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IN-VITRO ANTIOXIDATIVE AND ANTIBACTERIAL ACTIVITIES OF VARIOUS PARTS OF *STEVIA REBAUDIANA* (BERTONI)

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ABSTRACT

Antioxidant activity of methanolic extracts from root, leaf, stem and flower of *Stevia rebaudiana* (Bertoni) was investigated. The high amounts of Phenols, Flavonoids and Tannins were found in methanolic root and leaf extract among all parts, at the value of 16.75±0.35, 1.68±1.38, 12.56±5.78 and 11.04±3.16, 2.73±1.44, 12.98±2.17mg/g respectively. Total antioxidant activity of root, stem, leaves and flower was assessed by 2,2'-azino-bis-3-ethylbenzo-thiazoline-6-sulfonic acid (ABTS) radical scavenging activity assay as well as 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay. Root extract showed highest (64.23±8.35 mM) TEAC 2,2'-azino-bis-3-ethylbenzo-thiazoline-6-sulfonic acid (ABTS) radical scavenging activity; and Leaf, stem, flower showed 56.26±16.87 mM, 49.28±12.87 mM and 46.49±13.13 mM respectively. SOD, Catalase and peroxidase enzymatic assays were carried out to determine antioxidative potential of extracts, and the root extract was found to have highest activities 4.84±0.22, 8.6±0.45 and 2.24±0.05 respectively. Zones of inhibition at 500 mg ml⁻¹ concentration are indicative of effective antibacterial activity of *S.rebaudiana* against *B.subtilis* NCIM 2708 and *E.coli* DM 4100. These results reveal that *S. rebaudiana* is an excellent antioxidant, and especially root and leaf show higher antioxidant effect and antibacterial activity.

Keywords: *Stevia rebaudiana* (Bertoni), Phenols, DPPH radical scavenging, ABTS radical scavenging, *E. coli* and *Bacillus subtilis*.

INTRODUCTION

Since the beginning of the 20th century, free radicals were considered as an intermediate compound in the organic and inorganic chemistry. Daniel Gilbert and Rebecca Gersham in the year 1954 were first to suggest free radicals to be an important part of the biological system. Reactive oxygen species (ROS) are a class of highly reactive molecules derived from the metabolism of oxygen. These species react with free radicals to become radical themselves. The various forms of these activated oxygen, include free radicals such as superoxide anion radicals (O₂⁻) and hydroxyl radicals (OH[•]), as well as non-free radical species (H₂O₂) and the singlet oxygen (¹O₂)^{1,2}. ROS exert oxidative stress, rendering each cell to face about 10000 oxidative hits per second³. When ROS production overhaul the endogenous antioxidative defence system, then these radicals start damaging the cell bio molecules such as lipids, proteins and carbohydrates^{4,5,6} ultimately leading to various physiological disorders, including diabetes, cancer, Alzheimer's and aging⁷.

Antioxidants delay or inhibit the oxidation of lipids or other molecules by inhibiting the initiation or propagation of scavenging oxidative chain reactions. Polyphenols, such as flavonoids⁸, phenolic acids, and phenolic diterpenes⁹ exhibit antioxidative effect. The phenolic groups play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen or decomposing peroxides¹⁰. Antioxidants principally from natural resources are versatile in their multitude and magnitude of activity and provide enormous scope in correcting imbalance¹¹. Recently interest has been increased considerably in finding natural occurring antioxidants for use in foods or medicinal materials to replace synthetic antioxidants which are being restricted due to their side effects such as carcinogenicity¹².

The genus *Stevia* belongs to Asteraceae family, tribe Eupatorieae and comprises of 240 species, growing mostly at the altitude of 500-3000 m in semidry mountainous terrain. Different species of *Stevia* contain several potential sweetening compounds, with *Stevia rebaudiana* (Bertoni) being the sweetest of all^{13,14}. It is commonly known as *Stevia*, Sweet leaf, Sweet herb of Paraguay, Honey leaf and Candy leaf. *S. rebaudiana* contains in its leaves all of the eight entkaurene glycosides¹⁴, with stevioside being the major constituent i.e., 3-8% by weight of the dried leaves¹⁵. Other constituents are steviolbioside (trace), the rebaudiosides A (2-4%), B (trace), C (1-2%), D (trace), E (trace) and dulcoside A (0.4-0.7%)^{16,17,18}. In addition to its sweetening properties, it has therapeutic values such

