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Full Length Research Paper

Analysis of stevioside in *Stevia rebaudiana*

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Analysis of stevioside from *Stevia rebaudiana* was carried out using systematic procedures of various chromatographic techniques including column chromatography yielding the crude stevioside in 12.5%. This procedure was followed by thin layer chromatography and examination of the spots under UV light at 254 and 366 nm, and spraying reagent followed by mixing of fractions on the basis of Rf values. Semi purified fraction obtained from crude stevioside by column chromatography and further evaluation by thin layer chromatography appeared as a mixture showing the presence of four major components (Rf values 0.27, 0.34, 0.42 and 0.60). These finding were similar to reported values for the constituents namely rebaudiosides A, stevioside, rebaudiosides B and steviolbioside, and stevia glycoside reported to occur in *Stevia rebaudiana.*

Key words: Stevioside, *Stevia rebaudiana,* chromatography, column chromatography.

INTRODUCTION

Stevia rebaudiana bertoni is a plant of the compositae family native to Paraguay whose leaves have been used for centuries as a sweetener. *Stevia* is a perennial shrub growing up to 1 m tall. Stems are weak and semi-woody, it produces upward branches. The sessile leaves are oppositely arranged, lanceolate to oblancoelate and serrated above the middle. The flowers are small, white and arranged in indeterminate heads. The seed is an achene with a feathery pappus. Great interest in *S. rebaudiana* as a low calorie sweetener has induced researches not only to characterize, isolate and extract the sweet constituents (Kinghorn et al., 1982; Kinghorn et al., 1984; Kasai et al., 1987; Liu et al., 1997) but also to verify the lexicological safety and to characterize the productive attitude (Brandie and Rosa, 1992). In addition to its interesting sweetening property, *Stevia* extract shows many pharmacological properties. Among the therapeutic activities attributed to it, we can list hypotensive regulation (Melis 1992), hypoglycemic (White et al., 1994), antimicrobial (Nardi, 1996) and contraceptive activities (Planas and Kuc, 1968). Recently, it has been introduced as a crop in the United States and Canada in response to increased interest in natural foods. In its leaves, *Stevia* produces several sweet diterpene glycosides, which are non-glycemic, yet range in sweetness from 30 to 320 times that of sucrose (Garvey et al., 1999). Four major *Stevia* diterpene glycosides are recognized in the literature: stevioside, rebaudioside A, rebaudioside C and dulcoside A. In addition to these, previously identified leaf constituents include volatile oil components, sterols, triterpenes, flavonoids, coumarins, and non-glycosidic diterpenes (sterebins). Research interest in stevia has been oriented toward developing genetic lines in which levels of sweet-tasting glycosides are maximized and non glycosidic diterpenes are minimized (Garvey et al., 1999; Brandle et al., 1998). For major screening and determination of major constituents responsible for therapeutic effects, *S. rebaudaiana* indigeneous to Paraguay and Brazil which has gained importance in the food industry in Japan, Korea, France, Europe, USA was analyzed.

Keeping in view its importance, the herb has been successfully introduced in Pakistan through Tissue Culture Techniques and Stevioside, the active ingredient has been isolated from the leaves through laboratory developed procedure.

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MATERIALS AND METHODS

Plant materials

The herb was collected at appropriate time and was dried under shade, powdered and utilized for screening.

Determination of steviosides in *S. redaudiana*

Extraction of plant material

Crushed leaves of 50 g of *S. rebaudiana* were soaked in one litre of water. The contents were heated on a water bath for 1 h with occasional shaking and stirring. The solution was filtered on Buckner funnel and residue pressed between layers of gauze. The filtrate transferred to a separating funnel, extracted with about 1400 ml isobutyl alcohol by shaking in portions. The organic extract was separated, and the solution filtered and concentrated under vacuum in a rotary evaporator till the solution become turbid. The residue was washed with acetone and then dissolved in 100 ml methyl alcohol while heating on a water bath. Thin layer chromatography (TLC) of the constituents was performed using silica gel G-flurecent plates. Developed in the mobile phase consisting of chloroform, ethyl alcohol, ethyl acetate and water (30:15: 5: 2), and visualized under UV light and treatment with sulphuric acid and methanol (50:50) spraying reagent followed by heating at 105°C in oven for ten minutes.

