Comparative Standardization and Physicochemical Evaluation of the Leaves of *Stevia rebaudiana* Bertoni from Different Geographical Sources

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ABSTRACT

Stevia rebaudiana Bertoni, a natural non-caloric substitute to conventional sugar, is also popular as the "sweet herb of Paraguay". It is a storehouse of various bioactive constituents mainly, the ent-kaurene diterpene glycosides namely- stevioside, rebaudioside A, B, C, D and E. The plant is known to exhibit a wide range of biological activities like hypoglycemic, anti-oxidant, anticancer, antibacterial activities. The present research is based on a comparative standardization and physicochemical analysis of the dried leaves of five varieties of *Stevia rebaudiana* procured from five different geographical locations of India viz., Delhi, Surat, Kangra, Bangalore and Indore. Fluorescence analysis of the powdered leaves was carried out as a means for identification. The standardization parameters included determination of foreign matter, ash values, loss on drying, extractive values. Preliminary phytochemical screening was also performed. The results from the current study can prove to be an indicator to differentiate the five varieties based on their standardization parameters.

Key words: *Stevia rebaudiana*, non-caloric substitute, ent-kaurene glycosides, comparative standardization.

INTRODUCTION

The modern era faces a number of growing ailments and diseases that are a serious concern to normal sustenance of an individual in this scenario. These include hypertension, diabetes mellitus, premature aging, cancer, dental caries, skin diseases like acne and pruritis, bacterial and fungal infections and many more. Control and cure of these diseases require a source that can overcome these health concerns and that has a minimal potential to cause adverse effects.

This situation and need has brought "*Stevia rebaudiana*" (Family:-Asteraceae) into the picture which is a substitute to conventional sugar existing in nature. It is a non-caloric sweetener which is consumed in many countries.[1] It is a small perennial shrub growing upto 1m tall and with leaves 2-3cm long[2] and native to regions of Paraguay and Brazil. It is popular as the "sweet herb of Paraguay", as the leaves have been traditionally used by natives of Paraguay and Brazil for hundreds of years to sweeten local teas, medicines and as a 'sweet treat'. The plant is also known as sweet herb, honey leaf, or sweet chrysanthemum as it possesses sweet tasting glycosides.[3] It is a storehouse of various bioactive constituents mainly, the ent-kaurene diterpene glycosides (the sweet tasting glycosides) namely- stevioside, steviolbioside, dulcoside A and rebaudioside A, B, C, D and E.[4] These compounds stevioside and rebaudioside are 250-300 times sweeter than sucrose, heat stable, pH stable, and non-fermentable. With reference to its sweetening power, it is estimated that 30ml of *Stevia* extract is equivalent to 3 kg of sucrose.[3] The plant's leaves, the aqueous extract of the leaves, and purified steviosides are used as sweeteners. The sweetener extractives have been known to exert beneficial effects on human health- antihypertensive,[5,6] antidiabetic,[7-10] non-carcinogenic,[11,12] antioxidant,[13,14] anti-inflammatory activities.[15,16] They are also thought to effect glucose metabolism and renal function.[17] Apart from these it also exhibits antimicrobial activities.[18,19] It also plays a beneficial role as a dentifrice as it inhibits the development of plaque and cavities.[20]

The current investigation is aimed at a comparative standardization of five varieties of *Stevia rebaudiana* procured from five different geographical locations of India viz.,

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DOI: 10.5530/pj.2011.25.4

Delhi, Surat, Kangra, Bangalore and Indore to find out which variety best complies with the standardization parameters so that it can be effectively used in manufacturing of various *Stevia* based products with maximum quality.

MATERIALS AND METHODS

Collection

Dried leaves of *Stevia rebaudiana* were procured from different suppliers of India: Saico Healthcare Pvt. Ltd. (Delhi), Keshal Nursery (Surat), Deepak Trading Co.(Bangalore), Shri Krishna Herbal (Indore) and locally field grown leaves from Chachiyan Village (Kangra) between the months of September to November, 2010. The identity of the leaves was verified by Dr. H. B. Singh, Head, Raw Materials Herbarium and Museum, NISCAIR, New Delhi and a voucher specimen for the leaves was deposited at the Herbarium of National Institute of Science Communication and Information Resources, New Delhi respectively.