as antihyperglycemic, anticancerous,^{19, 20} and also act as antihypersensitive agent^{21,19}. Along with these, this plant also has contraceptive properties²². It helps in the prevention of dental cavities²³. It can also inhibit bacterial and fungal growth^{24,25}. Moreover, it has been reported that *S. rebaudiana* shows ability to maintain blood glucose level with glucose tolerance enhancement in diabetic patients²⁶. Therefore, it is attractive as a natural sweetener to diabetics and others on carbohydrate-controlled diet²⁷.

Thus the present study has been directed to investigate the antioxidant activity of different parts of plant *Stevia rebaudiana* (Bertoni) in various *in vitro* models.

MATERIAL AND METHODS

Materials

All reagents used were of analytical grade. Bacterial strain of *Escherichia coli* DM 4100 and *Bacillus subtilis* NCIM 2708 were bought from MTCC, Chandigarh, India.

Plant Collection and extract preparation

Taxonomically identified *Stevia rebaudiana* (Bertoni) plants were collected from Meerut. Plant parts viz., stems, roots, leaves and flowers were shade dried, grounded and pass through sieve no. 80. The uniform particle sized powder was packed in thimble and extracted in soxhlet apparatus with methanol till the solution in the thimble becomes clear. The extract so obtained was then filtered through Whatman filter paper no.40 and concentrated to dryness under reduced pressure at 70-80° C in rotary vacuum evaporator. The extract was then dried in hot air oven at 30-40° C till solid to semisolid mass was obtained. It was then stored in refrigerator below 10° C for further use.

Determination of phytoconstituents

Phytoconstituents in herbal medicine are important to manage oxidative stress resulted due to certain pathological conditions. Therefore, it is necessary to estimate amount of the major class of constituents present in the crude extract.

Total phenols²⁸

1ml of the sample was collected from the supernatant of the extract; it was dried in the petriplate. The dried residue was then dissolved in 1ml of distilled water. 100 µl of the dissolved residue was then again dissolved in distilled water to make up 3 ml volume. To this

0.5 ml of folin-ciocalteau reagent was added. Then after 2 min, 20% of Na₂CO₃ was added and mixed thoroughly. The tubes were kept in boiling water for about 1 min. Then, were cooled in running tap water and the absorbance of the blue coloured complex was recorded against blank at 765nm. The total phenol content was calculated and expressed in mg Gallic Acid Equivalent, GAE using a standard curve of Gallic acid.

Flavonoids ²⁹

To 100 µl of sample 0.1 ml of aluminium chloride (10%), 0.1 ml of potassium acetate (1M) and 2.7 ml of distilled water was added. The reaction mixture was then kept at room temperature for 30 min. The absorbance was measured at 414 nm. The calibration curve was prepared using different concentrations of quercetin which is expressed in mg/gm dry weight.

Tannins ²⁸

7.5 ml of distilled water was added to 100 µl of sample. After that 0.5 ml of Folin Denis Reagent (FDR), followed by 1 ml of 35 % Na₂CO₃ were added. The final volume was made up to 10 ml with distilled water. The blue colour produced was measured spectrophotometrically at 700 nm. The calibration curve was prepared using Tannic acid, and expressed in mg/gm dry weight.

Enzymatic Assay

Superoxide dismutase Activity ³⁰

This method relies on the reduction of Nitroblue tetrazolium (NBT). 1 ml of sodium carbonate, 0.4 ml NBT, 0.2 ml EDTA were added to 0.5 ml plant extract. The reaction was initiated with the addition of 0.4ml of Hydroxylamine hydrochloride. Zero absorbance was taken at 560 nm using spectrophotometer followed by recording the absorbance after 5 minutes at 25°C. Units of SOD were expressed as amount of enzyme required for inhibiting the reduction of NBT by 50%. The specific activity was expressed in terms of units per milligram of protein.

