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Journal of Food Composition and Analysis 20 (2007) 323–329

JOURNAL OF FOOD COMPOSITION AND ANALYSIS

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Original Article

In vitro antioxidant activities of *Stevia rebaudiana* leaves and callus

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Received 2 June 2006; received in revised form 1 August 2006; accepted 24 August 2006

Abstract

Leaf extract of *Stevia rebaudiana* promotes effects on certain physiological systems such as the cardiovascular and renal and influences hypertension and hyperglycemia. Since these activities may be correlated with the presence of antioxidant compounds, leaf and callus extracts of *Stevia rebaudiana* were evaluated for their total phenols, flavonoids content and total antioxidant capacity. Total phenols and flavonoids were analyzed according to the Folin–Ciocalteu method and total antioxidant activity of water and methanolic extracts of stevia leaves and callus was assessed by ferric reducing/antioxidant power (FRAP) assay as well as 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay. The total phenolic compounds were found to be 25.18 mg/g for stevia leaves and 35.86 mg/g for callus on dry weight basis. The flavonoids content was found to be 21.73 and 31.99 mg/g in the leaf and callus, respectively. The total antioxidant activity was expressed as mg equivalent of gallic acid, ascorbic acid, BHA and trolox per gram on dry weight basis. Total antioxidant activity found was ranged from 9.66 to 38.24 mg and 11.03 to 36.40 mg equivalent to different standards in water and methanolic extract of stevia leaves, respectively. In case of stevia callus, it was found to be 9.44 to 37.36 mg for water extract and 10.14 to 34.37 mg equivalent to standards for methanolic extract. The concentrations required for 50% inhibition (IC_{50}) of DPPH radicals were 11.04, 41.04 and 57.14 μg/mL for gallic acid, trolox and butylated hydroxyanisole (BHA), respectively. The percent inhibition of DPPH radical of various extracts of stevia leaves and callus found were ranged from 33.17% to 56.82%. The highest percent of inhibition was observed in methanolic extract of callus.

Keywords: *Stevia rebaudiana*; Antioxidant activity; Total phenols; Flavonoids; FRAP; DPPH

1. Introduction

Active (or reactive) oxygen species and free radical-mediated reactions have been implicated in degenerative or pathological processes such as aging (Ames et al., 1993; Harman, 1995), cancer, coronary heart disease and Alzheimer's disease (Ames, 1983; Gey, 1990; Smith et al.,1996; Diaz et al., 1997). To protect their possible damages to biological molecules, especially to DNA, lipids and proteins, all oxygen-consuming organisms are endowed with a well-integrated antioxidant system, including enzymatic and non-enzymatic components. The superoxide dismutase (SOD), glutathione peroxidase (GSHPx) and catalase are the major antioxidant enzymes frequently mentioned in the literature. The non-enzymatic components consist of macromolecules, such as albumin, ceruloplasmin and ferritin as well as an array of small molecules, such as vitamin C, E, β-carotene, reduced glutathione (Fang et al., 2002; Jacob, 1995). Plants constitute an important source of active natural products which differ widely in terms of structure and biological properties. They have played a remarkable role in the traditional medicine of various countries. In recent years, the prevention of cancer and cardiovascular diseases has been associated with the ingestion of fresh fruits, vegetables or teas rich in natural antioxidants (Virgili et al., 2001; Johnson, 2001). The protective effects of plant products are due to the presence of several components which have distinct mechanisms of action; some are enzymes and proteins and others are low molecular weight compounds such as vitamins (Halliwell, 1996; Head, 1998), carotenoids (Edge et al., 1997), flavonoids (Zhang and Wang, 2002), anthocyanins and other phenolic compounds (Sanchez-Moreno et al., 1998).