Fluorescence Analysis

1-2 mg of the dried leaf powder of all the five varieties of *Stevia* were taken and placed on a microscopic slide and

Table 1: Fluorescence analysis of powdered leaves of *Stevia rebaudiana* from Delhi

Treatment	*Stevia rebaudiana* (Delhi)		
	Day light	UV light	
		254 nm	366 nm
Powder as such	Light green	Greyish brown	Dark brown
Powder + 1N NaOH (aq.)	Brownish yellow	Bright green	Blackish green
Powder + 1N NaOH (alc.)	Yellowish green	Yellowish green	Brownish green
Powder + 1N HCl	Yellowish green	Bright green	Greenish black
Powder + NH_3	Dark green	Blackish green	Purplish black
Powder + 5% iodine	Greyish green	Silvery green	Blackish green
Powder + 5% $FeCl_3$	Blackish green	Greenish black	Dark green
Powder + acetic acid	Light brown	Blackish green	Blackish brown
Powder + 1N H_2SO_4	Yellowish green	Bright green	Blackish green
Powder + 1N HNO_3	Yellowish green	Light green	Blackish green

Table 2: Fluorescence analysis of powdered leaves of *Stevia rebaudiana* from Surat

Treatment	*Stevia rebaudiana* (Surat)		
	Day light	UV light	
		254 nm	366 nm
Powder as such	Dark green	Dark green	Brownish green
Powder + 1N NaOH (aq.)	Dark brown	Light green	Dark brown
Powder + 1N NaOH (alc.)	Dark green	Dark green	Brownish green
Powder + 1N HCl	Brownish green	Light green	Purplish black
Powder + NH_3	Blackish green	Blackish green	Black
Powder + 5% iodine	Blackish green	Dark green	Purplish green
Powder + 5% $FeCl_3$	Dark green	Blackish green	Brownish green
Powder + acetic acid	Dark green	Light green	Brownish green
Powder + 1N H_2SO_4	Dark green	Blackish green	Blackish green
Powder + 1N HNO_3	Orange green	Dark green	Black

Table 3: Fluorescence analysis of powdered leaves of *Stevia rebaudiana* from Bangalore

Treatment	*Stevia rebaudiana* (Bangalore)		
	Day light	UV light	
		254 nm	366 nm
Powder as such	Yellowish green	Yellowish green	Brownish green
Powder + 1N NaOH (aq.)	Brownish green	Dark green	Purplish black
Powder + 1N NaOH (alc.)	Brownish green	Bright green	Brown
Powder + 1N HCl	Brownish yellow	Light green	Grey
Powder + NH_3	Blackish green	Dark green	Black
Powder + 5% iodine	Greyish green	Bright green	Purplish grey
Powder + 5% $FeCl_3$	Yellowish green	Dark green	Purplish black
Powder + acetic acid	Dark brown	Dark green	Purplish brown
Powder + 1N H_2SO_4	Yellowish green	Light green	Black
Powder + 1N HNO_3	Orange brown	Blackish green	Black

Table 4: Fluorescence analysis of powdered leaves of *Stevia rebaudiana* from Kangra

Treatment	*Stevia rebaudiana* (Kangra)		
	Day light	UV light	
		254 nm	366 nm
Powder as such	Dark green	Light green	Blackish green
Powder + 1N NaOH (aq.)	Blackish green	Blackish green	Black
Powder + 1N NaOH (alc.)	Dark green	Dark green	Black
Powder + 1N HCl	Light green	Light green	Blackish green
Powder + NH_3	Blackish green	Greenish black	Brownish green
Powder + 5% iodine	Dark green	Dark green	Black
Powder + 5% $FeCl_3$	Blackish green	Brownish green	Blackish green
Powder + acetic acid	Dark green	Dark green	Purplish green
Powder + 1N H_2SO_4	Light green	Light green	Blackish green
Powder + 1N HNO_3	Dark brown	Dark green	Blackish green

Table 5: Fluorescence analysis of powdered leaves of *Stevia rebaudiana* from Indore

Treatment	*Stevia rebaudiana* (Indore)		
	Day light	UV light	
		254 nm	366 nm
Powder as such	Light green	Yellowish green	Dark green
Powder + 1N NaOH (aq.)	Brownish green	Blackish green	Dark green
Powder + 1N NaOH (alc.)	Light brown	Bright green	Dark brown
Powder + 1N HCl	Yellowish green	Dark green	Brownish green
Powder + NH_3	Dark green	Bright green	Blackish green
Powder + 5% iodine	Brownish green	Light green	Blackish green
Powder + 5% $FeCl_3$	Yellowish green	Blackish green	Black
Powder + acetic acid	Brownish green	Dark green	Blackish green
Powder + 1N H_2SO_4	Yellowish green	Bright green	Blackish green
Powder + 1N HNO_3	Dark brown	Light green	Black

