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Preliminary Phytochemical Screening and Evaluation of Free Radical Scavenging Activity of *Stevia rebaudiana* Bertoni From Different Geographical Sources

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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 preliminary phytochemical screening and comparative evaluation of *in vitro* antioxidant activity 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. Total phenolic and total flavonoid content was also determined using Folin-Ciocalteu reagent method and aluminum chloride colorimetric. The result showed that the variety from Kangra showed the highest phenolic and flavonoid content of 5.87 and 62.22 mg GAE/L respectively.

Keyword: *Stevia rebaudiana*, Non-Caloric Substitute, Ent-Kaurene Glycosides, Comparative Standardization.

1. Introduction

Oxidation reactions occur when life essential oxygen combusts within the human body and produces by-products referred to as oxygen free radicals which steal electrons from other molecules, like proteins, lipids and DNA, causing damage. In case of DNA, the problem intensifies and genetic cell mutations may occur which may become a common cause of cancer. Uninhibited over time, free radical damage builds in the body, thus causing aging. An overload of free radicals has been linked to certain diseases, including heart disease, liver disease and some cancers.

Stevia rebaudiana, (*S. rebaudiana*) a non-caloric substitute to conventional sucrose, distinctly possesses good antioxidant activities [1-3] and thus has the ability to boost the immune system and prevent free radical mediated diseases. It contains micronutrients like selenium, zinc, manganese which play an important role as antioxidants [4]. It is a small perennial shrub growing up to 1m tall and with leaves 2-3cm long [5] and native to regions of Paraguay and Brazil. It is popular as the "sweet herb of Paraguay" [6].

The current research involves the phytochemical screening and antioxidant activities of *S. rebaudiana* procured from five different geographical locations of India viz., Delhi, Surat, Kangra, Bangalore and Indore, to find out which variety contains the highest content of rebaudioside A, the bioactive glycoside, as well as to conclude which variety possesses the most potent antioxidant activity amongst all the varieties.

2. Materials and Methods

2.1 Collection of Plant Material

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.

2.2 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 [7] [8]. The different chemical tests included the tests for alkaloids, saponins, carbohydrates, glycosides (general), anthraquinone glycosides, cardiac glycosides, coumarin glycosides, cyanogenetic glycosides, tannins, proteins, steroids, waxes, flavonoids, amino acids and acidic compounds and the results were taken.

Table 1: Preliminary phytochemical screening of the methanolic extracts of the different varieties of *Stevia rebaudiana*

Stevia rebaudiana					
Test	**Delhi**	**Kangra**	**Surat**	**Indore**	**Bangalore**
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

2.3 Chemicals

1-Diphenyl-2-picryl hydrazyl (DPPH) were obtained from Sigma Aldrich Co., St. Louis, USA. Folin Ciocalteu's reagents and rutin were purchased from SD Fine chemicals, India. Naphthyl ethylene diamine dihydrochloride (NEDD) was obtained from Roch-Light Ltd., Suffolk, UK. *p*-nitroso dimethyl aniline (*p*-NDA) were obtained from Across Organics, New Jersey, USA. All chemicals used were of analytical grade.

2.4 Equipment

UV/VIS spectrophotometer (Beckman DU 640, USA)

2.5 Preparation of Plant Extracts

1 gm of coarsely powdered air dried leaves from each sample were accurately weighed and allowed to leave for cold maceration in 25 ml of methanol for 24 hours.