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doi:10.1016/j.jfca.2006.08.004

Phenolic compounds are commonly found in both edible and non-edible plants. They are important in the plant for normal growth development and defense against infection and injury. The presence of phenolic compounds in injured plants may have an important effect on the oxidative stability and microbial safety. Although phenolic compounds do not have any known nutritional function, they may be important to human health because of their antioxidant potency (Hertog et al., 1995; Shadidi and Nazck, 1995; Hollman et al., 1996). The importance of the antioxidant constituents in the maintenance of health and protection from coronary heart disease and cancer is also raising interest among scientists, food manufacturers and consumers as the trend of the future is moving toward functional food with specific health effects (Velioglu et al., 1998; Kahkonen et al., 1999; Robards et al., 1999). The beneficial health-related effects of certain phenols or their potential antioxidant properties, especially when these compounds are present in large quantities in foods, are of importance to consumers. Natural antioxidants such as α-tocopherol and L-ascorbic acid are widely used because they are seen as safer and causing fewer adverse reactions but their antioxidant activities are lower than the those one synthetic antioxidants such as BHA and butylated hydroxytoluene (BHT) (Seong et al., 2004) which have possible activity as promoters of carcinogenesis (Barlow, 1990). Therefore, the need exists for safe, economic antioxidants with high activity from natural sources to replace these synthetic antioxidants. The antioxidant compounds present in edible plants have recently promoted as food additives because they display little or no toxic side effects (Seong et al., 2004).

Stevia rebaudiana Bertoni, belonging to the family Compositae, is a sweet herb native to South America. The plant has also been cultivated in China and Southeast Asia (Koyama et al., 2003). Stevia sweeteners, crude extract from leaves, have been used for a few decades to sweeten soft drinks, soju, soy sauce, yogurt, and other foods in Japan, Korea and Brazil (Kinghorn et al., 2001). The dry extract from the leaves of stevia also contains flavonoids, alkaloids, water-soluble chlorophylls and xanthophylls, hydroxycynnamic acids (caffeic, chlorogenic, etc.), neutral water-soluble oligosaccharides, free sugars, amino acids, lipids, essential oils and trace elements (Komissarenko et al., 1994). Stevia sweetener extractives have been suggested to exert beneficial effects on human health, including antihypertensive (Chan et al., 2000; Lee et al., 2001), antihyperglycemic (Jeppesen et al., 2000, 2002) and anti-human rotavirus (Das et al., 1992) activities.

Therefore, it is of great interest to evaluate the total phenolic compounds, flavonoids and total antioxidant activity of *Stevia rebaudiana*. For this purpose, *Stevia rebaudiana* leaves and callus were selected in the present study for the analysis of the total phenolic compounds, flavonoids and their total antioxidant activity.

2. Materials and methods

2.1. Experimental materials

Plants of *Stevia rebaudiana* and dried stevia leaves were supplied by Growmore Biotech Ltd. Hosur, Tamilnadu. The plants were maintained under laboratory conditions and leaves were packed in polyethylene bags and stored at −18 °C until used. For standard, gallic acid (Sigma), BHA (Sigma), trolox (Fluka) and ascorbic acid (Sigma) were used.

2.2. Induction of callus

Leaves of *Stevia rebaudiana* were surface sterilized on laminar airflow cabinet, treated with 70% alcohol for 2 min followed by treatment with 0.1% mercuric chloride for 2 min. Finally, the explants were washed with sterile distilled water successively three times and were inoculated on Murashige and Skoog (1962) medium supplemented with 2.0 mg/L NAA (1-napthaleneacetic acid), 0.3 mg/L BA (6-benzyladenine), 30 g/L sucrose, 8 g/L agar at pH 5.8 in glass tubes for the production of the callus. The cultures were incubated at 25 ± 2 °C, 3000 lux intensity for 16 h photoperiod. Callus was subcultured onto fresh medium of the same composition for a period of six weeks before being analyzed.

