



Original Research Article

Phytochemical Screening and Antimicrobial Activity of *Stevia rebaudiana* Leaves

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ABSTRACT

The herb *Stevia rebaudiana* is fast becoming a major source of sweetener which produces sweet taste but has no calorific value. With an objective of understanding the Physio-chemical, Phytochemical and antimicrobial aspects of the Stevia plant research was carried out. Different extracts were obtained by Ayurvedic Pharmacopeial Method (water and alcohol), Soxhlet method and by column extraction using adsorbant resin were subjected to antimicrobial assay against bacteria *Staphylococcus albus*, *Klebsiella aerogenes*, *Enterobacter aerogenes*, *Bacillus subtilis*, *E. coli* and fungi *Penicillium chrysogenum*, *Aspergillus niger*, *Candida albicans*. Highest rate of susceptibility was exhibited by *E. aerogenes* (maximum 18.6mm) invariably by all the extracts obtained by various methods. Based on the findings alcohol extract obtained by Ayurvedic Pharmacopeial Method showed maximum activity against all the bacteria used (range 13.8mm to 18.6mm). The TLC-bioautography was also performed which was found to be quick method for preliminary antibacterial activity study. This study may be explored for a value addition as natural food preservative to sugar substitute property of Stevia powder.

Keywords

Stevia rebaudiana,
Plant extracts,
Phytochemicals,
Anti-microbial
activity
Bioautography

Introduction

Stevia plant belongs to Aster family which is a perennial shrub that grows up to 1m tall. Leaves are sessile, 3-4cm long, elongate-lanceolate or spatulate shape with blunt-tipped lamina, serrate margin from the middle to the tip and entire below (Swati *et al.*, 2010). The leaves of this plant are used to extract a natural, non-caloric sugar substitute, 200-300 times sweeter than table sugar (Giuffre *et al.*, 2013). *Stevia* plant was officially discovered by Dr.M.S.Bertoni

(Ahmad *et al.*, 2014). *Stevia* as a sweetener also have bitter aftertaste due to the presence of essential oils, tannins, and flavanoids. *Stevia* has many properties such as anti-fungal, anti-bacterial, anti-oxidant, anti-microbial, anti-inflammatory, anti-diabetic, hypertensive, and hyperglycaemic (Goyal *et al.*, 2010). It is used to sweeten many things such as soya sauce, pickles, ice-creams, cookies, soft drinks, and skincare products. There is high need of natural sweetener in

future of food market. Stevia is 100% natural flavour enhancer and non-fermentable (Reshu *et al.*, 2014).

Stevia is native to the valley of the Rio Monday in highlands of North-eastern Paraguay in South America where it grows in sandy soils near streams on the edges of marshland, acid infertile sand or muck soils. It is found growing wild in the highlands of the Amambay at Iguac districts (a border area between Brazil and Paraguay). Stevia is a semi-humid subtropical plant that can be grown easily like any other vegetables crop even in the kitchen garden. The soil should be in the pH range 6.5-7.5; well-drained red soil and sandy loam soil. Stevia has been successfully cultivated in recent years in many areas of Indian states: Rajasthan, Maharashtra, Kerala and Orissa. Micropropagation is a conventional technique that can help us to cultivate the disease free and resistant plant in many countries for the manufacturing of artificial non-caloric sweetener (Swati *et al.*, 2010; Goyal *et al.*, 2010).

There are many natural and artificial sweeteners that are sold in market as a dietary supplement. Today humans are switching to safe and alternative sweetener that doesn't have any negative health impact. As there are no side-effects of Stevia, it can be used as natural sweetener. Six sweet tasting compounds are present in a leaves in *S. rebaudiana* Bertoni-rebaudioside A, D and E, stevioside, dulcoside A and B (Goyal *et al.*, 2010).

Japan began marketing stevioside as a sweetener in the 1970s, when chemical sweetener were banned and replaced with Stevia. Japanese have conducted more than 40,000 clinical studies on Stevia and concluded that it is safe for human use. This plant has been used in several areas of the world, such as in Brazil and Paraguay, as a

natural control for diabetes. Stevia also has been used to help control weight in obese persons. Besides it is well known for its application in treatment of many diseases like diabetes and High blood pressure in various traditional systems of medicine (Goyal *et al.*, 2010). In recent times the extract has been subjected to rigorous chemical, biochemical, pharmacological, clinical and toxicological investigations and many new therapeutic applications have been emerged out.

