ISOLATION, PURIFICATION AND CHARACTERIZATION OF SWEETNERS FROM STEVIA REBAUDIANA (BERTONI) FOR THEIR ANTICANCEROUS ACTIVITY AGAINST COLON CANCER

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ABSTRACT

Stevioside, an abundant component of Stevia rebaudiana leaf, has become well-known for its intense sweetness and is used as a non-caloric sweetener beside sweetness, it may also offer therapeutic benefits. It is natural sweetner and contains phytochemicals including well characterized glycosides such as Stevioside, Reboudioside A-F and Steviol. The present study was deal with extraction of Steviosides and its derivatives from Stevia rebaudiana which is followed by its identification, analysis of its quality, quantity and biological activity. Stevioside was extracted from the dried Stevia leaves. Further it was purified, recovered using Calcium hydioxide and Ion exchange chromatography. In fresh leaves extract the percentage of carbohydrates is higher than reducing sugars. TLC was carried out for qualitative analysis. Confirmatory test for identification and presence of Stevioside was positive. Steviosides have excellent heat stability which is up to 100°C for 1 h at pH range 3 -9. The HPLC analysis of water extract had shown the presence of Rebaudioside-D, Stevioside and Rebussoside. Methanolic leaf extract had shown the presence of Rebaudioside D and rebaudioside. Etahnolic extract had shown a type of peak which was remained unidentified. Along with this, it showed presence of Rebaudioside A and Rebussoside. The data obtained by 1H NMR showed isolated compound was Stevioside. The methanol and ethanol extract of Stevioside showed anticancer activity against Caco cell line with IC\textsubscript{50} value 10 and 12µg/ml respectively whereas these extract had shown the cytotoxicity against Caski cell line with IC\textsubscript{50} value of 20 and 5µg/ml respectively.
**Keywords:** Stevioside, TLC, HPLC, 1H NMR, anticancer activity, LDH activity.

1. INTRODUCTION

Sweeteners are alternative substances to sugars, which give food a sweet taste and are used to partially or totally replace sucrose. The discovery of great number of sweeteners during the last decade has triggered the development of sugar free products, particularly for diabetics. Pharmaceutical sweeteners like aspartame and saccharin qualify as calorie-free but come with significant limitations and health risks. Various side effects have been associated with the ingestion of aspartame and include migraines, memory loss, slurred speech, dizziness, stomach pain, and even seizures.

The sweet herb of Paraguay, *Stevia rebaudiana* Bert., added special advantage that as sweeteners are natural plant products. It is high demanding antidiabetic medicinal plant belonging to Asteraceae family. It is a perennial and endemic, medicinal shrub (Sivaram *et al*., 2003). It is also called as honey leaf, sugar leaf 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 leaves of *S. rebaudiana* contain diterpene glycosides namely Stevioside and Rebaudioside A-F and ducloside A, which are responsible for the typical sweet taste. Human physiology cannot metabolize the sweet glycosides present in stevia leaves; therefore they are eliminated from the body with nocaloric absorption (Mantovaneli *et al*., 2004). 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 (Yasukawa *et al*., 2002) diabetes obesity, cavities, hypertension (Dyrskog *et al*., 2004) fatigue, depression, and in cosmetic and dental preparations. It possesses hypoglycemic, hypotensive, vasodilating, taste improving, sweetening, anti-fungal, anti viral, anti inflammatory, anti bacterial 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. It can be safely used in herbal medicines, tonics for diabetic patients and also in the daily usage products.

The demand of Stevia is increasing widely due to its non caloric (300-400 times sweeter than sugar) and usages as natural supplement for sugar. The plant was domesticated in India in last 20th century from the wide source. So there is a need to set up certain work on Stevia extract.
by various techniques. Hence, it might help in preventing diabetic complications and may serve as a good alternative in the present armamentarium of antidiabetic drugs. Therefore, stress should be on collection of more relevant Indian data for pharmacological and stability of Stevia and its products. Considering the medicinal potential of this plant and the increasing demand of huge population in India, the main objective of the proposed research work was to extract Stevioside and its derivatives from *Stevia rebaudiana* which is followed by its identification, analysis of its quality, quantity and biological activity.