Column chromatography of crude steviosides

For purification and separation, 3 g of the dried crude extract was added to a column packed with silica gel. The column was run with a solvent system of methanol and chloroform from 5 to 80% with increasing polarity. Sixty fractions were collected. Thin layer chromatography of each fraction was performed in the mobile phase consisting of chloroform, ethyl alcohol, ethyl acetate and water (30:15:5:2). After chloroform, ethyl alcohol, ethyl acetate and water (30:15, 5:2). After development of chromatograms were evaluate by TLC Scanner Densitometer and visualized with conc. sulphuric acid and methanol (50:50) spray reagent followed by heating at 105°C in oven for ten minutes.

Crystallization of constituents

Dried crude product 2 g was suspended in 20 ml methanol. The suspension heated on a water bath for 10 min and then allowed to cool, precipitated residue was separated by filtration and clear extract stoppered, and allowed to stand over night at room temperature for crystallization of constituents. Mother liquor from the crystalline product was transferred to another flask and the product deposited at the bottom washed with a further increment of methanol (10 ml), the process was repeated with additional quantities of crude product and colorless products separated were recovered by filtration and drying in vacuum desiccators.

RESULTS AND DISCUSSION

Stevioside content of *S. rebaudiana*

Maceration of plant material phase separation, filtration of extracts partitioning, removal of suspended matter, concentration of organic extract under reduced pressure, recycling of organic solvents, recovery of crude product, purification by column chromatography and crystallization were conducted under controlled conditions. Partition coefficient of constituents between immiscible layers was optimized, and a convenient, improved and refining method, yielding 15.2% crude stevioside product was perfected as detailed in the scheme (Figure 1). Scheme for extraction of Steviosides, steps involved: (i) Maceration; (ii) filtration (iii) partitioning (iv) layer separation (v) concentration under reduced pressure for solvent recovery; and (vi) dried product.

TLC examination of a fraction of crude product appeared as a mixture of five components (three major and one minor). For resolution of the mixture, the product was applied to column chromatography, using gradient elution system prepared from chloroform and methanol mixture. Sixty fractions collected were concentrated, evaluated on TLC by UV light 254 and 366 nm, and spraying reagent followed by mixing of fractions on the basis of Rf values. Semi purified fraction obtained from crude stevioside by column chromatography and further evaluation by thin layer chromatography, appeared as a mixture showing the presence of four major components (Rf values: 0.27, 0.34, 0.42 and 0.60) these finding were similar to reported values for the constituents namely rebaudiosides A, stevioside, rebaudiosides B and steviolbioside, respectively (Garvey et al., 1999) All these compound are listed in Table 1.

Attempts for total purification of these components by preparative chromatographic techniques have not yielded pure compounds. As a consequence, purification of main constituents from individual fractions by crystallization from methanol was tried, which successfully furnished two constituents that stayed as a single entity on multiple TLC development. Melting points and IR absorption bands of the compounds were consistent to Stevioside and Rebaudioside B.

Other constituents purified exhibited close Rf values and as a result these were not separable into a single component by classical chromatographic and crystallization procedures. These partially purified compounds were subjected to centrifugal chromatography (Chromatotron), a technique used for the resolution of components, possessing very close Rf values. Examination of the recovered constituents on TLC Scanner/Densitizer showed that about 85% purity was attained. Since the ideal approach for the quantitative estimation of ingredients of crude stevioside extract was through application of HPLC and spectroscopic techniques, HPLC analysis of crude/ purified constituents was performed but due to lack of Normal Phase Lichrosorb NH2 (5 µm) column, satisfactory results were not derived.