The present study aims at standardisation of Stevia plant, and phytochemical study of the plant leaves. Further the extraction of stevioside was done in water and ethanol as a solvent by Ayurvedic Pharmacopoeial Methods. The extraction was also carried out by using Soxhlet apparatus using alcohol as a solvent. Stevia extract was also obtained by passing the crude extract through a column containing adsorbent resin. Antimicrobial assay was carried by using Standard Kirby-Bauer method for the above extracts of Stevia, against different food spoiling and disease causing micro-organisms. In the present study, the ethanolic extract of leaf was analysed by Thin-layer chromatography to check the presence of steviosides using commercial Stevia powder as a standard. Later the bioautography analysis of the TLC plate was done against *E. coli* which showed maximum zone of inhibition.

Materials and Methods

Procurement and standardization

Dried leaves were collected from the local commercial supplier from Pune (Maharashtra). The leaves were cleaned of fibrous matter and foreign material, and dust was removed. The leaves were then powdered by mortar and pestle.

Physical evaluation (The Ayurvedic Pharmacopeia of India, 2008)

Determination of foreign matter: 10gm of the sample was weighed and spread on a white tile uniformly without over lapping. Then the sample was inspected to check any fibrous matter and the foreign organic matter. The foreign matter includes mud, stones, small dried leaf stalks etc. After complete separation the matter was weighed and percentage w/w was determined.

Determination of total ash value: Total ash content was determined by weighing 3gm of the dried Stevia leaves in the crucible. Incinerate the sample using muffle furnace set at a temperature 450°C. Cool the crucible at room temperature. Weigh the crucible and calculate the total ash value.

Determine the moisture content: Total moisture content was determined by weighing accurately the empty evaporating dish. Accurately weighed 10gm of the powdered Stevia leaves was placed it in evaporating dish. It was placed in the oven at temperature 105±1°C. Continue weighing and drying at one hour interval until the difference between two constructive weighing is less than or equal to 1mg normally. 5hrs are sufficient. Cool the evaporating dish. Record the constant weight of the dish and sample.

To determine the water soluble & alcohol soluble extractives: 5g of powdered Stevia leaves was macerated with 100ml of water and alcohol and mixed it gently for 3hr. Stopper the flask and allow it to stand for 24hr. Filter the mixture by Whatman filter paper, No 1. After filtration the filtrate was evaporated in evaporating dish on boiling water bath and dry at 105°C to constant weight. Later the residue was weighed carefully.

Extraction of Stevia

Extraction of Stevia was done by 3 methods.

Ayurvedic pharmacopoeial method: The leaves were cleaned of fibrous matter and foreign material, and dust was removed. Then leaves were powdered by mortar and pestle. 10g of Stevia powder was soaked in 100ml of water and alcohol separately. Keep stirring for 3-4hrs and allow it to stand for 24hrs, filter the mixture with Whatman filter paper. Later the water and alcohol was evaporated on boiling water bath. The dried aqueous and alcoholic extract was collected separately in flat bottom shallow dish (The Ayurvedic Pharmacopeia of India, 2008).

Soxhlet extraction: A total 40g of weighed dried leaves was taken for the extraction in the Soxhlet apparatus using 150ml of 70% ethanol as solvent. The dried leaves of Stevia were placed inside a thimble made from thick filter paper, which is loaded into the main chamber of the Soxhlet extractor. The Soxhlet extractor is placed onto a flask containing 70% ethanol as extraction solvent. The Soxhlet is then equipped with a condenser. The solvent is heated to reflux. The solvent vapour travels up a distillation arm and floods into the chamber housing the thimble of dried Stevia leaves. The condenser ensures that any solvent vapour cools, and drips back down into the chamber housing the dried Stevia leaves. The chamber containing the dried Stevia leaves slowly fills with warm solvent. Some of the desired compound will then dissolve in the warm solvent. When the Soxhlet chamber is almost full, the chamber is automatically emptied by a siphon side arm, with the solvent running back down to the distillation flask. This cycle may be allowed to repeat 4 times, 4-5 hours. During each cycle, a portion of the non-volatile compound dissolves in the solvent. After 4 cycles the

desired compound is concentrated in the distillation flask. After extraction the solvent is removed, typically by means of a rotary evaporator under reduced pressure, yielding the extracted compound (The Ayurvedic Pharmacopeia of India, 2008).