2. MATERIALS AND METHODS

2.1 Plant Material

The plants of *Stevia rebaudiana* were collected from the Jamna Biotech, Pune district of Maharashtra, State of India. All the botanical aspects of the whole plant were studied in detail. After the samples were identified and authenticated by the scientists of the institution and the voucher specimens were deposited in our laboratory collections.

2.1.2 Cell Lines

The various cancerous as well as normal cell lines were used to analyze the anticancerous activity of leaves of *Stevia rebaudiana*. The cell lines, used for the present study were Caco 2 (human colon cancer) and Caski (human cervical cancer) and Cord blood stem cell line. The cancerous cell lines were purchased from NCCS, Pune.

2.3 Preparation of leaves powder of *Stevia rebaudiana*

Leaves were removed from the plants then, washed in clean water and spread on trays. Leaves were dried under shade at room temperature ranged from to 25 - 30°C for 24-48 h. Dry leaves were packed in polyethylene bags and stored at freeze condition until used. By using mortar and pestle, fine powder of leaves was prepared. The dry leaves were used for extract preparation.

2.4 Extraction of Stevioside from Stevia plant

*Stevia* sweetener (Stevioside) was extracted from the dried ground leaves of Stevia plant by using water, methanol and ethanol extraction. The dried ground leaves were mixed with hot water (65°C) at different percentage of powder leaves/water ratio of 1:45 (w/v). Stevia leaves were extracted by using hot water for 3 h. The crude extract containing Stevioside was filtered through Whatman No. 1 filter paper. It was named as filtrate A (Abou-Arab *et al.*, 2010).
Dried ground Stevia leaves were extracted by using methanol according to the method of Nikolai et al. (2001). Methanol was added to ground leaves at ratio (4:1 v/w) and remained for 7 h, then filtered through Whatman No.4 filter paper. The filtrate containing solvent was evaporated to dryness by using rotary evaporator at 45°C. The residue was washed with ether and then extracted with butanol (three times). The organic phase was evaporated, and the residue was recrystallized in freezer. Same procedure was followed for extraction of stevioside with 80% ethanol. Purification steps were determined by addition of Calcium hydroxide and Ion exchange chromatography.

2.5 Purification of Steviosides
Water extract was purified by addition of 5% Ca(OH)2 (based on weight of dried leaves). The addition of Ca(OH)2 was repeated twice (filtrate B and C) and the filtrates were collected, passing through ion exchange column (packed with glass wool) to remove the undesirable colors at a rate of 1 ml/sec. The elute (clear and colorless solution) containing Stevioside was collected (in which pigments were adsorbed on resin) and then concentrated by using rotary evaporator at 45°C to the maximum concentration value.

The ion exchange chromatography was carried out using slurry of the diethyl amino ethyl (DEAE) resin in the equilibration buffer. Glass wool was packed in column. The glass column was then filled with the equilibration buffer with the nozzle of the column closed. The nozzle was opened with a slow flow rate. Using a pipette, load the resin suspension onto the column. The material was allowed to settle till the required level.0.5N HCL and 0.5M NaOH were passed one by one. The column was washed thoroughly with 2 to 3 column volumes of equilibration buffer before loading the sample onto the column. Fractions (3ml) were collected in 30 test tubes. Absorbance was taken on UV-VIS spectrophotometer for each fraction to detect the protein fraction.

