Research Article

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# STUDIES ON BIOCHEMICAL AND MEDICINAL PROPERTIES OF *STEVIA REBAUDIANA* GROWN *IN VITRO*

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**ABSTRACT**
*Stevia rebaudiana* commonly called candy leaf belongs to the family of Asteraceae. In this preliminary research, we describe *in vitro* grown stevia plants in the basal MS medium for the quantification and identification of steviol glycosides by Agilent 1260 High Performance Liquid Chromatography (HPLC). The *in vitro* plants showed the presence of glycosides, stevioside and rebaudioside A and its percentage by mass were 6.79 % and 3.91 % in 0.01 g of leaf powder. These *in vitro* plants can be used as a raw material for the production of glycosides in high yield which is of medicinal importance. The different *in vitro* stevia leaf extracts prepared by using different solvents have shown better antioxidant and antimicrobial activity. For the antimicrobial activity, the ethanolic extract showed zone of inhibition with diameter of 3 to 7 mm on *B. subtilis* and maximum 1 mm on *S. aureus* and for the methanolic extract it was 3 to 4 mm for *B. subtilis* ATCC 6633 and maximum 1 mm for *S. aureus*. The $IC_{50}$ value using DPPH assay method for studying antioxidant property of aqueous, ethanolic and methanolic stevia leaf extracts were found to be 68%, 77.77% and 71.17% respectively. The results obtained in the present study indicate that *in vitro* plants grown only in basal medium possess some similar properties like *in vivo* plants.
**Keywords:** Steviol glycosides, Antioxidant, Antimicrobial, DPPH assay, $IC_{50}$.

## INTRODUCTION

Medical plants constitute an effective source of both traditional and modern medicines[1]. *Stevia rebaudiana* (Bertoni), often referred to as ''the sweet herb of Paraguay'', is a perennial shrub belonging to Asteraceae (Compositae) family. It is native to certain regions of South America like Paraguay and Brazil. The major sweet component present in the leaves of *Stevia rebaudiana* (Bertoni), Stevioside, tastes about 300 times sweeter than sucrose (0.4 % solution). The leaves are found to contain a complex mixture of eight sweet diterpene glycosides, including stevioside, steviolbioside, rebaudioside (A, B, C, D, and E) and dulcoside A[2]. The whole extracted leaves of the *Stevia rebaudiana* plant claims have been made for health benefits, both internally and externally. It is not only the natural sweetener but also cholesterol free. It plays vital role in total sugar demand in India, around 70 % of the sugar is reported to be used for industrial purposes. On the other hand, the steviol given the hope to the diabetic patients who craves for the sweets where it regulate the blood glucose level by enhancing the insulin secretion and also insulin utilization in insulin deficient animal[3]. The search for new antimicrobial compounds with different mechanisms of action is carried out aggressively nowadays due to the bloom of re-emerging infectious diseases and the incidence of antibiotics-resistance in clinical use. Antioxidant-based drug formulations can be used for the prevention and treatment of complex diseases like atherosclerosis, stroke, diabetes, Alzheimer's disease and cancer. Plant extracts become a part of major concerns in the discovery of new antimicrobial compounds. This preliminary research mainly focuses on *in vitro* plants which are grown in basal MS medium without any plant growth regulators and contributes for biochemical and medicinal properties. The quantification and identification of steviol glycosides were done using Agilent 1260 High Performance Liquid Chromatography (HPLC). Different extracts were prepared using solvent extraction procedure and its antimicrobial properties were studied. Stevia leaf extracts, which is recovered from *in vitro* grown *Stevia rebaudiana*, has been found to possess both antimicrobial and antioxidant properties. Free radical scavenging activity wasevaluated *in vitro* using 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) assay method.