2.6 Preparation of Test and Standard Solutions

The extracts and the standard antioxidants, ascorbic acid and rutin, were dissolved in distilled dimethyl sulphoxide (DMSO) separately and used for *in-vitro* antioxidant study. The stock solutions were serially diluted with DMSO to get required dilutions

2.7 Determination of Total Phenolic Content by Folin-Ciocalteu Reagent

Total phenol estimation was carried out with folin-ciocalteu reagent (FCR) method[9]. 5 mg of the sample was weighed and dissolved in 1 ml of 50% methanol using a vortex mixer (Touch Type) followed by adding to it 4 ml of 50% methanol and finally mixing through sonication to prepare a sample of concentration 1 mg/ml. 0.5 ml of this solution was pipetted out in a test tube to which was added 3.5 ml of distilled water followed by addition of 0.25 ml of Folin-Ciocalteu reagent (FCR). This was left for incubation for 1-8 minutes at room temperature. Lastly, to this was added 0.75 ml of 20% sodium carbonate solution and the final sample solution in the test tube was left to incubate for 2 hours. The sample was prepared in duplicate. Finally, the absorbance was measured at 765 nm against a reagent blank. The procedure was repeated for all the five varieties of *Stevia rebaudiana*. The standard curve was obtained using gallic acid monohydrate as shown in Fig 1. The total phenol content was expressed as gallic acid equivalent to % w/w of the extracts [10].

2.8 Determination of Total Flavonoid Content by Aluminium Chloride Colorimetric Method

The total flavonol content of the extracts was determined by aluminium chloride colorimetric method[11]. 10 mg of the sample was weighed and dissolved in 1 ml of 80% ethanol using a vortex mixer (Touch Type) followed by adding to it 9 ml of 80% ethanol and finally mixing through sonication to prepare a sample of concentration 1 mg/ml. 0.5 ml of this solution was pipetted out in a test tube to which was added 1.5 ml of methanol, 0.1 ml of 10% aluminium chloride, 0.1 ml of 1M potassium acetate aqueous solution and 2.8 ml of distilled water. A yellow color indicated the presence of flavonoids. The final sample solution in the test tube was left to incubate for 30 minutes at room temperature. The sample was prepared in duplicate. Finally, the absorbance was measured at 415 nm against a reagent blank. The procedure was repeated for all the five varieties of *S. rebaudiana*. The standard curve was obtained using quercetin using solution in the range of 1-10 µg/ml as shown in fig. 2. The results were expressed as mg quercetin/g dry weight by comparison with quercetin standard curve, which was made under the same conditions.

Table 2. Results of the total phenolic content of different varieties of *S. rebaudiana*

S. rebaudiana	**Total phenol (mg GAE/L)**	**Average (mg GAE/L)**
Delhi	3.80	3.93
	4.06	
Surat	3.53	3.64
	3.76	
Bangalore	5.38	5.66
	5.94	
Kangra	5.90	5.87
	5.84	
Indore	3.62	3.72
	3.82	

Table 3. Results of the total flavonoid content of different varieties of *S.rebaudiana*

S. rebaudiana	**Total flavonoid (mg QE/L)**	**Average (mg QE/L)**
Delhi	54.16	54.21
	54.27	
Surat	43.45	42.90
	42.35	
Bangalore	52.83	52.87
	52.91	
Kangra	61.72	62.22
	62.72	
Indore	41.21	39.86
	38.51	

2.9 Determination of Antioxidant Activity by DPPH Radical Scavenging Assay

The antioxidant activity of the different methanolic extracts was evaluated using the method of Schmeda-Hirschmann, 1999 [12]. 10 mg of the sample was weighed and dissolved in 1 ml of methanol using a vortex mixer (Touch Type) followed by adding to it 19 ml of methanol and finally mixing through sonication to prepare a sample of concentration 0.5 mg/ml. DPPH solution was prepared by dissolving 4 mg of DPPH in 100 ml of methanol. Various dilutions of the sample were prepared with methanol resulting in concentrations 5 µg/ml, 10 µg/ml, 20 µg/ml, 30 µg/ml, 40 µg/ml, 50 µg/ml, 100 µg/ml and 150 µg/ml. To each dilution was added 2 ml of the prepared DPPH solution in methanol. A control was also prepared simultaneously consisting of 2 ml methanol and 2ml DPPH solution. The prepared dilutions were then left for colour development in the dark for 20 minutes. Finally, the absorbance was measured at 517 nm against a reagent blank. The procedure was repeated for all the five varieties of S*tevia rebaudiana*. The standard curve was obtained using ascorbic acid. A plot of concentration vs. the percentage inhibition of DPPH radical gave the IC_{50} value which is the concentration of sample required to inhibit 50% of DPPH radical.

