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Original Article

PHYTOCHEMICAL STUDIES AND ESTIMATION OF MAJOR STEVIOL GLYCOSIDES IN VARIED PARTS OF *STEVIA REBAUDIANA*

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ABSTRACT

Objective: Determination of physicochemical parameters, comparativephytochemical screening of leaves, stem and root extract of *S. rebaudiana* and qualitative and quantitative analysis of secondary metabolites (Stevioside and Rebaudioside A) through HPLC (High Performance Liquid Chromatography).

Methods: Standards methods were followed for physicochemical and phytochemical analysis and HPLC was used for estimation of Stevioside and Reb A. Quantification of metabolites was done by plotting a standard curve for standards.

Results: Leaves, stem and root possesses different phytochemicals however, leaves showed all tests positive for phytochemicals. Leaves of *S rebaudiana* possess higher stevioside (1.13±0.02 mg/ml) than stem (0.88±0.008 mg/ml). Concentration of Reb A in the leaves (0.071±0.005 mg/ml) was also higher than stem (0.052±0.002 mg/ml).

Conclusion: Qualitative and quantitative estimation of various parts of *S rebaudiana* revealed that leaf showed the maximum type and amount of secondary metabolites which are of immense medicinal use of mankind.

Keywords: *Stevia rebaudiana*, Stevioside, Rebaudioside A.

INTRODUCTION

Stevia rebaudiana of Asteraceae family is a perennial herbaceous plant. It is a natural sweetener plant known as "Sweet Weed", "Sweet Leaf", "Sweet Herbs" and"Honey Leaf [1]. It is native of Eastern Paraguay and widely used in Latin America. It grows easily in tropical and subtropical areas. Reports of therapeutic effect in human body [2]favor its commercialization in several countries, including Latin America, Canada, China, Japan, Indonesia, and the USA, Paraguay, Brazil, Mexico, Australia, Norway, Russian Federation, New Zealand and Singapore. In these countries use of Stevia is approved by regulatory authorities. Although, In some other countries Stevia is available in different forms, but not verified by regulatory agencies, which includes Argentina, Chile, Colombia, Korea, Malaysia, Peru, Philippines, Saudi Arabia, Taiwan, Thailand, Turkey, United Arab Emirates, Uruguay, and Vietnam [3] In India, *Stevia* is now approved in the food industry [4] and being cultivated successfully in the states of Rajasthan, Maharashtra and Kerala.

The leaves of *Stevia* are the source of diterpene glycosides, viz. Stevioside and rebaudioside [5]. Stevioside content in leaf was found to vary from 3.17 to 9.94% and that in stem from 1.54 to 3.85%. In terms of weight fraction, the four major steviol glycosides found in *Stevia* plant tissues are 5–10% stevioside, 2–4% rebaudioside A, 1–2% rebaudioside C and 0.5–1% dulcoside A [6]. Stevioside are 110 to 270 times sweeter than sucrose, rebaudioside A are 150 to 320 times, rebaudioside C is 40 to 60 times and Dulcoside A is 30 times sweeter than sucrose [7]. *Stevia* leaves also contain numerous all-natural foods that help regulate blood sugar, including chromium, magnesium, manganese, potassium, selenium, zinc, and vitamin B3 (Niacin).

Diet conscious and diabetic persons with hyperglycemia can use steviosides as an alternative sweetener [8] as it regulates the blood glucose level by stimulating insulin secretion [9]. Stevioside can also be used as an antihyperglycaemic [10], antihypertensive [11], anti-tumor [12], vasodilator [13] drugs. Products can be added to tea and coffee, prepared or baked goods, processed foods and beverages, fruit juices, tobacco products, pastries, chewing gum and sherbets. In Japan alone, 50 tons of stevioside are used annually with sales valued in order of 220 million Canadian dollars [14]. Stevioside is established as a valuable natural sweetening agent due to its relatively good taste and chemical stability [15].