Table 6: Foreign matter of the different varieties of *Stevia rebaudiana*

Stevia rebaudiana	Weight of sample taken (g)	Foreign matter (%)
Delhi	100	2.35
Surat	100	0.58
Bangalore	100	1.65
Kangra	100	1.80
Indore	100	0.85

Table 7: Total ash, acid insoluble ash and water soluble ash of the different varieties of *Stevia rebaudiana*

Stevia rebaudiana	Total ash (%w/w)	Acid insoluble ash (%w/w)	Water soluble ash (%w/w)
Delhi	9.00	1.25	6.25
Surat	13.50	2.25	7.25
Bangalore	12.75	1.25	7.75
Kangra	7.75	0.75	4.25
Indore	11.50	1.75	6.75

Table 8: Loss on drying of the different varieties of *Stevia rebaudiana*

Stevia rebaudiana	Weight of sample taken (g)	Loss on drying (%w/w)
Delhi	10	5.75
Surat	10	7.90
Bangalore	10	5.35
Kangra	10	8.15
Indore	10	7.25

observed in day light as well as in short wave UV light (254nm) and long wave UV light (366 nm). The powdered drugs were then treated with different reagents as 1 N sodium hydroxide (aqueous), 1 N sodium hydroxide (alcoholic), 1 N hydrochloric acid, ammonia, 5% iodine, 5% ferric chloride, acetic acid, 1 N sulphuric acid, 1 N nitric acid[21,22,23] and the results were noted. (Table 1,2,3,4,5)

Standardization and physicochemical parameters

Physicochemical parameters of the leaves which included determination of foreign matter, ash values (total ash, water soluble ash and acid insoluble ash) and loss on drying[24,25,26] and the results were taken. (Table 6,7,8)

Extraction

The five varieties of the leaves collected were taken and subjected to both hot soxhlation as well as cold maceration using petroleum ether (b.p. 40°-60°), chloroform, methanol, methanol:water(1:1) and chloroform:water (1:99) as solvents. The different extracts were concentrated using rota vapor. Extractive values in different solvents (petroleum ether soluble, chloroform soluble, methanol soluble, diluted methanol soluble and water soluble) were then determined

according to the method[24,27] and noted. (Table: 9 and Table 10)

Successive solvent extraction

Successive solvent extraction of the air-dried drug powdered leaves was carried out using the same solvents as earlier successively in increasing order of polarity starting with petroleum ether (b.p. 40°-60°), chloroform, methanol, methanol:water(1:1) and finally with chloroform: water (1:99) by cold maceration.[28] Before extracting with a new solvent, the powdered material was dried in hot air oven at temperatures below 50 °C.[29] The different successive solvent extractive values were then recorded. (Table 11)

Preliminary phytochemical screening

The methanolic extracts of all the five varieties were subjected to preliminary phytochemical screening to judge the presence of various classes of phytoconstituents as per the method.[17,30] The different chemical tests included the tests for alkaloids, saponins, carbohydrates, glycosides (general), anthraquinone glycosides, cardiac glycosides,

Table 9: Extractive values in different solvents by hot soxhlation

Stevia rebaudiana	Petroleum ether (%w/w)	Methanol (%w/w)	Methanol-water (%w/w)	Chloroform-water (%w/w)
Delhi	2.40	31.15	29.50	21.85
Surat	2.55	38.10	33.80	26.50
Bangalore	3.60	35.25	32.00	24.20
Kangra	6.00	28.55	35.40	24.90
Indore	3.25	29.00	32.65	23.40

Table 10: Extractive values in different solvents by cold maceration

Stevia rebaudiana	Petroleum ether (%w/w)	Chloroform (%w/w)	Methanol (%w/w)	Methanol-water (%w/w)	Chloroform-water (%w/w)
Delhi	3.15	11.95	39.95	14.95	18.60
Surat	3.85	10.55	45.30	20.95	25.15
Bangalore	4.90	13.80	47.00	17.05	24.50
Kangra	2.55	10.10	41.95	19.55	20.25
Indore	5.45	14.25	44.30	15.50	21.20

Table 11: Successive solvent extractive values of the different varieties of *Stevia rebaudiana*

Stevia rebaudiana	Petroleum ether (% w/w)	Chloroform (% w/w)	Methanol (% w/w)	Methanol-water (% w/w)	Chloroform-water (% w/w)
Delhi	2.00	4.90	19.20	11.20	10.60
Surat	2.90	4.00	24.50	10.40	12.70
Bangalore	3.00	6.40	22.40	7.20	15.40
Kangra	3.20	6.30	27.20	12.00	11.30
Indore	2.40	5.80	21.20	9.60	12.20