Column extraction by color removal resin- A total 40g weighed dried leaves powder was taken and soaked in 100ml of water and stirred for 3-4hrs and was allowed to stand for 24hrs. The mixture was filtered through Whatman filter paper, No 1. 40ml of calcium hydroxide (for making the solution clear by flocculation) was added to the filtered extract. The column containing colour removal resin (Microporous type I anion, Thermax) was setup for the extraction of above mentioned water extract. Filtrated extract, 100ml was passed through 50ml of absorbent resin in a column of 50cmX20mm with a specific flow rate of 10-11ml/min. The eluent so obtained was evaporated with boiling water bath. The dried extract was collected in a flat bottom shallow dish (Rongfu *et al.*, 2002).

Phytochemicals (Abu *et al.*, 2014)

Primary phytochemical screening

The crude extracts were subjected to classical tests for the detection of the presence or absence of the unsaturation, phenolic group, carboxylic acids and sugar molecules.

Preliminary phytochemical screening

Standard screening test of the extract was carried out for various plant constituents. The crude extract was screened for the presence or absence of secondary metabolites such as alkaloids, steroidal compounds, flavanoids, saponnins, tannins and anthraquinines using standard procedure.

Thin Layer Chromatography (TLC)

A TLC plate used in this study was a sheet of metal which is coated with a thin layer of a solid adsorbent (silica gel-60 plate, Merck). Each of the crude extracts was applied separately 1.0 cm above from the lower edge of the activated silica gel plates along with the standard reference compound of stevioside. It was then placed in a shallow pool of a solvent in a developing chamber so that only the very bottom of the plate is in the liquid. The plates were developed in an air tight chamber containing chloroform: methanol: water (6:2.5:1.5) as a solvent. When the solvent reached the top of the plate, the plate was removed from the developing chamber and dried. The developed plates were air dried, sprayed with 50% sulphuric acid and subsequently heated at 100°C for 15 min. It showed spots which coincided with that of the standard reference stevioside (bluish grey). When plates were placed in a chamber saturated with Iodine vapours, it also showed deep brown colour of stevioside (Roop and Amla, 2013). Later the plate was observed for the spots with the help of UV chamber at 365nm. The R_f values were calculated further.

Bioautography

Bioautography can be employed in the target directed isolation of active constituents. Paper chromatography followed by bioautography was used for the first time in 1946 by Goodal and Levi to estimate the purity of penicillin. Thin-layer chromatography - bioautography was introduced by R. Fisher and H. Lautner in 1961. There are three different approaches for bioautography to localize antimicrobial activity on a TLC chromatogram - agar diffusion or contact bioautography, direct bioautography and immersion or agar overlay bioautography. Those assays supply

a quick screen for new antimicrobial compounds through bioassay-guided isolation (Patil *et al.*, 2013). The antimicrobial activity of an isolate can be detected by bioautography methods. Bioautography is sensitive method for detection of antimicrobial compounds even in small amounts. Hence for detection of antimicrobial compounds, bioautography was employed (Irena *et al.*, 2010).

TLC plates were completely dried for 36hrs. The dried TLC plates (on which spots are developed) were kept in the petri plates. *E.Coli* (5ml) culture was inoculated in 100ml of nutrient agar. Thin layer of inoculated nutrient medium was poured in the petri plates to cover the TLC plates completely. The plates were incubated at 37°C for 24hrs. Zone of inhibition was observed around the developed spots.

Antimicrobial activity

Procurement of cultures- All the microbial cultures were procured from National Collection of Industrial Microorganisms (NCIM), NCL, Pune. Four bacteria [*Staphylococcus albus* (ATCC 2178), *Klebsiella aerogenes* (ATCC 2239), *Enterobacter aerogenes* (ATCC 2340), *Bacillus subtilis* (ATCC 2239), *E.coli* (ATCC 25744)] and three fungi [*Penicillium chrysogenum* (ATCC 709), *Aspergillus niger* (ATCC 504), *Candida albicans* (procured from Microbiology department Sinhgad Dental college)].