Studies on the absorption and metabolism of ent-kaurene glycosides in rats showed that stevioside is not readily absorbed from the upper small intestine owing to its high molecular weight. However, stevioside is degraded by bacteria of the colon, resulting in free steviol, part of which is absorbed by the colon and transported to the liver and part is excreted in feces. Steviol is then converted into its glucuronide derivative in the liver and excreted from the body through urine [6].

India has a suitable climate for *Stevia* cultivation. In spite of this, *Stevia* cultivation has not been taken up on a large scale. Studies have been done on its importance and metabolism and purification of stevioside from the leaves through HPLC. Some studies showed the presence of stevioside was very less or negligible in root but, Quantitative comparison of stevioside content in root and stem with leaf was not present. Thus,in the present study, preliminary phytochemical screening of leaves, stem and root was performed and secondary metabolite estimation was done using HPLC to test the presence of secondary metabolites in stem and root.

MATERIALS AND METHODS

Collection of plant material

Stevia rebaudiana plants were collected in the months of November from Birla Institute of Technology, Mesra, Ranchi, Jharkhand, India. Whole plants were washed thoroughly with water to get rid of dirt and clay and then root, stem and leaves were separated for further usage.

Drying and storage

Collected and washed plant materials were dried under shade for 3-4 days with frequent rotation and dried leaves were milled to make a coarse powder using mortar and pestle. Dried powder was packed tightly in an airtight PVC jar and stored.

Physicochemical analysis

Physicochemical parameters viz. Moisture content determination, Ash value determination, Water soluble ash value, Determination of acid insoluble ash and extractive value estimation was performed using standard protocols by Indian pharmacopeia [16].

Extraction of plant material

The powdered plant materials were extracted using water and methanol as solvent. Both the extracts were then treated with tri-carboxylic acid to lower the pH at 4. Subsequently $CaCO_3$ was added to remove the acidity. The aqueous extract was neutralized with weak acids and sample mixture was filtered using filter paper (Whatman No. 1). The filtrate was further separated by using 250 ml separatory funnel with 25 ml of diethyl ether to remove the green color. Lower transparent layer was collected and extracted again with Butanol (25 ml). Finally the butanol extract (upper layer) was collected and left overnight for drying. The extraction method was optimized to form fine crystals

Preliminary phytochemical screening

Phytochemical screening of active plant extracts was done by following the established standard methods [17]. Preliminary screening included testing for the presence of alkaloids, glycosides, steroids, saponins, tannins, flavanoids and carbohydrates.

Estimation of secondary metabolites: Stevioside and rebaudioside a

Dried extracts of samples were dissolved in water and filtered through a 0.2 µm filter and steviol glycosides were separated using a Waters HPLC system (Waters Corporation, Milford, MA) in dC18 column (Waters Atlantis, 4.6 mm × 150 mm; 4 µm particle size). 20 µl of extracts were injected in an HPLC system. The solvents optimized for isocratic elution consisted of acetonitrile andMilliQ with 0.1% orthophosphoric acid in the ratio of 20:30 with a flow rate of 0.5 ml/min. The detector was set at 210 nm. The compounds from samples were identified by comparing the retention time with the corresponding retention time of standards. Quantification of compounds was performed using standard curves. All experiments were replicated at least three times. The results were presented as mg/ml of extracts.

RESULTS AND DISCUSSION

Secondary metabolites of *S. rebaudiana* were well recognized for their beneficial health effect. In the present investigation, evaluation of different physicochemical parameters with comparative estimation of secondary metabolite content in the leaves, stem and root was done. Moisture content may be ascertained as the quantity of water present in the plant parts showed in (table 1).

Ash content of a crude drug is the parameter to decide the quantity of minerals present in the specific drug. Different types of ash values are applied for detection of crude drugs like total ash, acid insoluble ash and water soluble ash. Total ash value is useful for detection of any siliceous contamination, chalk powder, lime or other earthy matter.