## MATERIALS AND METHODS

### Identification and quantification of steviol glycosides by Agilent 1260 HPLC

### Collection and storage of *in vitro* leaf sample

The *in vitro* plants were cultured in basal Murashige and Skoog's medium in GA7 for approximately two months in the plant tissue culture room, conditioned with 27ºC temperature, 16 hours photoperiod and > 2200 lumen light intensity. The leaves sizes were tiny and the diameter was approximately 3 mm. Then the samples were washed with tap water and distilled water twice. The leaf samples were air-dried for 3-4 days kept in a sterile plastic tray. Once the samples were dried, they were ground into fine powder, sieved and stored at -20 $^0$ C[4,5]. (Figure 1)

### Preparation of standard glycosides

1 mg of standard glycosides were weighed and dissolved in 30 % of distilled water and 70 % of acetonitrile. Different standards were prepared like 0.5, 0.25, 0.1 and 0.05. These standards were filtered using syringe disk filter (0.2 µm) and kept in the vials for HPLC analysis.

**Preparation of unknown leaf sample**

0.01 g of *in vitro* leaf powder were weighed and dissolved in 30 % of distilled water and 70 % of acetonitrile and they were sonicated. Then the samples were centrifuged for 10 minutes at 15,000 rpm. The supernatants were collected and filtered using syringe disk filter and kept in the vials for HPLC analysis.

**Identification and Quantification of Steviol glycosides by HPLC**

Agilent 1260 High-Performance Liquid Chromatography was used for the analysis of steviol glycosides. Acetonitrile and HPLC grade water were used as a mobile phase. HPLC specifications used in this experiment were quaternary pump flow rate: 1 ml/min, injection volume: 20 µl, pressure: 300 bars, column: C18, 4.6 x 50 mm and DAD signal: 205 nm. 20 µl of the standards and unknown samples were injected for the analysis[6-8]. From the chromatogram, the percentage mass of Steviol glycosides present *in vitro* leaf samples were calculated using the following formula,

$$\text{Standard content} = \frac{A2}{A1} \times \frac{M1}{M2} \times P$$

Where, A1 = peak area of glycosides in reference standard solution, A2 = peak area of glycoside in sample solution, M1 = mass, in mg of the reference standard glycoside, M2 = mass, in mg, of the sample, P = Purity of reference standard

**Antimicrobial assay**

**Test Microorganism**

The bacterial cultures used for antimicrobial testing were *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6633 and *Staphylococcus epidermidis* ATCC 12228.

**Extract preparation**

For aqueous extract, 0.1 g of Stevia leaf powder was dissolved in 1 mL of sterile water. Without incubation, the samples were transferred into water bath at 70ºC for 120 minutes. The extracts were then filtered by using sterile Whatman filter paper and dried in oven at 60ºC. For ethanolic and methanolic extract, 0.1 g of Stevia leaves powder was weighed and dissolved in 1.25 mL of methanol and ethanol. The tubes were sealed and kept in room temperature for four days. These tubes were transferred into water bath at 70ºC for 90 minutes. The extracts were then filtered by using sterile Whatman filter paper and dried in oven at 60ºC. The dried extracts were then dissolved in double the volume of 0.25 % dimethyl sulphoxide (DMSO)[9-16].

**Disc diffusion method**

Antimicrobial test was done by disc diffusion method. The sterile discs were prepared. In the fresh culture, the bacterial concentration were adjusted to approximately 1 × $10^6$ CFU/mL, the sterile cotton swab was dipped into the bacterial suspension and pressed against the wall of the bottle to get rid of excess solution. The bacteria were then swabbed on Mueller-Hinton agar (MHA) three times, at about 60º each time. The sterile discs were then dipped in the extracts and placed into the petri dishes that have been inoculated with bacteria. 3 µL of 50 mg/mL ampicillin was used as the positive control and 3 µL of 0.25 % DMSO was used as the negative control for this assay. All the plates were incubated in refrigerator at 4ºC for one hour to allow the extract to diffuse into the agar. Then they were incubated at 37ºC for 20 hours. Results were observed by measuring the diameter of the zone of inhibition[9,12,17].

**Antioxidant assay using DPPH**

**Standard preparation**

Different concentration of ascorbic acid was prepared. 0.5, 0.4, 0.3, 0.2 and 0.1 mg of standard ascorbic acid dissolved in 1 ml of sterile water. Then added 3 ml of 100 mM DPPH in methanol and kept in a water bath at $37^0$C for 30 minutes and read absorbance at 517 nm.