2.3. Analyzes

2.3.1. Total phenolic compounds

Total phenolic compounds were estimated according to the method described by Malik and Singh (1971). Known quantity of stevia leaf or callus powder was taken in 100 mL conical flask. To this, 25 mL 0.3 N HCl in methanol was added and kept on environmental shaker (Brunswick, USA) at 150 rpm for an hour. After shaking, crude extract was filtered through Whatman No.1 filter paper. The filtrate obtained was evaporated to dryness in a boiling waterbath. To the residue, hot water was added and final volume was adjusted to 100 mL with distilled water. From this, 1 mL aliquot was taken in a test tube. To this, 1 mL each of Folin–Ciocalteu reagent (diluted 1:2) and 35% sodium carbonate were added and then mixed. After 10 min, 2 mL of distilled water was added and intensity of the color was recorded at 620 nm in the UV spectrophotometer (Hitachi 220S) against the reagent blank. The content of total phenolic compounds was determined using a standard curve prepared with gallic acid.

2.3.2. Total flavonoids

Known quantity of stevia leaf or callus powder was taken in 100 mL conical flask. To this, 25 mL 0.3 N HCl in methanol was added and kept on environmental shaker (Brunswick, USA) at 150 rpm for an hour. After shaking, crude extract was filtered through Whatman No.1 filter paper. The filtrate obtained was evaporated to dryness in a

water bath. To the residue, hot water was added and final volume was adjusted to 100 mL with distilled water. From this, 1 mL aliquot was taken in a test tube and 1 mL of 20% HCl and 0.5 mL formaldehyde were added and the tubes were allowed to stand for overnight. After 24 h, the content was centrifuged and 1 mL from supernatant was taken and treated as described for phenolic compounds.

2.3.3. Total antioxidant activities

Sample preparation: For the preparation of sample, 150 mg of fine ground powder of stevia leaves or callus was taken in 250 mL conical flasks. To this, 50 mL water added and were kept on environmental shaker (Brunswick, USA) at 150 rpm for an hour. After removing flask, content were filtered using filter paper (Whatman No.1). Similarly, methanol extracts were also prepared. The filtrate was used directly for FRAP and DPPH assay without storage.

FRAP assay: The procedure described by Benzie and Strain (1996) was followed. The principle of this method is based on the reduction of a ferric-tripyridyl-triazine complex to its ferrous, colored form in the presence of antioxidants. Briefly, the FRAP reagent contained 2.5 mL of a 10 mmol/L TPTZ (2,4,6- tripyridyl-s-triazine, Sigma) solution in 40 mmol/L HCl plus 2.5 mL of 20 mmol/L $FeCl_3$ $6H_2O$ and 25 mL of 0.3 mol/L acetate buffer, pH 3.6 and was prepared freshly and warmed at 37 °C. Aliquots of 40 μL sample filtrate were mixed with 0.2 mL distilled water and 1.8 mL FRAP reagent and the absorbance of reaction mixture at 593 nm was measured spectrophotometrically after incubation at 37 °C for 10 min. Gallic acid, ascorbic acid, BHA and trolox were used as the standard. The final result was expressed as the concentration of antioxidants having a ferric reducing ability equivalent to that of mg of standard used per gram stevia leaves and callus on dry weight basis.

DPPH assay: Assay for DPPH free radical scavenging effect, the method was adopted from Germano et al., (2002). Methanolic and water extracts of stevia leaves and callus were evaluated in terms of their hydrogen donating or radical scavenging ability using DPPH radical. For assay, 200 μL filtrate was taken in test tubes and volume made up to 1 mL with methanol. Three milliliters of the freshly prepared solution of DPPH (200 μM) in methanol was added to the sample tube and mixed vigorously for 15 s. The sample tube was then kept in a water bath at 37 °C for 20 min. The absorbance of the sample was measured at 517 nm by UV spectrophotometer (Hitachi 220S). Gallic acid, BHA and trolox were used as standard references. The DPPH radical scavenging effect was calculated as "inhibition of percentage" according to the following formula:

Inhibition of percentage (%) $= [A_{c(0)} - A_{a(t)}/A_{c(0)}] \times 100$ where $A_{c(0)}$ is an absorbance of control DPPH solution at 0 min and $A_{a(t)}$ is an absorbance of test sample after 20 min.