3. Results and Discussion

The result of the 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 and coumarin), saponins, carbohydrates, flavonoids, tannins, amino acids, steroids, waxes supporting the reason for its wide range of biological activities as showed in Table 1.

Table 4. Results of *In Vitro* antioxidant activity by DPPH free radical scavenging assay of different varieties of *S. rebaudiana*

S. rebaudiana	**IC_{50} (µg/ml)**
Delhi	68
Surat	63
Bangalore	56
Kangra	54
Indore	60

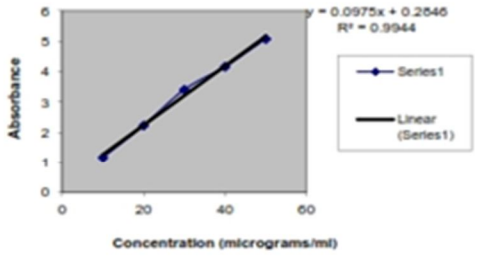

Fig 1. Standard curve for total phenol estimation with Gallic acid

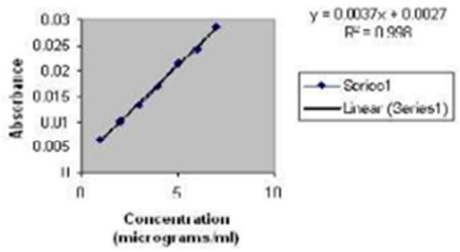

Fig 2. Standard curve for total flavonoid estimation with Quercetin

The current study also aimed at finding out the the variety of *S. rebaudiana* with the highest content of rebaudioside A among the five varieties procured from five different geographical locations of India viz., Delhi, Surat, Kangra, Bangalore and Indore. It also focused on finding out the best variety with highest antioxidant potential which can fight against various oxidative stresses in the human body and hence included a comparative evaluation of antioxidant activity of these five varieties. The climatic conditions like temperature, rainfall, humidity as well as soil conditions, altitude etc. that vary from region to region as well as the time of collection may be responsible for such a variation in the glycosidal content of rebaudioside. Folin-Ciocalteu reagent method was used to evaluate the total phenolic content, Total flavonoid content was also determined through aluminium chloride colorimetric assay and the variety from Kangra showed the highest phenolic and flavonoid content of 5.87 and 62.22 mg GAE/L respectively (Table 2-3). A. DPPH free radical scavenging assay was used to evaluate the antioxidant activity of the different varieties and the variety from Kangra showed the maximum potency of activity with an IC_{50} of 54 µg/ml (Table 4) and hence, this can serve as a source for curbing various health problems related to oxidative damage. As these compounds contribute to the antioxidant efficacy of natural products hence this observation can be directly correlated to the leaves from Kangra showing the most potent antioxidant activity.

4. Conclusion

Hence, the current research enables to find out which variety is the best with the highest content of the bioactive glycoside, rebaudioside A, responsible for various pharmacological activities of *S. rebaudiana*. Further, it also helps to find out the variety having the most potent antioxidant activity by a comparative evaluation of their antioxidant activities which was estimated with the help of DPPH free radical scavenging assay, according to which the variety from Kangra was found to possess the highest antioxidant activity which can be used to curb various health problems related to oxidative stress. Furthermore, the total phenolic content (Folin Ciocalteu reagent method) and the total flavonoid content (aluminium chloride colorimetric assay) which was found to be the maximum in the variety from Kangra also gave an idea of correlating the antioxidant activity to the flavonoid and phenolic content.

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