2.6 Biochemical Analysis of Steviosides
Total soluble carbohydrates were estimated quantitatively by using Anthrone’s method. Green to dark green color was read at 630 nm using spectrophotometer. Total soluble carbohydrate was calculated with the help of a reference curve using D- glucose as standard. The reducing sugar content was estimated as per the method of Lindsay (1973). For the estimation of reducing sugar, DNSA method was used. Samples were cooled and the intensity of dark red color was read at 510 using spectrophotometer. Also series of standards glucose was read and graph was plotted.
2.7 Characterization of isolated Stevioside and related compounds

2.7.1 Thin Layer Chromatography
The qualitative analysis of major groups of Steviosides from methanolic and ethanolic extracts of Stevia rebaudiana was initially done by thin layer chromatography (TLC) technique on preparative silica gel (silica gel-60). Mobile phase or solvent system used for stevioside estimation was Methanol:Chloroform:Water (25:65:4). Bands were visualized using iodine vapour also by observing the plates under UV-transilluminator. Identification was done on the basis of color of bands and their Rf values under UV light.

The isolated and purified compounds which were obtained from column chromatography were subjected to thin layer chromatography using solvent system viz. Ethanol: Methanol : Water (4:4:2). Identification of separated compounds was done by using iodine vapour. In some fractions the bands were not observed. The fractions showing same Rf value were mixed concentrated and refrigerated. Quantitative estimation of stevioside from each obtained fractions was done by spectrophotometric technique.

2.7.2 Nuclear Magnetic Resonance (NMR)
For structural analysis of steviosides, NMR was carried out in Department of Chemistry, University of Pune, Maharashtra, India.

2.7.3 High Performance Liquid Chromatography
HPLC method was performed at Reliable’s Shree Industrial Training Center Jalgaon. All the necessary experimental conditions were provided. The column used for performing HPLC of crude extract of Stevia rebaudiana plant was Lichrosorb C – 18 (25 X 0.5cm 10A). Mobile phase used was Mobile phase of Acetonitrile:10 mmol/l sodium phosphate buffer with pH-2.6 (32:68). 20 µl of the volume was injected with the flow rate of 1ml/min. Detection wavelength was 210nm where as the method was carried out at ambient temperature. Isocratic method was used for obtaining chromatogram of metabolites of Stevia rebaudiana. The Steviosides and related compounds were quantified by comparing the peak area and retention time of standard samples.

2.7.4 Confirmatory test of Stevioside
Stevioside of 1 gm was dissolved in 100ml of distilled water by heating in a water bath. After cooling, it was shaken with100ml of N-butyl alcohol. It was then set aside to separate two phases. The aqueous phase was discarded and N-butyl alcohol layer was used as test solution.
When 10ml of test solution was mixed with 10 ml of anthrone solution and heated in water bath, it turns to green.

2.8 Stability of Stevioside at Elevated Temperatures and at Different pH range
Fifty milligrams of solid stevioside were incubated in a sealed glass vial at different temperatures from 40 - 200°C for 1 h for evaluate stevioside degradation at the specific temperatures according to the method of Chang and Cook (1983).

Aqueous solutions of stevioside 0.5 g/L water were heated in a sealed glass vial at different temperatures of 60 and 80°C for time periods of 1 and 2 h at different pH values of 1 - 10, which were individually adjusted by appropriate buffer systems. Losses in stevioside content were determined followed by Molisch’s test according to the method of Chang and Cook (1983).

2.9 Determination of biological activities of plant by using cancerous and normal cell lines
2.9.1 Cytotoxicity assay (MTT assay)
The cancerous cell lines, Caco-2 and Caski along with normal cord blood stem cell lines were used to analyze the anticancerous activity of water, extracts of Stevia rebaudiana. Small aliquots of 250 µl of culture medium were taken in sterile vials. Then 50 µl of different concentrations of crude extracts (25, 50, 100, 250, 500, 750, 1000 µg/ml of ethanol) were added. Then these vials were incubated at 37°C for 48 hours in CO2 incubator. The vials were centrifuged and supernatant was discarded. Then 600 µl of fresh BSS media and 30 µl of MTT working solution were added. Vials were then incubated for 1 to 4 hours. Small aliquots of 450 µl of solubilization solution (usually dimethyl sulfoxide, an acidified ethanol solution or a solution of detergent sodium dodecyl sulphate in dilute hydrochloric acid) were added. The vials were then mixed properly to dissolve the formed formazan crystals and absorbance was measured at 595 nm on UV- visible spectrophotometer. Finally, the 50% reduction in cell number or IC50 was estimated.