Catalase ³¹

To 1ml of plant extract, 5ml of 300 µM phosphate buffer (pH 6.8) containing 100 µM Hydrogen peroxide was added and left at 25°C for 1 minute. 10 ml of 2% sulphuric acid was added to arrest the reaction, and the residual H₂O₂ was titrated with potassium permanganate (0.01 N) till pink colour was obtained. Units of enzyme activity were expressed as ml of 0.01N potassium permanganate equivalent of hydrogen peroxide decomposition per min per mg of protein.

Peroxidase activity ³²

3.5 ml of phosphate buffer, pH 6.5, was taken in a clean dry cuvette, 0.2ml of plant extract and 0.1 ml of freshly prepared 10 µM ianisdine solution was then added to it. The temperature of assay was brought to 28-30° C, and absorbance was recorded at 430 nm. Then 0.2ml of 0.2M hydrogen peroxide was added and mixed. Initial absorbance was recorded and then, at every 30 sec intervals up to 3min. A graph was plotted with increase in absorbance against time. The enzyme activity was expressed per unit time per mg of protein.

Antioxidant ability assay

Determination of Total Antioxidant Capacity ³³

This assay is based on the reduction of Mo(VI) to Mo(V) by the extracts with the subsequent formation of a green phosphate complex/ Mo (V) at acidic pH. An aliquot of 0.3ml of each extract was combined with 3 ml of reagent mixture (0.6 M sulphuric acid, 28mM sodium phosphate and 4mM ammonium molybdate) in appendroff tube, and incubated at 95° C for 90 minutes. The absorbance of the extracts was measured at 695nm after cooling them to room temperature.

The antioxidant activity is expressed as the number of gram equivalent of ascorbic acid.

ABTS Free radical Scavenging Assay ³⁴

Total antioxidant status of the extracts was measured using 2,2'-azinobis[3ethylbenzthiazoline]-6-sulfonic acid (ABTS) assay. The

assay relies on the antioxidant ability of the samples to inhibit the oxidation of ABTS to ABTS radical cation. ABTS radical cations were produced by the reaction of aqueous ABTS (7mM) with 2.45 mM potassium persulfate. The reaction mixture was left to stand at room temperature overnight (12-16 hrs) in dark before use. The resultant intensely coloured ABTS radical cation was diluted with 0.01 M, pH 7.4 PBS (Phosphate buffered saline) to give absorbance of ~0.70 at 734 nm.

The plant extracts were diluted 100 times with the ABTS solution to a total volume of 1 ml. The absorbance was measured at time interval of 1 min after addition of each extract. The assay was performed in triplicate. The total antioxidant activities were expressed as mM trolox equivalent antioxidant capacity (TEAC).

Free radical Diphenylpicrylhydrazyl (DPPH) Antioxidant Activity ³⁵

DPPH is a stable nitrogen centred free radical, upon reduction either by hydrogen or electron donation, its colour changes from violet to yellow. The anti-oxidant potential of the extract is based on the determination of the concentration of 2, 2-diphenyl-1-picryl hydrazyl (DPPH) at steady state in a methanol solution after adding the plant extracts. DPPH absorbs at 515nm, and its concentration is reduced by the existence of antioxidants. The absorption gradually disappears with time, and hence the quantity of the mixture of antioxidants needed to reduce the initial DPPH concentration was evaluated.

The DPPH radical- scavenging activity in terms of percentage was calculated according to the following equation³⁶ (Lo and Chung, 2005):

$$\text{DPPH scavenging activity (\%)} = \{1 - (\text{Abs}_{515} \text{ sample} / \text{Abs}_{515} \text{ DPPH solution})\} \times 100 \%$$

Anti-bacterial Activity ³⁷

The antibacterial activity of plant extracts was tested against two bacterial strains i.e., *Bacillus subtilis* NCIM 2708 and *E. coli* DM 4100. Bacterial isolates were maintained in 15 % (v/v) glycerol at -20° C then cultured and sub cultured in a modified Luria Bertani broth at 37° C until the desired cell population was obtained. Bacterial strains were nourished in nutrient broth (Difco) and incubated for 24 h. According to the agar diffusion method bacteria were incubated on Mueller-Hinton Agar. The wells were dug in the media with the help of a sterile steel borer. 100-500 mg ml⁻¹ of sample concentrations were prepared by using dilution method. Then 100 µl of each sample was introduced in corresponding well and ampicillin was used as a standard. The resulting zones of inhibition were measured in millimetres.