2.3.4. Statistical analysis

All data are reported as mean ± standard error of mean for three independent samples ($n = 3$). One-way analysis of variance (ANOVA) was used and the least significant difference (LSD) at $P < 0.05$ was calculated using the SPSS 10.0 for windows package.

3. Results and discussion

Phenolic compounds are commonly found in both edible and non-edible plants, and they have been reported to have multiple biological effects, including antioxidant activity (Kahkonen et al., 1999). Contents of flavonoid and other phenolic substance have been suggested to play a preventive role in the development of cancer and heart disease (Kahkonen et al., 1999). In the present study, the Folin–Ciocalteu method was used to determine the total phenolic compounds and flavonoids content of stevia leaves and callus. The phenolic compounds in stevia leaves and callus were extracted by using HCl-methanol. Fig. 1 shows the total phenolic compounds and flavonoids content of the leaf and callus of *Stevia rebaudiana*. Total phenolic compounds was found to be 25.18 and 35.86 mg per gram of stevia leaves and callus on dry weight basis, respectively. Flavonoids content was 21.73 mg per gram for stevia leaf and it was 31.99 mg per gram for stevia callus on dry weight basis. Content of total phenolic compounds and flavonoids were found to be higher in callus compared to the leaf.

There are many different antioxidants present in plants and it is very difficult to measure each antioxidant component separately. Therefore, several methods have been developed to evaluate the total antioxidant activity of fruits or other plants and animal tissues. Among them, trolox equivalent antioxidant capacity (Van den Berg et al., 1999), total radical absorption potentials (Evelson et al., 2001), oxygen radical absorption capacity assays (Cao and Prior, 1999; Ou et al., 2001), the ferric reducing ability of plasma (FRAP) assay (Benzie and Strain, 1996) are commonly used and are the representative methods frequently used in various investigations. We selected the FRAP and DPPH assay to evaluate the antioxidant activities of the leaves and callus of stevia. The FRAP assay showed comparable sensitivity with concentration range from 1 to 4 μg, calculated in test system. Figs. 2 and 3 show the absorbance at 593 nm against various concentrations of gallic acid, ascorbic acid, BHA and trolox as standards in water and methanol, respectively. Gallic acid was the strongest antioxidant in both water and methanol whereas trolox was proved to be a weak antioxidant in water. Ascorbic acid and trolox have similar antioxidant activity in methanol. The FRAP values of stevia leaf water (SLW) extract, stevia leaf methanol (SLM) extract, stevia callus water (SCW) extract and stevia callus methanol (SCM) extract are presented in Table 1. All the values are expressed as mg equivalent to various standards per gram on dry weight basis.

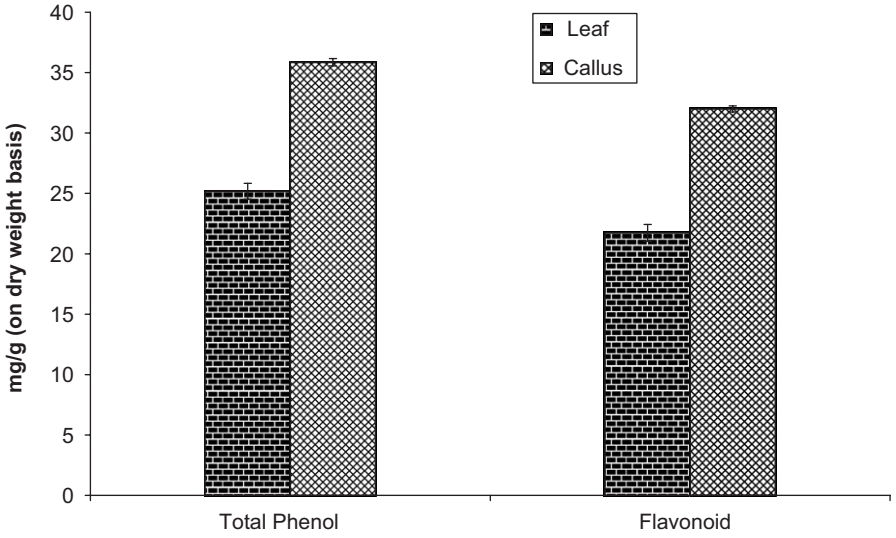

Fig. 1. Total phenolic compounds and flavonoids content of *Stevia rebaudiana* leaves and callus.