Table 12: Preliminary phytochemical screening of the methanolic extracts of the different varieties of *Stevia rebaudiana*

Test	*Stevia rebaudiana*				
	Delhi	Surat	Bangalore	Kangra	Indore
Alkaloids	+ve	+ve	+ve	+ve	+ve
Saponins	+ve	+ve	+ve	+ve	+ve
Carbohydrates	+ve	+ve	+ve	+ve	+ve
Glycosides (general)	+ve	+ve	+ve	+ve	+ve
Anthraquinone glycosides	+ve	+ve	+ve	+ve	+ve
Cardiac glycosides	+ve	+ve	+ve	+ve	+ve
Coumarin glycosides	+ve	+ve	+ve	+ve	+ve
Cyanogenetic glycosides	–ve	–ve	–ve	–ve	–ve
Tannins	+ve	+ve	+ve	+ve	+ve
Proteins	–ve	–ve	–ve	–ve	–ve
Steroids	+ve	+ve	+ve	+ve	+ve
Waxes	+ve	+ve	+ve	+ve	+ve
Flavonoids	+ve	+ve	+ve	+ve	+ve
Amino acids	+ve	+ve	+ve	+ve	+ve
Acidic compounds	–ve	–ve	–ve	–ve	–ve

coumarin glycosides, cyanogenetic glycosides, tannins, proteins, steroids, waxes, flavonoids, amino acids and acidic compounds and the results were taken. (Table 12)

RESULTS AND DISCUSSION

The current investigation assessed in a detailed and comparative standardization and physicochemical analysis of the dried leaves of five varieties of *Stevia rebaudiana* procured from five different geographical locations of India viz., Delhi, Surat, Kangra, Bangalore and Indore. From the current study, it was possible to differentiate the five varieties based on their standardization parameters. The results may prove to be a valuable indicator in finding out a suitable variety that best matches in accordance with the standardization parameters so that it can be effectively used in manufacturing of various *Stevia* based products with reasonable and fair quality.

The various physicochemical parameters carried out for the purpose of standardization and authentication included determination of foreign matter, loss on drying, ash values (total ash, acid insoluble ash, water soluble ash), extractive values in different solvents as petroleum ether (b.p. 40°-60°), chloroform, methanol, methanol-water (1:1), chloroform-water (1:99). Both hot soxhlation, cold maceration and successive solvent extraction were carried out for all the five varieties in all the five solvents and it was found that successive solvent extraction led to lower extractive values compared to hot soxhlation and cold maceration. The foreign matter was found to be the highest in the leaves from Delhi with a value of 2.35% w/w and lowest in the leaves from Surat with a value of 0.58% w/w. Presence of moisture which was determined through loss on drying (LOD) was found to be the maximum in Kangra variety i.e., 8.15% w/w and the minimum in the Bangalore variety i.e., 5.35% w/w. Ash values were mainly determined with the purpose of estimating the inorganic salts naturally occurring in the drug and adhering to it as well as the inorganic matter added for the purpose of adulteration and it was found that the total ash and acid insoluble ash was found to be the maximum in the Surat variety with a value of 13.50% w/w and 2.25% w/w respectively and minimum in the Kangra variety with a value of 7.75% w/w and 0.75% w/w respectively. However, the water soluble ash was found to be the highest in the Bangalore variety i.e., 7.75% w/w and the lowest in the Kangra variety i.e., 4.25% w/w.

Additionally, fluorescence analysis for the powdered leaves was carried out using various reagents in day light and UV light (254 nm and 366 nm) which served as a parameter for identification of the plant material.

Preliminary phytochemical screening was carried out on the methanolic extracts of all the varieties and revealed the presence of a wide range of phytoconstituents including alkaloids, glycosides (anthraquinone, cardiac, coumarin), saponins, carbohydrates, flavonoids, tannins, amino acids, steroids, waxes supporting the reason for its wide range of biological activities.

CONCLUSION

Hence, the current research assists to differentiate the five varieties of *Stevia rebaudiana* based on their standardization and physicochemical parameters. The fluorescence analysis of the powder, various physicochemical parameters like foreign matter, loss on drying, ash values, extractive values as well as phytochemical studies including preliminary phytochemical screening supported the identification and authentification of the five varieties for the present study. The results may thus, be helpful in obtaining the variety of best quality to be used in manufacturing of various *Stevia* based products.

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