Statistical analysis

The data are expressed as Mean ± Standard Deviation (S.D.) from triplicate determination. Linear regression analysis was used to calculate IC₅₀ values whenever needed. Data were analyzed by one-way analysis of variance (ANOVA) comparison test with equal sample size. The difference was considered significant when *p* value < 0.05.

RESULTS & DISCUSSION

Free radicals and reactive oxygen species are well known inducers of cellular and tissue pathogenesis leading to various diseases like diabetes, cancer etc³⁸. Many constituents of plants have antioxidant activity and serve as a protective agent against the damage caused by such diseases ³⁹. In the present study, phenolic content in the methanolic root extract of *S. rebaudiana* was found to be higher than that of other parts (Table 1). The order of phenolic concentration in different extracts of *S. rebaudiana* was root>leaves>stem>flower. Phenolic compounds have been reported with multiple biological effects including antioxidant activity⁴⁰. Anti-oxidative property of these polyphenols is due to their high reactivity as hydrogen or electron donor. Polyphenols have the ability to stabilize and delocalize the unpaired electron, and have the potential to chelate metal ions ⁴¹. Flavonoids and Tannins are also known to possess antioxidant activity. In Flavonoids, this is due to certain mechanisms such as free radical

scavenging, chelation of metal ions viz., iron and copper, and inhibition of enzymes responsible for free radical generation. Depending upon the structure, flavonoids are able to scavenge practically all known

reactive oxygen species^{42,43}. The pattern of amount of tannins and flavonoids observed in plant extracts was Leaf>root>stem>flower and leaf>stem>root>flower respectively.

Table 1: Total phenolic compounds, flavonoids and Tannins of methanolic extracts of various parts of *Stevia rebaudiana* (Bertoni)

Extracts	Phenols (mg GAE)	Tannins (mg TAE)	Flavonoids (% Quercetin)
Root	16.75±0.35	12.56±5.78	1.68±1.38
Stem	6.5±2.31	7.21±2.95	2.53±2.75
Leaf	11.04±3.16	12.98±2.17	2.73±1.44
Flower	5.93±5.52	2.43±0.66	0.73±0.38

Values are mean±SD (n=3); Values are significantly different at p<0.05.

Biological system counterpoised the inevitable generation of ROS by selection of enzymatic defence system. These enzymatic antioxidants SOD, catalase, peroxidase, plays an important role in protecting cell wall against the lipid peroxidation and other cellular damage. SOD protects cell wall by catalyzing superoxide ions into H₂O and H₂O₂. Catalase works at high concentration of H₂O₂, and it

readily detoxify it into H₂O and O₂. Peroxidases catalyze the degradation by oxidizing glutathione with the formation of its conjugates⁴⁴. The levels of enzymatic antioxidants are collectively compiled in Table 2. The highest activity of SOD, catalase and peroxidase was observed in root, depicting its higher antioxidative potential⁴⁵.

Table 2: Enzymatic antioxidant levels of different parts of *Stevia rebaudiana* (Bertoni)

Plant parts	SOD (μ/mg)	Catalase (μ/mg)	Peroxidase (μ/mg)
Root	4.84±0.22	8.6±0.45	2.24±0.05
Stem	3.35±0.17	3.13±0.35	1.22±0.26
Leaf	1.98±0.16	7.3±0.36	1.68±0.061
Flower	2.23±0.20	1.06±0.15	0.46±0.044

Values are mean±SD (n=3); Values are significantly different at p<0.05.

Plant contains different antioxidants, and it is difficult to measure each antioxidant separately. Therefore several methods have been developed to measure the antioxidant activity. ABTS, DPPH and Total scavenging ability assays were used to measure scavenging activity of different extracts of plant parts. It was observed that there was a difference in antioxidant activity of different extracts of plant *S.rebaudiana*. Percentage of radical scavenging activity is

depicted in Fig 1, Root and leaf extracts of *S.rebaudiana* at a concentration of 800 μg / ml DPPH, exhibited equally high radical scavenging activity of 82.36 % and the least of 47.05 % by the flower extract. Steady state absorbance value for pure Ascorbic acid and BHA (reference sample) was 0.19 and 0.25, exhibiting the highest antioxidant activity followed by high pressure extracted *Stevia*.