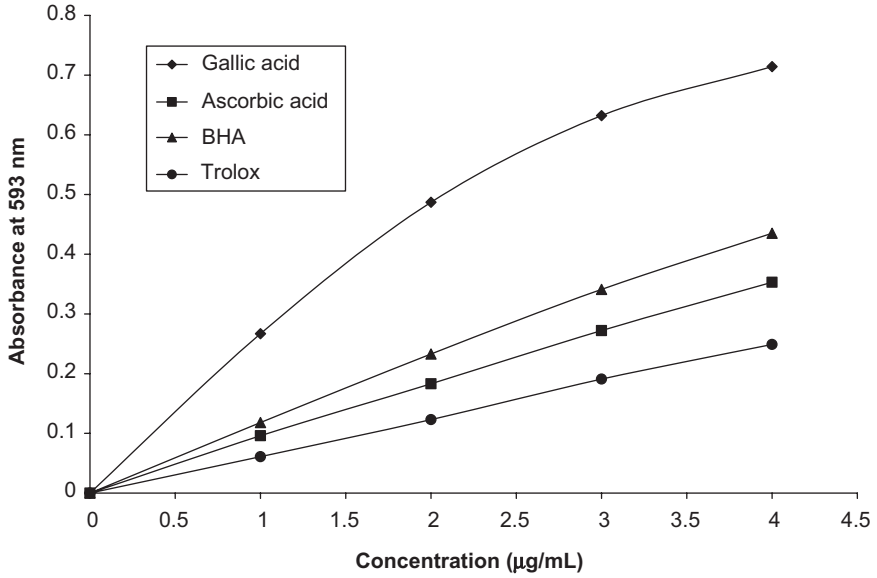

Fig. 2. Absorbance of the four standards antioxidant gallic acid, ascorbic acid, BHA and trolox in water with different concentrations in the FRAP assay.

To the best of our knowledge and from the cited literature, this is first time that the antioxidant activity of stevia leaves and callus has been reported. Methanolic extract of stevia leaves showed significantly ($P<0.05$) high antioxidant activity equivalent to gallic acid (11.03 mg equivalent) and BHA (35.16 mg equivalent) compared to the other three extracts. Water extract of stevia leaves and callus did not show significant difference in antioxidant activity when expressed in terms of ascorbic acid but it was significantly ($P<0.05$) lower compared to the methanolic extract of leaves and callus. Water extract of leaf showed the highest antioxidant activity equivalent to trolox compared to all other extracts. Methanolic extract of callus showed the least antioxidant activity compared to all other samples when expressed in terms of trolox as the standard. On expressing the antioxidant activity in terms of gallic acid or ascorbic acid as standards the methanolic extracts of leaf and callus showed slightly higher values whereas on expressing in terms of BHA the methanolic leaf extract showed higher value compared to the water extract whereas in trolox the water extract of callus showed higher value compared to the methanolic extract.

DPPH radicals are widely used in the model system to investigate the scavenging activities of several natural compounds. When DPPH radical is scavenged, the color of the reaction mixture changed from purple to yellow with decreasing of absorbance at wavelength 517 nm. Fig. 4 shows the percent inhibition of DPPH radicals with gallic acid, trolox and BHA as standards. Among the three antioxidants used in the experiment, gallic acid was found to be the strongest one. The IC_{50} for gallic acid, trolox and BHA observed was 11.04, 41.04 and 57.14 µg/mL,