**Extract preparation**

Weighed 0.1 g of stevia leaf powder and dissolved in 1 ml of water, methanol and ethanol separately and incubated in a room temperature for two days.

**DPPH assay**

The incubated samples were centrifuged at 10,000 rpm for 15 minutes. To the 200 ul of supernatant, 800 ul of methanol and 3 ml of 100 mM DPPH in methanol were added, kept in a water bath at $37^0$C for 30 minutes and absorbance were read at 517 nm. The percentage of inhibition were calculated using the formula,

$$I = [(A_o - A_t)/A_o \times 100]$$

Where, I = DPPH inhibition (%), $A_o$ = absorbance of control sample (t = 0 h) and $A_t$= absorbance of a tested sample[18-20].

**RESULTS AND DISCUSSION**

**Identification and quantification of glycosides by HPLC analysis**

High performance liquid chromatography, being more sensitive and accurate, was used for the estimation of rebaudioside A content in the samples. The identification and quantification of steviol glycosides content in the extracted samples was done by comparing the retention time and peak area of sample with that of the standard. The retention time ($R_t$) of stevioside in standard was found to be 0.466 minute (Figure 2a) and $R_t$ of rebaudioside A was 0.793 minute (Figure 2b). The retention time ($R_t$) of stevioside and rebaudioside A *in vitro* leaf sample was found to be 0.466 minutes and 0.766 minutes respectively (Figure 3).

**Quantification of Steviol glycosides present *in vitro* leaf sample** (Figure 2)

***In vitro* leaf sample**

Concentration of Stevioside in *in vitro* sample:

$$\frac{100.66899}{35.54254} \times \frac{0.25}{10} \times 96\% = 6.79\%$$

Concentration of Rebaudioside in *in vitro* sample:

$$\frac{67.59796}{40.97965} \times \frac{0.25}{10} \times 95\% = 3.91\%$$

**Table 1: Minimum inhibitory concentrations (MIC) of the crude extract of *Stevia rebaudiana* against the test bacterial strains**

| | Aqueous | | Methanolic | | Ethanolic | | Control | |
|---|---|---|---|---|---|---|---|---|
| | | *In vitro* | | *In vitro* | | *In vitro* | Positive | Negative |
| *E. c* | | NA | | NA | | NA | 18 ± 1 | NA |
| *B. s* | | NA | | 9 ± 1 | | 9 ± 3 | 34 ± 3 | NA |
| *S. a* | | NA | | NA | | 6 ± 1 | 33 ± 1 | NA |
| *S. e* | | NA | | 7 ± 1 | | 8 ± 3 | 24 ± 2 | NA |

Table 1 show s the zone of inhibition resulted from *in vitro* samples. Measurements were taken in millimetre (mm) and were inclusive of the diameter of disc which is 6 mm. Datas were collected from three replicates and were presented as means ± standard error (SE). NA: no activity;
*E. c: Escherichia coli* ATCC 25922; *B. s: Bacillus subtilis* ATCC 6633; *S. a: Staphylococcus aureus* ATCC 25923; *S. e: Staphylococcus epidermidis* ATCC 12228.

**Table 2: DPPH free radical scavenging activity of standard and different solvent extracts of *Stevia rebaudiana***

| S. No. | Sample | $IC_{50}$ |
|---|---|---|
| 1. | Standard ascorbic acid | 91.48 % |
| 2. | Aqueous | 68.01 % |
| 3. | Ethanol | 77.77 % |
| 4. | Methanol | 71.17 % |

**(a) (b) (c)**

**Figure 1: (a) *In vitro* plants (b) Size of *In vitro* leaves used (c) Leaf powder**

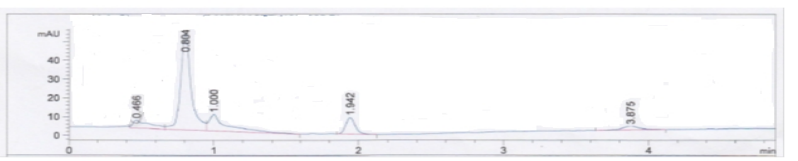
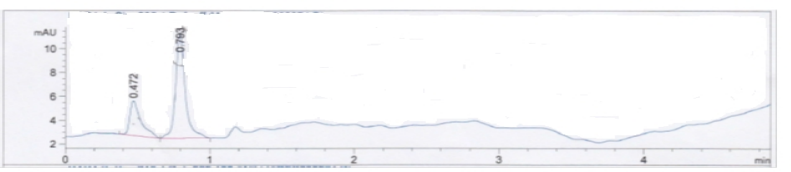