Table 1: Moisture content of *S rebaudiana*leaves, stems and root

Plant parts	Moisture content value determined in % (w/w)
Leaves	7.17±0.15
Stem	6.28±0.08
Root	6.44±0.11

Acid insoluble ash is applied to detect excessive earthy materials, which have varying amounts of calcium oxalate crystals in the cells while water soluble ash is applied to discover the presence of water exhausted material. Total ash, acid insoluble ash and water soluble ash was shown in (table 2).

Table 2: Ash values of different parts of *S rebaudiana*

Plant parts	Total ash % (w/w)	Acid insoluble ash% (w/w)	Water soluble ash% (w/w)
Leaves	9.27±0.08	1.92±0.01	4.81±0.01
Stem	9±0.05	1.22±0.04	4.93±0.01
Root	8.5±0.15	0.95±0.02	4.96±0.02

Secondary metabolites present in the plant material could be dissolved in the different solvent for extraction and their yield of extraction may vary according to the polarity of the solvent. Therefore, two solvents, water and alcohol were selected to determine their extractive values. Results of extractive values of alcohol and water in the leaves, stem and root was summarized in (table 3).

Table 3: Extractive values of plant parts in water and methanol

Plant parts	Water soluble % (w/w)	Alcohol soluble % (w/w)
Leaves	34.3±0.25	35.66±0.33
Stem	31.5±0.2	31.9±0.52
Root	26.6±0.6	30±0.33

Methanol extracts of leaves, stem and root of *Stevia* were subjected toa preliminary phytochemical screening. Phytochemical compounds such as alkaloids, glycosides, saponins, flavonoids, steroids, tannins and carbohydrates were found to be present in this plant. The results of phytochemical analysis of *S. rebaudiana* were summarized in (table 4). Leaves showed the entire test positive for the presence of phytochemicals.

Table 4: Preliminary phytochemical screening of *S. rebaudiana* leaves, stem and root.

S. No.	Chemical test		Leaves	Stem	Root
1.	Alkaloids				
	a.	Wagner's reagent	+	+	+
	b.	Dragendorff's reagent	+	-	-
	c.	Mayer's reagent	+	-	-
2.	Glycoside				
	a.	Keller-killiani test	+	+	-
	b.	Borntrager's test	+	+	-
	c.	Modified Borntrager's test	+	+	-
	d.	Legal's test	+	+	-
3.	Steroids				
	a.	Liebermann-burchard test	+	+	+
	b.	Salkowski test	+	-	-
4.	Saponins				
	Froth formation test		+	+	+
5.	Flavonoids				
	a.	Alkaline reagent test	+	+	+
	b.	Lead acetate test	+	+	+
6.	Carbohydrates				
	a.	Molisch's reagent	+	+	+
	b.	Fehling reagent	+	+	+
7	Tannins		+	-	-

'+'–Positive test, **'-'**–Negative test

Different methods viz. Thin-layer chromatography, near-infrared spectrometry, VIS spectrometry and capillary electrophoresis can be applied to determine diterpene glycosides [18, 19] however, HPLC was one of the most usual methods used for quantification of stevioside from plant material and nutrient samples. Joint Expert Committee on Food Additives (JECFA), the FAO/WHO in the 73rd meeting recommended an HPLC method for determination of steviol glycosides in 2010 [20]. Aranda-González *et al.* (2014) discovered HPLC as a rapid method for quantitative determination of Rebaudioside D from leaves of S rebaudiana and showed the accuracy of method upto 99%. Jaitak *et al.* (2008) showed HPLC as a rapid and effective method for extraction and screening of Rebaudioside A and Stevioside. They extracted *Stevia* leaves using microwave. A simple reversed-phase high-performance liquid chromatography (RP-HPLC) method has been developed by Woelwer-Rieck *et al.* (2010) for the determination of the major steviol glycosides, the diterpene sweeteners derived from Stevia rebaudiana. The method is based on a water extraction step and a solid-phase extraction cleanup.