2.9.2 Lactate Dehydrogenase (LDH )Assay
Materials like Phosphate buffer (0.1 mm/lit.) ph-7.4, Sodium pyruvate (21mm/lit prepared freshly in phosphate buffer), NADH2 (3.5 mm/lit) were prepared freshly.
Components | µl
--- | ---
Phosphate buffer | 1666
NADH2 (3.5mmol/lit) | 66
Cell supernatant LDH | 200
Sodium pyruvate | 66

Above mixture was equilibrated at 37°C for 10 min. in water bath. Rapidly 0.1 ml of the sodium pyruvate solution was added at 37°C, and transferred to a cuvette in a thermostatically heated cell housing of an ultraviolet spectrophotometer and the change in extinction at 340 nm per 30 seconds were observed. Enzyme activity for 2 ml reaction mixture used in the assay was carried out by using following formula:

Enzyme activity (µmol/min/ml) = extinction change /min/6.3×2

2.10 Statistical analysis
All experiments were conducted in triplicate and statistical analysis was done by using the MS Excel software (CORREL Statistical function). The data were presented as mean ± SD.

3. RESULTS AND DISCUSSION
3.1 Extraction and purification of Stevioside sweetener from Stevia leaves:
The quantity of crude extracts obtained by this method was 50%. Similar results obtained by Midmore and Rank (2006) who found that boiling water extraction can achieve 93 - 98% extraction of Stevioside. The quantity of extract was sufficient and was frequently used as per required for further use. The water extracted Stevioside at the optimum ratio (1:45) was purified by Ca(OH)2 and ion exchange treatments. Results showed that pigments present in the crude extract affected the purification process to great extent and also affected the purity of the produced Stevioside. Therefore, the removal of carotenoids and chlorophylls was increased as the purification steps preceded.

Extraction was carried out by using methanol and 80% ethanol. Methanol is preferable to extract Stevioside and showed better extraction ability for isolation of Stevioside from Stevia leaves than water extraction. However, the purity of Stevioside was lower than that produced by water extraction. These results are agreement with that reported by Jaroslav et al (2007). The quantity of crude extracts obtained by this method was 40% and 45% by methanol and ethanol respectively. This indicated that methanol and ethanol extraction were much easier and simple than water extraction. However, in respect to safety and it is of economically a great interest to use water for extraction than methanol followed by butanol.
3.2 Quantitative Estimation of Carbohydrates

The concentration of total carbohydrates present in one ml of sample was estimated as per Anthron et al. method and calculated by using a standard graph of glucose.

![Graph of Standard Glucose](image1)

**Figure 1: Estimated carbohydrate concentration by Anthrone et al. method**

The concentration of carbohydrates present in different samples is compared with the help of graph below. The amount of total carbohydrates was found to be 20mg/g.

The reducing sugar content was estimated as per the method of Lindsay (1973). For the estimation of reducing sugar, DNSA method was used. The concentration of total reducing sugar present in one ml of sample was calculated by using a standard graph of glucose and is presented in tabulated form. The amount of total carbohydrates was found to be 0.877 mg/g.

![Graph of Standard Glucose](image2)

**Figure 2: Estimated Reducing Sugar concentration by DNSA method**
3.3 Characterization of isolated Stevioside and related compounds

3.3.1 Thin Layer Chromatography (TLC) of plant methanol and ethanol extract

Thin layer chromatography was performed in order to estimate the qualitative analysis of Steviosides on preparative silica gel plates by using solvent system methanol:chloroform:water (25:65:4) (Shiwhare et al, 2011) which was specific to steviosides. Different bands which were obtained on the plate after iodine vaporization showed the presence of Steviosides. Rf value of purified sample was found to be 0.34 which is in the range of 0.30-0.34 specified in the literature for steviosides. Same method was used for the qualitative analysis of steviosides in ethanolic extract and its Rf value was found to be 0.89 which was matched with Rf value of standard sample (0.88) as given in some standard reference papers.