**Figure 2: (a) Stevioside standard**




**(b) Rebaudioside standard**

**Figure 2: HPLC Chromatogram of (a) stevioside and (b) rebaudioside A from standard solution, $R_t$ of stevioside was 0.466 minutes and rebaudioside A was 0.793 minutes under separation acetonitrile: water (70:30 v/v) as the elution solvent at flow rate of 1 ml/min and the detection wavelength 205 nm, column was Agilent ZORBAX Carbohydrate Analysis (Column 4.6 × 150 mm, 5 µm)**

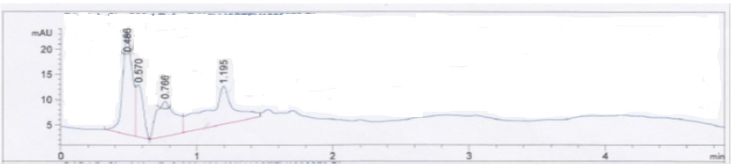



**Figure 3: HPLC Chromatogram of stevioside and rebaudioside A from *in vitro* leaf sample, $R_t$ of stevioside was 0.486 minutes and rebaudioside A was 0.766 minutes**

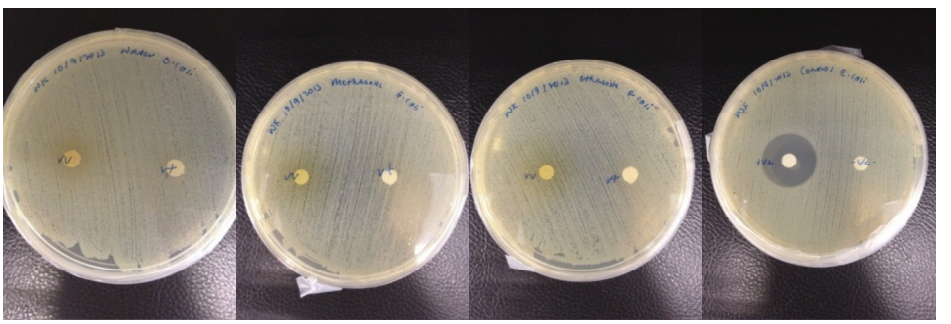


**Figure 4: Antibacterial activity against *Escherichia coli* ATCC 25922**
**(a) Aqueous extract (b) Methanolic extract (c) Ethanolic extract (d) Control**

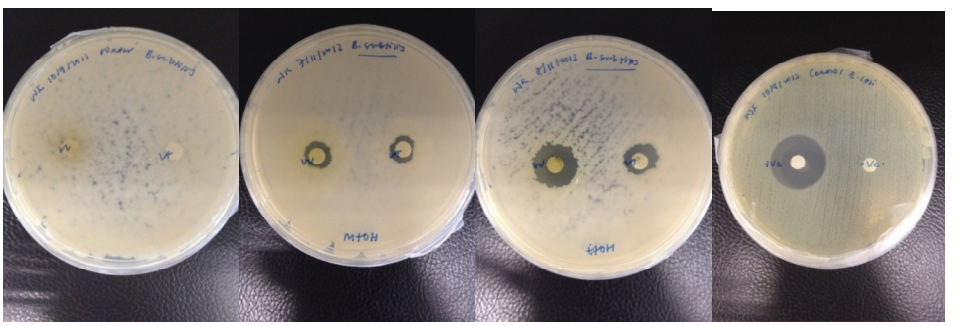


**Figure 5: Antibacterial activity against *Bacillus subtilis* ATCC 6633**
**(a) Aqueous extract (b) Methanolic extract (c) Ethanolic extract (d) Control**

