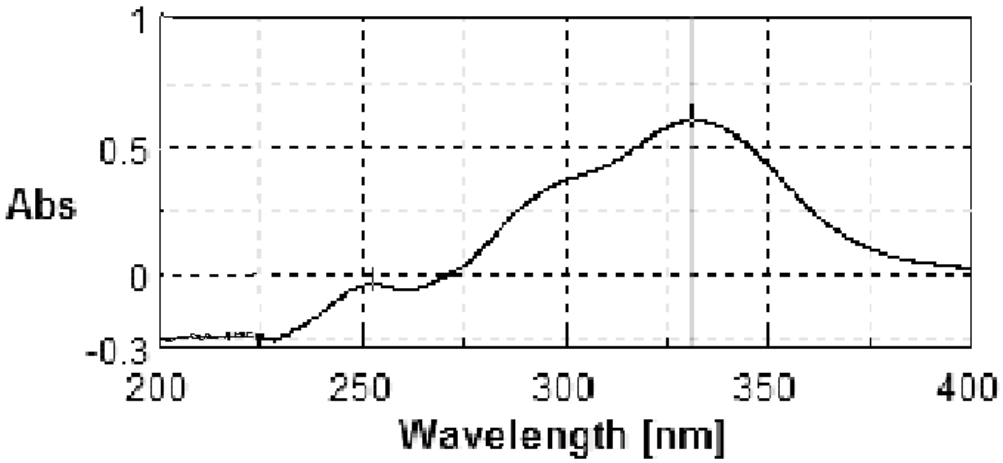

Fig 4: U.V spectra of standard

The light absorption maxima for product and standard was found to be 333 & 331.2nm (λ_{max}) in distilled water.

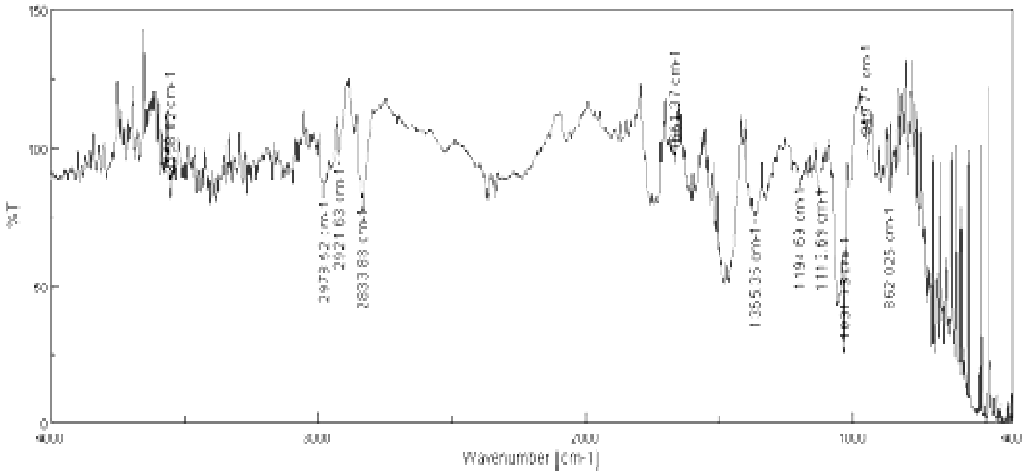

Fig 5: FTIR Studies of sample

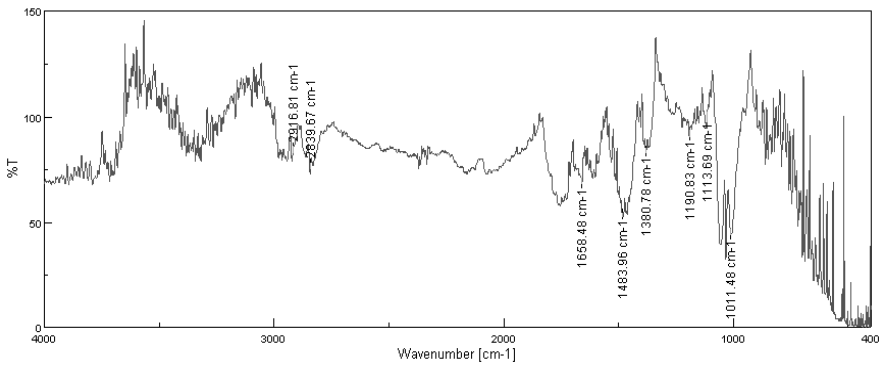

Fig 6: FTIR Studies of standard stevioside

FTIR was performed on isolated product, the spectra was compared with that of the standard and was found to be in accordance with the standard.

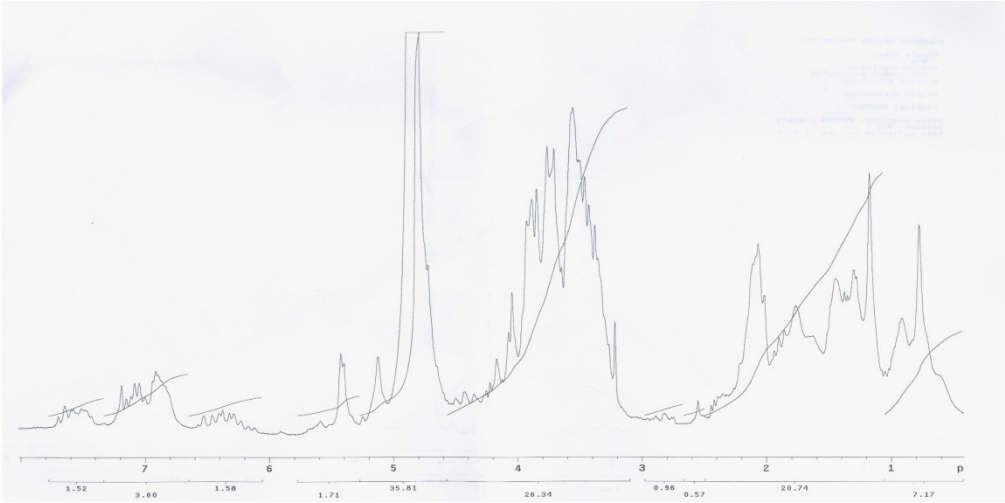

Fig 7: ^{1}H NMR Data Enlarged View

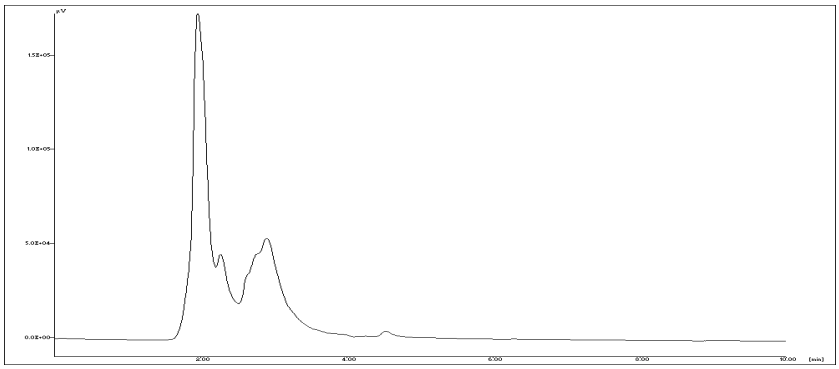

Fig 8: HPLC chromatogram for isolated product

The sharp and highest sharp peak with 1.958 retention time is of isolated stevioside.

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