![TLC of isolated Stevioside in methanolic and ethanolic extracts](image)

Figure 3: TLC of isolated Stevioside in methanolic and ethanolic extracts

3.3.2 Ion-Exchange Chromatography

Water extract was loaded on DEAE column and pH range was maintained at 7.0. Total 30 elutes were collected from the column using 0.5 N NaCl as eluent. All the elutes were subjected to Thin Layer Chromatography on steviosides specific Ethanol: Methanol: Water (4:4:2) solvent system for their further analysis. The elutes which showed the Rf value of 0.8 were mixed with each other because they might be contained same derivatives of Stevioside since that matched with Rf values of standard Stevioside. In this way, three samples were collected. Same type of procedure was followed by Abou-Arab et al (2010). Then, these three samples were used for further experimentation of Stevioside.
After identification by TLC method, the isolated fractions were dried and crystallized by deep freezing. These crystals were used for UV-VIS spectrophotometry and HPLC analysis.

### 3.3.3 Confirmatory test

Confirmatory test for identification and presence of Stevioside in crystalline and crude samples of *Stevia rebaudiana* was carried out. Test solutions were treated with anthrone solution followed by heating in water bath. The solutions turn to green color in each test tube which indicated the presence of Stevioside.

![Figure 5: Identification of Stevioside](image)

### 3.4 Stability of Stevioside at elevated temperatures and at different pH

Incubation of the solid sweetener stevioside at elevated temperatures for 1 h showed good stability up to 120°C while at temperatures exceeding 140°C, forced decomposition was seen which resulted in total decomposition by heating up to 200°C (Figure 6).
In aqueous solution, stevioside is remarkably stable over a wide range of pH values and temperatures. Under thermal treatment in a pH range of 1 -10 over 2 h, practically no degradation of stevioside could be observed at 60°C and only slight loss of up to 5% was observed at pH 2 to 10 on heating to a temperature of 80°C. Under strong acidic conditions i.e. at pH 1.0, forced decomposition of stevioside was observed which resulted in total decomposition after incubation at a temperature of 80°C for 2 h (Figure 3). These results are in accordance with that reported by Buckenhuskers and Omran (1997) who showed that the Steviosides have excellent heat stability which is up to 100°C for 1 h at pH range 3 - 9, but rapid decomposition occurs at pH level greater than 9 under these conditions.

3.5 High Performance Liquid Chromatography (HPLC):
The HPLC of water extract (A), metahanolic extract (B) and ethanolic extract(C) was done. The HPLC analysis of stevioside and related compounds was carried out on reverse
phase C18 column by using group specific isocratic methods. The isolated compounds were identified by comparing their retention times (Rt) with standards. HPLC of alcoholic extracts were performed. Also, HPLC of water extracts of which fraction obtained after column chromatography of leaf which showed maximum absorbance at 210 nm. Mobile phase used was Acetonitrile: 10mmol/l sodium phosphate buffer pH-2.6 (32:68).

The HPLC analysis of water extract had shown the presence of Rebaudioside-D, Stevioside and Rebussoside having the retention time of 3.53, 8.08, 14.68 min respectively. Methanolic leaf extract had shown the presence of Rebaudioside D having retention time of 3.83 min which matches to retention time 3.42 min of standard rebausdie. Ethahnolic extract had shown a type of peak at retention time 3.51 and 3.83 min which was remained unidentified. Along with this, it showed presence of Rebaudioside A and Rebussoside having retention time 7.33 and 13.43 min respectively. The HPLC chromatogram of water, Methanolic and etahnolic extract of Stevia rebaudiana are shown as fig. 7, 8, 9 respectively.