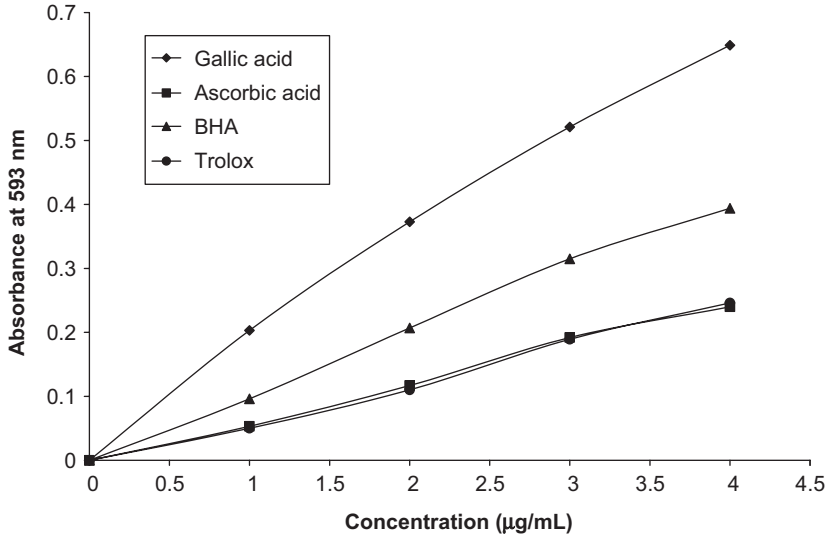

Fig. 3. Absorbance of the four standards antioxidant gallic acid, ascorbic acid, BHA and trolox in methanol with different concentrations in the FRAP assay.

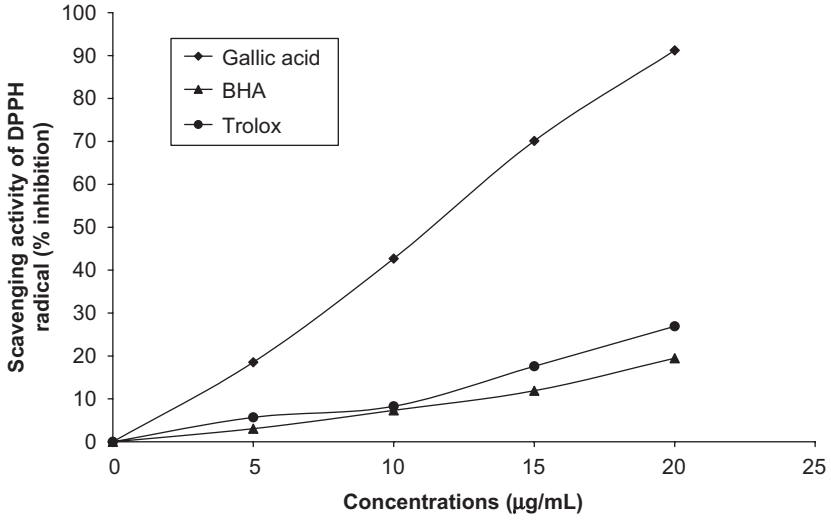

Fig. 4. Scavenging activity of the three standards gallic acid, BHA and trolox against DPPH radical.

Table 1
Antioxidant activity of water and methanolic extracts of *Stevia rebaudiana* leaves and callus equivalent to gallic acid or ascorbic acid or BHA or trolox

Mg equivalent per gram on dry weight basis	Leaf extract		Callus extract		*F*-value
	Water	Methanolic	Water	Methanolic	
Gallic acid	$9.66^{a} \pm 0.09$	$11.03^{b} \pm 0.47$	$9.44^{a} \pm 0.13$	$10.14^{a} \pm 0.18$	7.28*
Ascorbic acid	$25.70^{a} \pm 0.24$	$35.16^{c} \pm 0.49$	$25.11^{a} \pm 0.35$	$32.32^{b} \pm 0.56$	36.31*
BHA	$20.19^{a} \pm 0.19$	$35.16^{b} \pm 1.49$	$19.72^{a} \pm 0.27$	$18.27^{a} \pm 0.32$	107.79*
Trolox	$38.24^{b} \pm 0.36$	$37.40^{ab} \pm 1.58$	$37.36^{ab} \pm 0.51$	$34.37^{a} \pm 0.60$	3.54^{NS}

Values are a mean of three trials ± SEM ($n = 3$).
Mean values with the same superscript within a row do not differ significantly ($P > 0.05$).
NS: Non-significant.
*Indicates significant difference ($P < 0.05$).