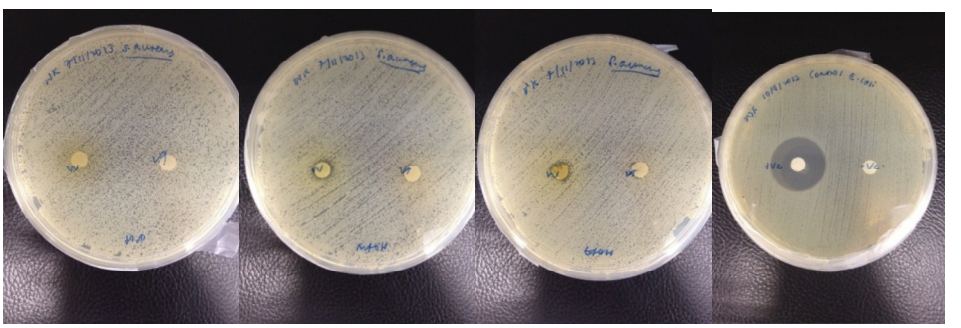


**Figure 6: Antibacterial activity against *Staphylococcus aureus* ATCC 25923**
**(a) Aqueous extract (b) Methanolic extract (c) Ethanolic extract (d) Control**

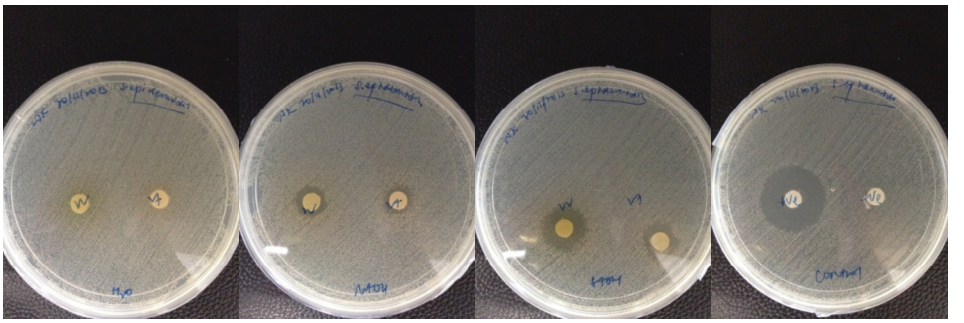


**Figure 7: Antibacterial activity against *Staphylococcus epidermidis* ATCC 12228**
**(a) Aqueous extract (b) Methanolic extract (c) Ethanolic extract (d) Control**

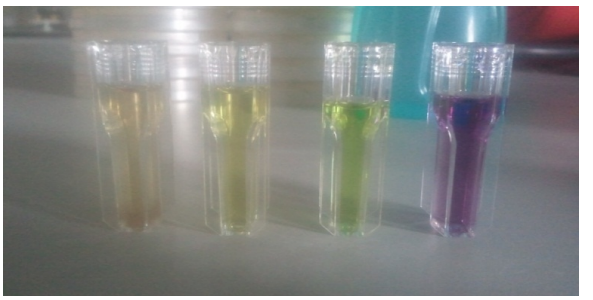


**Figure 8: Discoloration of DPPH from purple to yellow showing the presence of antioxidant compound in different leaf extracts**

**Antibacterial activity**

*In vitro* antibacterial activity of leaf extracts of *Stevia rebaudiana* was determined. Antibacterial activity of the crude extracts of *Stevia rebaudiana* against *E.coli, Bacillus subtilis, Staphylococcus aureus* and *Staphylococcus epidermidis* were represented. Standard antibiotics ampicillin was also tested for comparing with the results of crude extracts. Three leaf extracts of *Stevia rebaudiana* obtained by various organic solvents were investigated for antibacterial effects using the disc diffusion method. The plant parts used, extracts tested, standard antibiotics and the results of the bacterial sensitivity were tabulated. Among all the extracts, ethanolic extract showed high activity (3-7 mm of zone of inhibition) on all the organisms[11]. (Table 1, Figure 4 - 7)