![Figure 8: The HPLC chromatogram of water extract](image)

![Figure 9: The HPLC chromatogram of Methanolic extract](image)
3.6 Nuclear Magnetic Resonance (NMR)
NMR was used for structure determination of Stevioside. NMR was carried out in the Department of Chemistry, University of Pune, Pune. The 1H NMR of isolated Stevioside was recorded using NMR instrument. The signals obtained by NMR are mentioned in following figure.

3.7 Determination of cytotoxicity assay (MTT assay)
Cytotoxicity of water, methanolic and ethanolic extracts of *Stevia rebaudiana* was evaluated on colon and cervical cancer cells using Caco 2 and Caski cell lines. When the cells were treated for 48 hrs with various concentrations of extract, ethanolic and methanolic extracts
were shown highest activity as compared to water extracts. Results were obtained in dose dependent manner i.e. viability of 25µg/ml < % viability of 50µg/ml < % viability of 100µg/ml < % viability of 250µg/ml < 500% viability of 750 µg/ml < % viability of 1000 µg/ml. The results indicated that the leaves extract of Stevia appeared to be toxic towards cancer cells in a dose-dependent manner.

Figure 12: Cytotoxicity of methanol and Etahanol extract on Caco-2, Caski and Normal cell line
Thus, Stevioside showed anticancer activity against Caco 2 and Caski cell lines. The methanol and ethanol extract of Stevioside showed anticancer activity against Caco cell line with IC50 value 10 and 12µg/ml respectively whereas these extract had shown the cytotoxicity against 2 and Caski cell line with IC50 value of 20 and 5µg/ml respectively. Similar results were obtained by Mothanna et al. (2011) on HepG2 cell line originated from hepatoblastoma after the treatment of goniothalamine.

3.8 Lactate Dehydrogenase (LDH) Assay
Measurement of LDH activity is another indicator of cell viability through evaluation of the cell membrane permeability. The enzyme activity is measured externally as it leaks from dead cells which lose their membrane integrity. LDH leakage detection based on the loss of NADH due to its oxidation to NAD+ resulting in conversion of pyruvate to lactate.

As the concentration of Stevioside sample increased, it broken up the cell and LDH was released. The LDH activity of methanol extract was found to be greater than ethanol extract (fig.13) of steviosides.

![Figure 13: LDH activity of methanol and ethanol extract](image_url)

In this assay, the result showed that the untreated cells retained LDH in their cells and have minimal loss over the time of assay and so, they were having minimum LDH activity whereas stevioside treated cells showed the maximum loss of LDH with maximum LDH activity. Similar results were obtained by Mothanna et al. (2011) on HepG2 cell line originated from hepatoblastoma after the treatment of goniothalamine.
4. CONCLUSION
Stevia plants are a good source of carbohydrates and reducing sugar which are vital for human nutrition and maintains a good health. Stability of extracted Stevioside at different temperatures and pH value, indicated that this sweetener could be applied as substitute of sucrose in different drinks, beverages and bakery products.

Stevioside isolated from leaves of *Stevia rebaudiana* Bertoni and further structure was established on the basis of chromatographic and spectroscopic evidence. Isolated stevioside showed similar results of chromatographic and spectroscopic to those of standard stevioside. The data obtained from the TLC, UV, H1NMR and HPLC confirmed that the isolated compound was Stevioside.

As Stevioside showed anticancer activity against Caco-2 and Caski cell lines, the production of remarkably high levels of one class of secondary metabolite is become significant interest to chemists, biochemists and geneticists and may prove to be a foundation for the production of new metabolites in the future. Because the safety of stevia for human consumption remains controversial, there is a clear need for further experimentation with respect to the metabolic fate of steviol glycosides. Hence, it might help in preventing diabetic complications and may serve as a good alternative in the present armamentarium of antidiabetic drugs.

Thin layer chromatography of isolated compound from column chromatography was compared with the standard Stevioside showed same Rf value.

5. REFERENCES
5. Dyrskog SE, Jeppensen PB, Colombo M, Abudula R, Hermansen K. Preventive effects of


