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"Preliminary phytochemical screening of aqueous and alcoholic extracts of *Stevia rebaudiana* (Bertoni)"

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ABSTRACT:

Stevia rebaudiana (Bertoni) is high demanding antidiabetic plant belonging to Astaraceae family. Stevia has high nutritive value as it is non calorie sweetner containg calcium phosphorous, sodium, magnesium, zinc, vitamin A, vitamin C, proteins and other nutrients. This investigation was carried to study and compare presence of active phytochemical component in various leaf extracts of *Stevia*. Preliminary phytochemical screening revealed the presence of alkaloids and saponins. The other secondary metabolites like tannins, steroids and flavonides were present in trace amounts in aqueous extract as compared to alcoholic extract. Phytochemical analysis resulted in the presence of tannins in high concentration which was followed by alkaloids, flavonoids, saponins, steroidal compounds, reducing compounds and anthraquinones. This study will provide phytochemical information for preparation of concentrated and effective extract of *Stevia*.

Keywords: *Stevia rebaudiana*, Aqueous extract, Alcoholic extract, Phytochemical analysis

Introduction:

Stevia rebaudiana (Bertoni) is high demanding antidiabetic medicinal plant belonging to Astaraceae family. It is a perennial and endemic, medicinal shrub (Sivaram and Mukundan, 2003). It is natural medicinal herb which produces sweet steviol glycosides (Soejarta *et al*., 1982 and Savita *et al*., 2004). It is also called as honey plant

due to its sweetness. The fresh leaves have a nice liquorice taste. It is recommended for diabetes and has been extensively tested on animals and has been used by humans with no side effects (Megaji et al., 2005). The components obtained from *Stevia* are the best alternative natural sweertner for diabetes. Leaves of Stevia contain sweetening compounds viz., Stevioside, Rebaudieside-A, Rebaudieside-B,C and six other compounds which are said to be having insulin balancing properties (Farooqi and Sreeramu, 2001). Diabetic persons with hyperglycemia can use *Stevia* as alternative natural sweetner (Din *et al*., 2006). *Stevia* have versatile medicinal uses without any side effects that focus the interest towards *Stevia* in World wide. It is used for the treatment of various conditions such as cancer (K. Yasukawa et.al., 2002) diabetes (N. Lailerd et al., 2004), obesity, cavities, hypertension (Dyrskog et.al.,2004) fatigue, depression, and in cosmetic and dental preparations (Mowrey, 1992). It possesses hypoglycemic, hypotensive, vasodilating, taste improving, sweetening, anti-fungal, anti viral, anti inflammatory, anti bacterial (Ghosh, 2008) properties and increases urination function of the body. For Patients of diabetes, hypoglycemia, high blood pressure, obesity and chronic yeast infections, *Stevia* is the ideal sweetener. The purpose of this research work is qualitative investigation of phytochemicals present in various leaf extracts of *S. rebaudiana.* The demand of *Stevia* is increasing widely due to its non caloric nature and usages as natural supplement for sugar. The leaves are having commercial importance due to presence of di-terpene sweet glycosides which are 300-400 times sweeter than sugar without any side effects. The plant was domesticated in India in last 20th century from the wide source. So there is a need to set up certain protocols for purification of *Stevia* extract by various techniques. This investigation was carried to study and compare presence of active phytochemical component in various leaf extracts of *Stevia*.

Materials and methods:-

Young plants, dry leaves and commercial stevioside powder of *S. rebaudiana* were supplied by Jamna Biotech, Pune. Dry leaves were packed in polyethylene bags and stored in deep freezer until used. Chemicals used for investigation were purchased from Sigma, Hi-media and Qualigens companies. Sample preparation(A,B and C):