**Antioxidant activity determination by DPPH assay**

The DPPH method was evidently introduced nearly 50 years ago and it is widely used to test the ability of compounds to act as free radical scavengers or hydrogen donors, and to evaluate antioxidant capacity. DPPH is stable nitrogen centered free radical which can be effectively scavenged by antioxidants and shows strong absorbance at 517 nm. The change in absorbance of DPPH radical caused by the extracts was due to the reaction between the antioxidant molecules and the extracts, which resulted in the scavenging of the radical by hydrogen donation. It was visually noticeable as a discoloration from purple to yellow (Figure 8). Extent of DPPH radical scavenged was determined by the decrease in intensity of violet color in the form of $IC_{50}$ values. The 80 % methanol, aqueous and 70 % ethanolic extract showed significant DPPH scavenging activity and it was shown in Table 2. Our observation revealed the 90 % methanol extract had higher DPPH scavenging activity in comparison to aqueous extract.[18-20]

**CONCLUSION**

The preliminary study showed the *Stevia rebaudiana* grown *in vitro* were used for the identification and quantification of glycosides and also used to demonstrate the antibacterial and antioxidant activity. To the best of our knowledge there is not much research paper available on this biochemical and medicinal activity of *stevia rebaudiana* which is grown *in vitro*. The stevia grown in the basal MS medium showed the presence of glycosides from HPLC analysis. Using Disc diffusion method, the compounds present in the ethanolic extract has shown high antimicrobial activity. For antioxidant activity, the ethanolic extract showed high inhibition percentage by DPPH assay. Literature review revealed the production of glycosides from stevia which is grown in different environment, but the leaves from stevia grown *in vitro* only in the basal MS medium also showed the same property like the plants grown in the *in vivo* environment. It is further recommended to do detailed and thorough research on different parameters influencing on the biochemical and medicinal properties of *Stevia rebaudiana* grown *in vitro.*