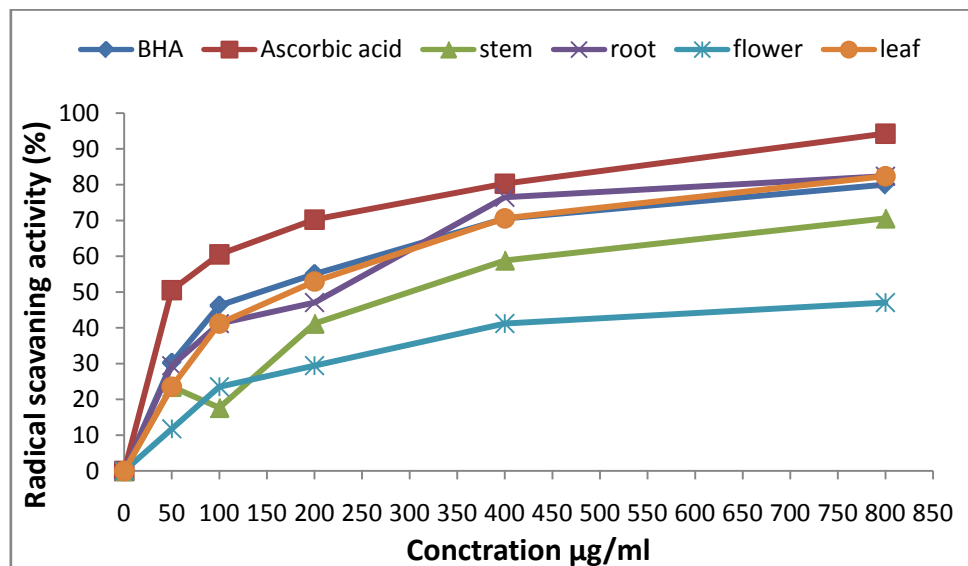


Fig. 1: DPPH radical scavenging activity of methanolic extracts of different parts of *Stevia rebaudiana*

ABTS radical scavenging (Figure2) and Total antioxidant ability assay (Table 3) showed the similar trend of antioxidative potential in methanolic extracts of *S.rebaudiana* i.e., Root>leaf>stem>flower. Higher phenol content has direct correlation with high radical scavenging activity⁴⁶. Thus, it can be concluded that root extract

having greater phenolic content, have higher enzymatic antioxidants and thus highest antioxidant activity. Roots of some plants like *Aporosa lindleyana* Baill⁴⁷, *Lannea velutina* A.⁴⁸ and *Smilax China*⁴⁹ also possess high phenol content with higher antioxidant potential.

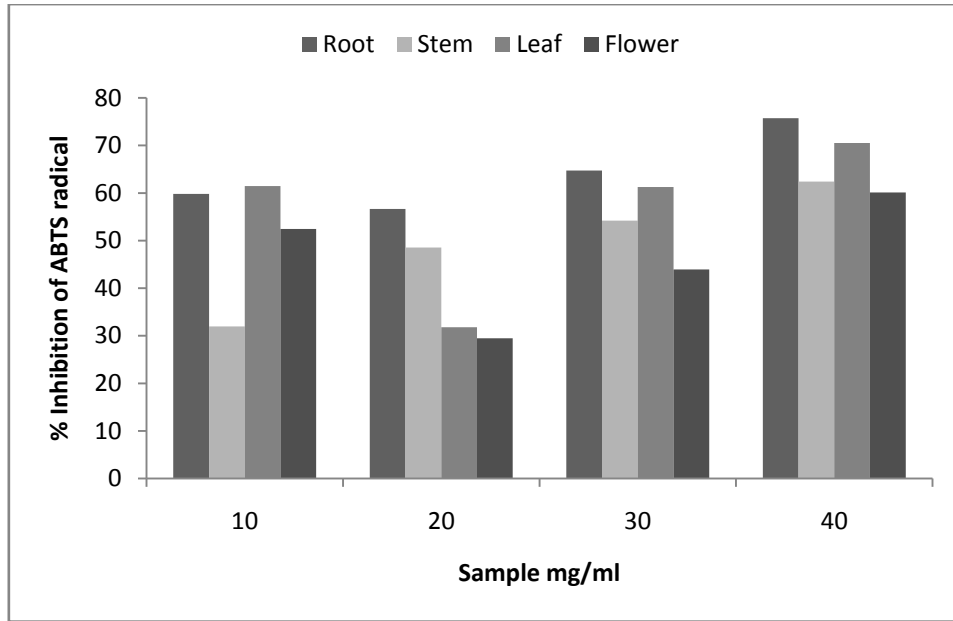


Fig 2: ABTS radical scavenging ability of Methanolic extracts of different parts of *Stevia rebaudiana*