Maintenance of cultures- The pure cultures were maintained by routine sub-culturing at one month interval on nutrient agar and potato dextrose agar slants for bacteria and fungi respectively. (Hi-Media laboratories private limited, Mumbai, India).

Disc Diffusion Assay by Kirby Bauer Method- Extraction of Stevia was done by 3

methods as mentioned above. All the extracts thus obtained were subjected to antimicrobial assay by measuring the diameter of zone of inhibition using disc diffusion assay by Kirby Bauer technique. Potato Dextrose agar and Muller Hinton agar plates were prepared by pouring 20ml each in sterile Petri plates for fungal and bacterial assay respectively and allowed to solidify. Standard cultures were grown in nutrient broth freshly during the assay. 24 hours old bacterial and 48 hour's old fungal cultures were used. The cotton swabs were used for inoculating the cultures. The cotton swab was lightly dragged across the agar surface in a zigzag pattern so that the surface is inoculated uniformly. Pre-sterilized filter paper discs of 5mm were dipped into individual extracts separately and placed on the swabbed agar plates before incubation. At the end of the incubation period of 24-48hr at 37°C for bacteria and 48-72hr at 20°C for fungi, the inhibition zones were observed. The zones of inhibition were measured in mm using measuring scale and the average was determined. The experiment was carried out in 5 replicates (Sumitt *et al.*, 2008; Abu *et al.*, 2014).

Results and Discussion

Physicochemical parameters of the Stevia leaves were determined. It was observed that foreign matter was 2.857%, total ash was 2.903% w/w, and moisture content was 9.4127%. Solvent extractive values of aqueous soluble and alcohol soluble were observed to be 27.278% and 30.573 % w/w respectively.

Preliminary phytochemical screening showed the most abundant compound in the stevia leaves were alkaloids and steroids followed by tannins, saponins and flavonoids. The results are shown in following table 1.

The signs “+” or “-” against different test indicates the presence and absences of respective functional groups or compounds. For the proper identification of the plant, physicochemical parameters provide useful information. From the ongoing study we have provided basic information regarding the physicochemical parameters as well as phytochemicals constituents present in the leaves of the Stevia plant of locally available variety. This is an agreement with the finding of Abu *et al.*, 2014 who recorded phytochemical screening of *Stevia rebaudiana* leaves.

TLC of ethanolic extract and standard reference compound was approximately the same. We observed that the spots in the ethanolic extract (Rf value 0.56) were very close to the standard stevioside compound (Rf value 0.59). Bio-autography of ethanolic extract obtained from soxhlet method and the available standard Stevia reference was successfully done and inhibition zones were observed around the developed spots. It was observed that the steviosides present in ethanolic extract showed the zone of inhibition against *E. coli*. The coupling of

TLC and bioautography provided primary information about the presence of antibacterial components. This process may provide a rapid technique for the detection of bioactive component on TLC and a quick antibacterial activity against the test bacterium (Fig. 1).

We carried out antibacterial and antifungal assay further with different extracts against different bacteria and fungi. The zone of inhibition obtained against the test microorganisms for various extracts are given in table 2. It is clearly depicted in table 2 that among the microorganisms selected for the study the highest rate of susceptibility was exhibited by *E. aerogenes* invariably by all the extracts obtained by various methods i.e. Ayurvedic Pharmacopeia Method in water, in alcohol, Soxhlet method and by column extraction. In case of other bacteria i.e. *S. albus*, *K. aerogenes*, *B. subtiles*, *E. coli* showed substantial inhibition with respect to first three extracts only. These four bacteria showed less or no inhibition by extract obtained by column extraction method.