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**REFERENCES**


1. Dey A, Kundu SB and Yopadhyay A and Bhattacharjee A. Efficient micropropagation and chlorocholine chloride induced stevioside production of *Stevia rebaudiana* Bertoni. Comptes Rendus Biologies 2013; 336(1): 17-28. http://dx.doi.org/10.1016 /j.crvi.2012.11.007
2. A Esmat Abou Arab, A Azza Abou Arab and M Ferial Abu Salem. Physico-chemical assessment of natural sweeteners stevioside produced from *Stevia rebaudiana* Bertoni plant. African Journal of Food Science 2010; 4(5): 269-281.
3. Liu J, Li JW and Tang J. Ultrasonically assisted extraction of total carbohydrates from *Stevia rebaudiana* Bertoni and identification of extracts. Food and Bio products Processing 2010; 88(2-3): 215-221. http://dx.doi.org/10.1016/j.fbp.2009.12.005
4. Muanda FN, Soulimani R, Diop B and Dicko A. Study on chemical composition and biological activities of essential oil and extracts from *Stevia rebaudiana* Bertoni leaves. Food Science and Technology 2011; 44: 1865-1872.
5. Hwang SJ. Rapid *in vitro* Propagation and Enhanced Stevioside accumulation in *Stevia rebaudiana* Bert. Journal of Plant Biology 2006; 49(4): 267-270. http://dx.doi.org/10.1007/BF03031153
6. Kaur H. Chromatographic determination of stevioside in leaf parts of *in vitro* and *in vivo* regenerated plants of *Stevia rebaudiana*. International Journal of Natural Products Research 2011; 1(4): 44-48.
7. M Amzad Hossain A, AB Siddique B, SM Mizanur Rahman B, M Amzad Hossain. Chemical composition of the essential oils of *Stevia rebaudiana* Bertoni leaves. Asian Journal of Traditional Medicines 2010; 5(2): 56-61.
8. Valerio Pieri, Andrea Belancic, Susana Morales, Hermann Stuppner. Identification and quantification of major steviol glycosides in *Stevia rebaudiana* purified extracts by 1H NMR spectroscopy. Journal of Agricultural and Food Chemistry 2011; 59(9): 4378-4384. http://dx.doi.org/10.1021/jf104922q
9. Jayaraman S, Manoharan MS and Illanchezian S. *In-vitro* antimicrobial and antitumor activities of *Stevia rebaudiana* (Asteraceae) leaf extract. Tropical Journal of Pharmaceutical Research 2008; 7(4): 1143-1149.
10. Choi YH, Kim I, Yoon KD, Lee SJ, Kim CY, Yoo KP, Choi YH and Kim J. Supercritical fluid extraction and liquid chromatographic-electrospray mass spectrometric analysis of stevioside from *Stevia rebaudiana* leaves. Chromatographia 2002; 55(9-10): 617-620. http://dx.doi.org/10.1007/BF02492911
11. Das K, Dang R and Gupta N. Comparative antimicrobial potential of different extracts of leaves of *Stevia rebaudiana* Bert. International Journal of Natural and Engineering Sciences 2008; 3(1): 65-68.
12. Debnath M. Clonal propagation and antimicrobial activity of an endemic medicinal plant *Stevia rebaudiana*. Journal of Medicinal Plants Research 2008; 2(2): 45-51.
13. Jaitak V, Bandna Singh B and Kaul VK. An efficient microwave-assisted extraction process of stevioside and rebaudioside-A from *Stevia rebaudiana* (Bertoni). Phytochemical Analysis 2009; 20(3): 240-245. http://dx.doi.org/10.1002/pca.1120
14. Puri M, Sharma D and Tiwari AK. Downstream processing of stevioside and its potential applications. Biotechnology Advances 2011; 29: 781-791. http://dx.doi.org/10.1016/j.biotechadv.2011 .06.006
15. Shukla S, Mehta A, Bajpai VK and Shukla S. *In vitro* antioxidant activity and total phenolic content of ethanolic leaf extract of *Stevia rebaudiana* Bert. Food and Chemical Toxicology 2009; 47: 2338-2343. http://dx.doi.org/10.1016/j.fct.2009.04.040
16. Zhang SQ, Kumar A and Kutowy O. Membrane-based separation scheme for processing sweeteners from stevia leaves. Food Research International 2000; 33(7): 617-620. http://dx.doi. org/10.1016/S0963-9969(00)00098-3
17. Tadhani MB and Subhash R. *In vitro* antimicrobial activity of *Stevia rebaudiana* Bertoni leaves. Tropical Journal of Pharmaceutical Research 2006; 5(1): 557-560.
18. Blois MS. Antioxidant determinations by the use of a stable free radical. Nature 2005; 3(6): 119-120.
19. Chitindingue K, Ndhlala AR, Chapano C, Benhura MA and Muchuweti M. Phenolic compound content, profiles and antioxidant activities of *Amaranthus hybridus* (Pigweed), *Brachiariabrizantha* (Upright Brachiaria) and *Panicum maximum* (Guinea grass). Journal of Food Biochemistry 2006; 31: 206-216. http://dx.doi.org/ 10.1111/j.1745-4514.2007.00108.x
20. Tadhani MB, Patel VH and Subhash R. *In vitro* antioxidant activities of *Stevia rebaudiana* leaves and callus. Journal of Food Composition and Analysis 2007; 20: 323-329. http://dx.doi.org/ 10.1016/j.jfca.2006.08.004
21. Shukla S, Mehta A, Bajpai VK and Shukla S. *In vitro* antioxidant activity and total phenolic content of ethanolic leaf extract of *Stevia rebaudiana* Bert. Food and Chemical Toxicology 2009; 47: 2338-2343. http://dx.doi.org/10.1016/j.fct.2009.04.040
22. Singh S, Garg V, Yadav D, Beg MN and Sharma N. *In vitro* antioxidative and antibacterial activities of various parts of *Stevia rebaudiana* (Bertoni). International Journal of Pharmacy and Pharmaceutical Sciences 2012; 4(3): 468-473.
23. Tepe B, Sokmen M, Akpulat HA and Sokmen A. *In vitro* antioxidant activities of the methanol extracts of four Helichrysum species from Turkey. Food Chemistry 2005; 2(90): 685-691. http://dx.doi.org/10.1016/j.foodchem.2004.04.030
24. Wei A and Shibamoto T. Antioxidant activities and volatile constituents of various essential oils. Journal of Agricultural and Food Chemistry 2007; 55: 1737-1742. http://dx.doi.org/10.1021 /jf062959x


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