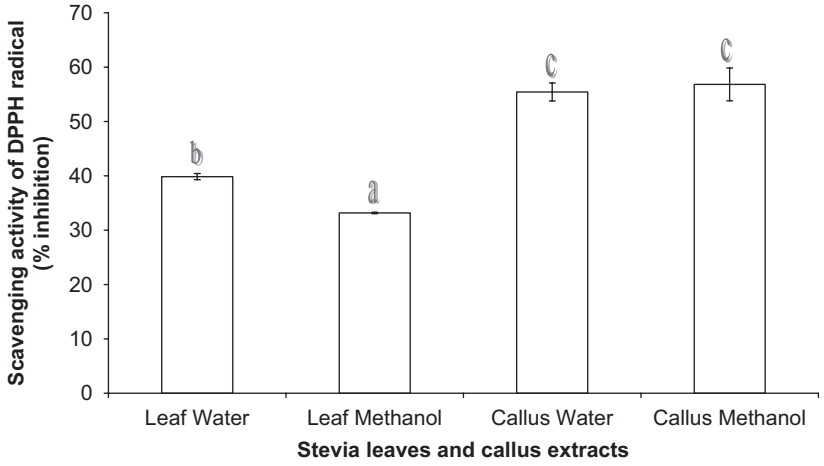

Fig. 5. Scavenging activity of water and methanolic extracts of *Stevia rebaudiana* leaves and callus (600 μg/mL) against DPPH radical. Mean values with the same superscript with in a row do not differ significantly ($P > 0.05$).

respectively. Fig. 5 shows the percent inhibition of DPPH radical with different extracts of stevia leaves and callus. The percent inhibition of DPPH radical with water extract of stevia leaves and callus were found to be 39.86% and 55.42%, respectively, whereas it was found to be 33.17% and 56.82% for methanolic extract of stevia leaves and callus, respectively. The IC_{50} was found to be 752.6 and 541.3 μg of sample for water extract whereas it was 904.4 and 527.9 μg of sample for methanolic extract of stevia leaves and callus, respectively. Percent inhibition of DPPH radical by water extract of stevia leaf was significantly ($P < 0.05$) differ from methanol extract of leaf. Water and methanolic extract of callus showed a significantly ($P < 0.05$) higher percent inhibition of DPPH radical compared to water and methanolic extract of stevia leaves. In the DPPH assay, callus showed higher antioxidant activity compared to the stevia leaves.

4. Conclusions

In summary, the antioxidant activities of stevia leaf and callus have been compared using the FRAP and DPPH assay in this study. FRAP assay of various extracts of the leaf and callus showed more or less similar antioxidant activity. In DPPH assay, callus showed a higher antioxidant activity than stevia leaves. Stevia leaves and callus have strong antioxidant activity and may be rich sources of antioxidants. This study suggests that the incorporation of the leaf and callus extracts instead of pure stevioside as sweetener during home consumption or processing could lead to increase in the amounts of all the major antioxidants in the final product. Consumer demands for healthy food products provide an opportunity to develop foods rich in antioxidants as new functional foods. The results of this study will be also of value in designing a unit on-site for the extraction of antioxidants from leaf and callus along with natural sweetener. The leaf and callus could be used as a value added ingredient in food products. Along with a diet rich in other plant produce, stevia extract could play an important role in improving antioxidant intake in the human diet. Further, studies on the effective antioxidants contained in these leaves and callus and the mechanisms by which they protect against disease development are highly warranted.

Acknowledgment

The authors are indebted to Growmore Biotech Ltd., Hosur, Tamilnadu for supplying stevia plants and dried leaves.

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