A) Green leaf extract: Alcoholic (Methanol) and aqueous extract of fresh *Stevia* leaves was prepared by vigorous grinding in 80% alcohol and distilled water respectively by using pestle and mortar. The mixture was centrifuged at 8000 rpm for 20 min. Supernatant was collected and used for analysis. B) Dry leaf extract: Same procedure was carried by using dry leaf powder. C) Stevioside powder: Comercially available stevioside powder was taken in 1mg/ml concentration. Alcoholic and water extract of pure stevioside powder were prepared and used for analysis. Sample preparation (D and E): 250 g of finely powdered dry leaves of *Stevia rebaudiana* were extracted with hot water (1 litre) for two hours at 70ºC. The extract was filtered through Whatman #1 filter paper and the clear green solution (800ml) was concentrated to 400ml by heating. Two methods were used for partial purification of extracts. D) pH of partially purified extract was brought down to pH 3.5 with fumaric acid. It was refiltered and the pH of the filtrate adjusted to 10.0 with dilute sodium hydroxide. A pasty mass separated out. It was filtered and the pH of the filtrate readjusted to 8.5 with addition of potassium aluminum sulphate (alum).The solution was clear and clarified. Thus the solution was partially purified. E) pH of the extract was adjusted to 11.5 with calcium oxide filtered and the pH of the filtrate adjusted to 6.5 with glucono-delta-lactone. The solution was clear and clarified. Both the extracts D and E were stand for 4-5 hours and supernatant was taken. The solution was further diluted 10 times with distilled water and used for analysis (1ml extract+9ml distilled water). Preliminary phytochemical analysis was carried out according to the methods described by Trease and Evans (1989). Various crude extract the *S. rebaudiana* leaves were used for estimation of phytochemicals and identification of components such as tannins, alkaloid, steroid, phenols, terpenoid, flavonoid and anthraquinones. Various screening test of the extract was carried out for estimation of plant constituents. Alcoholic and water extract of the sample was used for phytochemical analysis. The crude extract was screened for the presence or absence of secondary metabolites such as flavonoids, steroidal compounds, tannins, alkaloids and anthraquinones using standard procedures. Following tests were performed:

Test for flavonoids: a) Lead acetate test: To a solution of 1ml of the extract in water about 1 ml of 10% lead acetate solution was added. Production of yellow precipitate is considered as positive for flavonoids. b) Reaction with sodium hydroxide: Dilute sodium hydroxide solution was added to a solution of 1ml of the extract in water. The mixture was inspected for the production of yellow color which considered as positive test for flavonoids. c) Free flavonoids test: Five milliliters of ethyl acetate was added to a solution of 1ml of the extract in water. The mixture was shaken, allowed to settle and inspected for the production of yellow colour in the organic layer which is taken as positive for free flavonoids.

Test for steroidal compounds: Salkowski's test: 1ml of the alcoholic extract was dissolved in 2 ml chloroform in a test tube. Concentrated sulfuric acid was carefully added on the wall of the test tube to form a lower layer. A reddish brown colour at the interface indicated the presence of a steroid ring (i.e. the aglycone portion of the glycoside).

Test for tannins: a) Ferric chloride test: A portion of the alcoholic extract was dissolved in water. The solution was clarified by filtration. 10% ferric chloride solution was added to the clear filtrate. This was observed for a change in colour to bluish black.

b) Aqueous hydrochloric acid test: Deposition of a red precipitate when an aqueous extract of the plant part was boiled with 1% aqueous hydrochloric acid was taken as an evidence for the presence of phlobatannins.

Test for saponins: 10 ml of distilled water was added in 5 ml of aqueous extract and vigorously shaken for 10 min. Appearance of foam that persists for at least 15 min. Presence of saponins confirmed by formation of emulsion after addition of olive oil.

Test for alkaloids: 1ml of ethanol extract sample in a test tube was mixed with one ml of Mayer's reagents. The treated solutions were observed for any precipitation.

Test for anthraquinones: Free anthraquinones test (Borntrager's test): The hydro-alcoholic extract of the plant material was shaken vigorously with 10 ml of benzene, filtered and 5 ml of 10% ammonia solution added to the

filtrate. Shake the mixture and the presence of a pink, red or violet color in the ammonia (lower) phase indicates the presence of free anthraquinones.