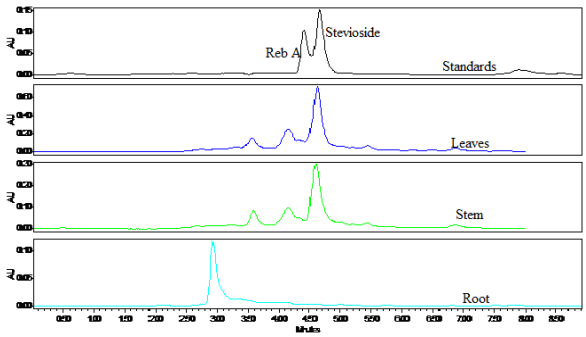

Fig. 1: HPLC profiles of standards with methanol extract of leaves stem and root sample

In the present study, two standard compounds viz. Stevioside and rebaudioside A (Reb A) procured from Sigma-Aldrich was used for qualitative and quantitative analysis of plant secondary metabolites. These two glycosides were selected for quantitative estimation on the basis of occurrence in the plant. Fig. 1 showed the qualitative estimation of Reb A and Stevioside where the presence of metabolites was inferred from the similar retention time of Standard compounds with samples. In stem and leaves extract of *Stevia* peaks of sample matches with the standard compounds, but root extract of *S rebaudiana* does not contain any of the metabolites as no peak matches with the standard compound.

Quantitative analysis (fig. 2) Showed that leaves of *S rebaudiana* possess highest stevioside (1.13±0.02 mg/ml) which was 1.2 fold higher than stem (0.88±0.008 mg/ml). Concentration of Reb A in the leaves (0.071±0.005 mg/ml) was also higher than stem (0.052±0.002 mg/ml). In most of the studies were focused on isolation of stevioside from leaves only and stem and roots remain unnoticed. Kour (2011) developed an HPLC method for determination of stevioside from the leafy parts of *in vitro* and *in vivo* regenerated plants of *S. rebaudiana.* The chromatographic separation achieved using mobile phase consisting of methanol: water with UV detection at 210 nm. On quantification, stevioside obtained was 0.0197 mg/ml for *in vivo* leaf which was still lower than our study.

In similar studies, acetonitrile/water (85:15) was optimized as the solvent system for HPLC analysis and quantification of stevioside and Rebaudioside A but the amount of stevioside was lower than our findings. Concentration of stevioside was 0.079 mg/ml and 39.6 mg/ml for rebaudioside A on a methanol extract of leaves [23]. They used the method based on a water extraction step and a solid-phase extraction (SPE) clean-up. Different SPE cartridges and two HPLC columns were tested in the separation of the main steviol glycosides stevioside and rebaudioside A. A good separation was performed on a Luna HILIC analytical column.

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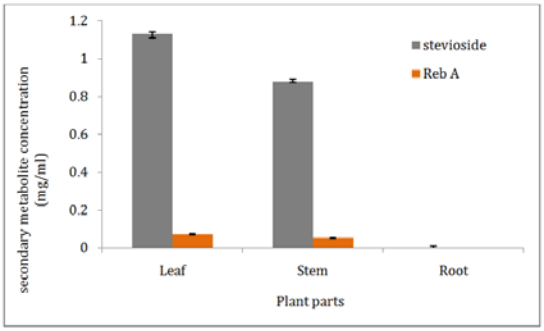

Fig. 2: Quantitative estimation of stevioside and Reb A in leaves and stem of *Stevia*

CONCLUSION

Current study reveals that different plant parts (leaves stem and root) possess phytochemicals which are responsible for plants therapeutic properties. *Stevia* leaf extract showed the presence of most of the phytoconstituents. These phytoconstituents possess different bioactivities which can be utilized further. Quantitative analysis through HPLC reveals that the leaves of *Stevia* contain both the secondary metabolites (Stevioside and Reb A) in higher amount when compared with stem extract. Leaf extract possesses 1.2 fold higher stevioside than stem extract. Further, isolated secondary metabolites can be purified and investigated for their bioactivities. Leaves of Stevia can be utilized for large scale production of stevioside and Reb A.

CONFLICT OF INTERESTS

Declared None

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