Table 3: Total Antioxidant ability of different parts of plant *Stevia rebaudiana* (Bertoni)

Extracts	Total antioxidant ability (gram equivalent of ascorbic acid)
Root	97.18 ± 1.04
Stem	72.63±0.92
Leaf	82.49 ± 3.07
Flower	53.10±1.86

Values are mean±SD (n=3); Values are significantly different at p<0.05.

Antibacterial activity of the prepared formulation was investigated against the standard gram positive strain *Bacillus subtilis* NCIM 2708, and gram negative *E. coli*. DM 4100. Clear inhibition zones revealed that, the compounds showed the antibacterial activity against these tested strains. Maximum antibacterial activity was observed in root, and the flower extract shows the minimum, judging from the zones of inhibition produced against the isolates.

Inhibitory zone was produced for *Bacillus subtilis* and *E. coli* at 100-500 mg/ml concentrations. Root extract of *S. rebaudiana* showed maximum zone of inhibition for *Bacillus subtilis* at 26 mm and *E. coli* 16 mm at 500 mg ml⁻¹ concentration. Results shown in Figure 3 signify that *S. rebaudiana* has effective antibacterial activity. Many medicinal plant extracts with effective antibacterial activity have been reported by various authors.^{50,51,52,53,54}

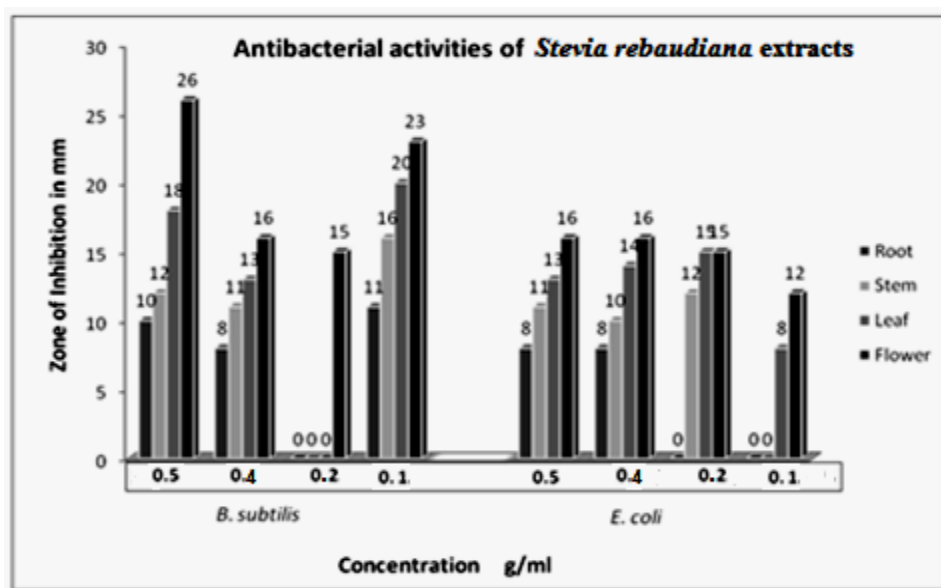


Fig. 3: Antibacterial activity of different parts of *Stevia rebaudiana* at different concentrations on bacterial strains: *Bacillus subtilis* and *E.coli*

Thus, it can be concluded that methanol extracts of *S. rebaudiana* can be used as an accessible source of natural antioxidants and antimicrobial drugs with consequent health benefits. Therefore, these plants extracts need to confirm for their *in vivo* activity and bioavailability, so that the good advantageous effects of the plant extracts may indeed be used in the management of diseases caused by these organisms, and thus supporting its ethno medicinal uses.

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