Table.1 Phytochemicals screening of *S. rebaudiana* Bertoni leaves

Phytochemical screening	Test name/ reagents	Response
Unsaturation	Baeyer’s test	+
Carbonyl group	2,4-dinitrophenylhydrazine and HCL	+
Phenolic group	Ethanolic FeCL3 solution	+
Acetyl group	Sodium bicarbonate	+
Sugar molecules	Fehling’s solution-I and II	+
Alkaloids	Dragendorff’s	+
Steroids	Salkowski’s test	+
Saponins	Froth test Ferric chloride test	+
Tannins	Phlobatannins	+
Flavonoids	Free flavonoids Borntrager’s test	+
		-

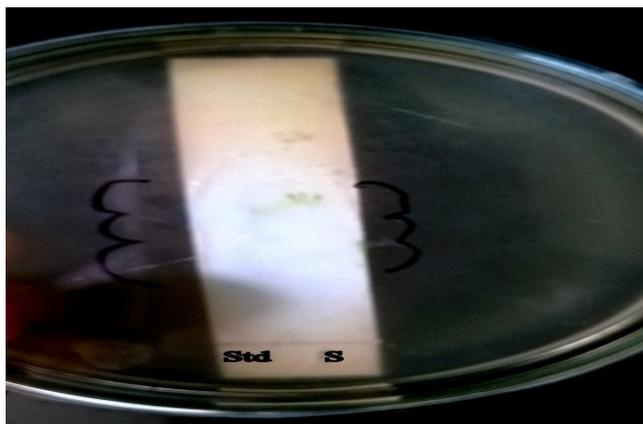
-: Negative result +: Positive result.

Table.2 DZI (Diameter of zone of inhibition in mm)

DZI (Diameter of zone of inhibition in mm)				
Microorganisms	Extracts			
	Water	Alcohol	Soxhlet	Column
S.A	8	14.2	8	-
K.A	11.2	17.2	9.8	-
E.A	13.6	18.6	12.4	10
B.S	18.6	13.8	10.6	-
E.C	-	15.6	11.8	7
P.C	6	-	-	6.6
A.N	-	-	-	6.6
C.A	6.5	6.2	5.2	7.2

Means- S.A: *Staphylococcus albus*; K.A: *Klebsiella aerogenes*; E.A: *Enterobacter aerogenes*; B.S: *Bacillus subtilis*; E.C: *E. coli*; P.C: *Penicillium chrysogenum*; A.N: *Aspergillus niger*; C.A: *Candida albicans*. -: no zone of inhibition

Fig.1 Bioautography plate for Standard stevioside (Std) and Alcohol extract (S)



The bacteria *B. subtilis* showed maximum zone of inhibition of 18.6 mm and the least zone of inhibition of 8mm was shown by *S. albus* against the water extract obtained by Ayurvedic Pharmacopial method. The bacteria *E. aerogenes* showed a maximum zone of inhibition of 18.6 mm and the *B. subtilis* showed the minimum zone of inhibition of 13.8 mm against the alcohol extract obtained by Ayurvedic Pharmacopial Method.

E. coli showed the maximum zone of inhibition of 11.8 mm and *S. albus* showed a minimum zone of inhibition of 8 mm against the extract obtained by Soxhlet method.

The extract obtained by column method showed very less inhibitory effect against bacteria *E. aerogenes* 10 mm and *E. coli* 7mm respectively.

Among the fungal strains used higher inhibitions was obtained by *P. chrysogenum* and *A. niger* during the first 24–48 hrs of the incubation period. But it was observed that after 48 hrs once the sporulation started the inhibition zone was not observed. *Candida* also showed very less zone of inhibition with respect to all the extracts tested.

The present investigation endows with the basic information about the antibiotic component present in the stevia leaves and

the extract obtained from it. It was observed that the stevia leaf extract obtained by Ayurvedic Pharmacopoeial Method using water and alcohol were found to be potent enough in exhibiting antibacterial activity. The extract obtained from column method showed very less antibacterial or antifungal activity indication the absence of any potent antibacterial components in it. The component probably got removed from the extract during the extraction process. Abu *et al.*, 2014 also studied the antimicrobial activity of the different stevia extracts against 16 food spoiling microorganisms. The value addition of stevia as natural food preservative was explored by them.

Based on the results it can be concluded that stevia extracts contains potent antibacterial as well as antifungal component. This study may be explored for a value addition as natural food preservative to sugar substitute property of stevia powder. More scientific research is needed to ensure the use of Stevia leaves and steviosides, related to toxicity and health effects, and its application in food industry.

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