Test for reducing compounds: 2 ml of aqueous extract was placed in test tube and 5 ml of equal volumes of Fehling's A and B were added and boiled in a water bath for 5 min. The test tube was observed for brick red precipitate.

Result and discussion

The qualitative test for presence of flavonoids, steroidal compounds, tannins, alkaloids and anthraquinones was carried using 5 different extracts of *S. rebaudiana.* For each test as per protocol either alcoholic or water extract of sample were used. The results are as follows.

Test for flavonoids: a) 10% lead acetate test- In sample A, B, D and E, yellow precipitate was obtained at the bottom of test tube whereas in case of sample C white precipitate was formed at bottom of test tube. b) Sodium hydroxide test-In this test yellow colour of solution was seen when dilute sodium hydroxide was added to the samples A, B, D and E, whereas no yellow colour was seen sample C. c) Ethyl acetate test- Yellow colour was produced in organic layer in samples of A, B, D and E,whereas no yellow colour was seen in sample C.

Test for steroidal compounds: a) Salkowski's test- A reddish brown colour was seen at interface of all the samples of *S. rebaudiana* analyzed.

Test for tannins: a) Ferric chloride test- Colour was changed to bluish black in A, B, D and E, samples whereas it was unchanged in sample C. b) Aqueous hydrochloric acid test-Red precipitate in very small amount was deposited at bottom in test tubes of samples A, B, D and E, i.e. in trace amount. But it was not seen in sample C.

Test for alkaloids:a) Mayer's test- Precipitate was seen in all the sample extracts except for sample C.

Test for anthraquinones: a) Free anthraquinones test: No presence of pink red or violet colour was seen in lower phase of ammonia in all the samples tested. This shows that anthraquinones are absent in fresh leaves, dry leaves, solution A, solution B and pure stevioside. Above result indicates that flavonoids are present in A, B, D

and E, whereas absent in sample E. The result showed that steroidal compounds are present in fresh leaves, dry leaves, partially purified extracts and pure stevioside sample. This indicated that tannins are present in A, B, D and E, whereas absent in sample C. Preliminary phytochemical screening revealed the presence of alkaloids and saponins. The other secondary metabolites like tannins, flavonoids, steroids, cardiac glycosides, etc. were present in trace amounts in some of the plants. There are differences in the antimicrobial effects of plant species, due to the phytochemical properties and differences among species. It is quite possible that some of the plants that were ineffective in this study do not possess antibiotic properties, or the plant extracts may have contained antibacterial constituents, just not in sufficient concentrations so as to be effective. It is also possible that the active chemical constituents were not soluble in methanol or water. The drying process may have caused conformational changes to occur in some of the chemical constituents found in these *Stevia.*

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Table 1: Phytochemical analysis of different sample extracts of *Stevia rebaudiana*

Test	**Reagent**	**Aqueous extract**					**Alcoholic extract**				
		A	**B**	**C**	**D**	**E**	**A**	**B**	**C**	**D**	**E**
Flavonoids	10% Lead acetate	+	++	+	+++	+++	++	++	++	+++	+++
	Sodium hydroxide	+	++	+	+++	+++	++	++	++	+++	+++
	Ethyl acetate	+	++	+	+++	+++	++	++	++	+++	+++
Tannins	Ferric chloride	±	±	-	±	±	+	++	-	±	±
	Aqueous HCl	±	±	-	±	±	±	±	-	±	±
Saponins	Olive oil	+	±	-	±	±	+	-	-	-	-
Alkaloids	Mayer's reagent	±	±	-	±	±	±	±	-	±	±
Steroidal compounds	Chloroform + Conc. H2SO4	+	++	+	++	++	+++	+++	++	++	++
Anthraquinones	Benzene & Ammonia solution	-		-	-	-	-		-	-	-
Reducing compounds	Fehling's A and B solution	+	+	±	+	+	++	++	+	++	++

Key: **+++=** High concentration, ++=medium concentration, + = low concentration, ± = Traces, **-** =Negative ressult