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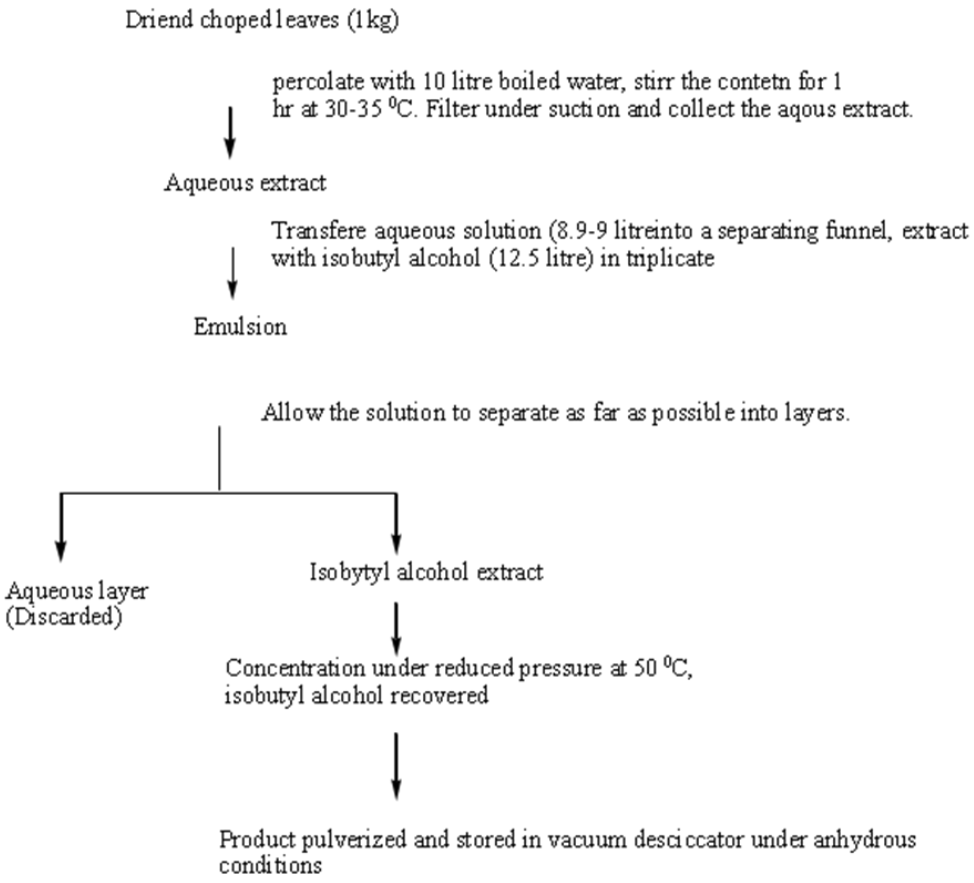

Figure 1. Flow chart for extraction of steviosides.

Table 1. Steviosides found in *S. rebaudiana*.

S/N	Compound name	R_1	R_2
1	Steviol	H	H
2	Steviolbioside	H	β-Glc- β-Glc (2→1)
3	Stevioside	β-Glc	β-Glc- β-Glc (2→1)
4	Rebaudiosides A	β -Glc	β-Glc- β-Glc (2→1) β-Glc (3→1)
5	Rebaudiosides B	H	β-Glc- β-Glc (2→1) β-Glc (3→1)
6	Rebaudiosides C (Dulcoside B)	β-Glc	β-Glc-α-Rha (2→1) β-Glc (3→1)
7	Rebaudiosides A	β-Glc- β-Glc (2→1)	β-Glc- β-Glc (2→1) β-Glc (3→1)
8	Rebaudiosides A	β-Glc- β-Glc (2→1)	β-Glc- β-Glc (2→1)
9	Rebaudiosides A	β-Glc	β-Glc- β-Xyl (2→1) β-Glc (3→1)
10	Dulcoside B	β-Glc	β-Glc-α-Rha (2→1)

Research Center.

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