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In vitro antioxidant activity and total phenolic content of ethanolic leaf extract of *Stevia rebaudiana* Bert.

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ABSTRACT

The aim of this study was to assess the *in vitro* potential of ethanolic leaf extract of *Stevia rebaudiana* as a natural antioxidant. The DPPH activity of the extract (20, 40, 50, 100 and 200 μg/ml) was increased in a dose dependent manner, which was found in the range of 36.93–68.76% as compared to ascorbic acid 64.26–82.58%. The IC_{50} values of ethanolic extract and ascorbic acid in DPPH radical scavenging assay were obtained to be 93.46 and 26.75 μg/ml, respectively. The ethanolic extract was also found to scavenge the superoxide generated by EDTA/NBT system. Measurement of total phenolic content of the ethanolic extract of *S. rebaudiana* was achieved using Folin–Ciocalteau reagent containing 61.50 mg/g of phenolic content, which was found significantly higher when compared to reference standard gallic acid. The ethanolic extract also inhibited the hydroxyl radical, nitric oxide, superoxide anions with IC_{50} values of 93.46, 132.05 and 81.08 μg/ml, respectively. However, the IC_{50} values for the standard ascorbic acid were noted to be 26.75, 66.01 and 71.41 μg/ml respectively. The results obtained in this study clearly indicate that *S. rebaudiana* has a significant potential to use as a natural antioxidant agent.

1. Introduction

Oxidation is essential to many living organisms for the production of energy to fuel biological processes. However, oxygen-centred free radicals and other reactive oxygen species (ROS), which are continuously, produced *in vivo*, result in cell death and tissue damage. The role of oxygen radicals has been implicated in several diseases, including cancer, diabetes and cardiovascular diseases, ageing, etc. (Halliwell and Gutteridge, 1999). Antioxidants are vital substances which possess the ability to protect the body from damage caused by free radical induced oxidative stress (Ozsoy et al., 2008). There is an increasing interest in natural antioxidants, e.g., polyphenols, present in medicinal and dietary plants, which might help in preventing oxidative damage (Silva et al., 2005). Polyphenols possess ideal structural chemistry for free radical scavenging activity, and they have been shown to be more effective antioxidants *in vitro* than tocopherols and ascorbate. Antioxidant properties of polyphenols arise from their high reactivity as hydrogen or electron donors, and from the ability of the polyphenol derived radical to stabilise and delocalise the unpaired electron (chain-breaking function), and from their ability to chelate transition metal ions (Rice-Evans et al., 1997).

Human body has multiple mechanisms especially enzymatic and non-enzymatic antioxidant systems to protect the cellular molecules against reactive oxygen species (ROS) induced damage (Anderson, 1999). However, the innate defense may not be enough for severe or continued oxidative stress. Hence, certain amounts of exogenous antioxidants are constantly required to maintain an adequate level of antioxidants in order to balance the ROS in human body. Many synthetic antioxidants such as butylated hydroxyl anisole (BHA) and butylated hydroxyl toluene (BHT) are very effective and are used for industrial processing but they possess potential health risk and toxic properties to human health and should be replaced with natural antioxidants (Anagnostopoulou et al., 2006). Hence, compounds especially from natural sources capable of protecting against ROS mediated damage may have potential application in prevention and/or curing of diseases.

The phenolic compounds in herbs act as antioxidants due to their redox properties, allowing them to act as reducing agents, hydrogen donors, free radical quenchers and metal chelators (Javanraedi et al., 2003).

Among the various medicinal and culinary herbs, some endemic species are of particular interest because they may be used for the production of raw materials or preparations containing phytochemicals with significant antioxidant capacities and health benefits (Exarchou et al., 2002). Crude extracts of fruits, herbs,

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vegetables, cereals and other plant materials rich in phenolics are increasingly of interest in the food industry because they retard oxidative degradation of lipids and thereby improve the quality and nutritional value of food (Javanraedi et al., 2003).

Researchers have studied polyphenolic constituents of various legume seeds and have reported that they contain potential medicinal/nutraceutical properties including antioxidant activities (Siddhuraju, 2006). Therefore, the study of the importance and role of non-nutrient compounds particularly phenolic acids, flavonoids and high molecular tannins of legumes as natural antioxidants have greatly increased (Siddhuraju and Becker, 2007).

Stevia rebaudiana (Bert.), Bertoni is an herbaceous perennial plant of the Asteraceae family. It is native of Paraguay, where it grows wild in sandy soils (Goenadi, 1983). *Stevia* leaf extracts are used in Japan, Korea and certain countries of South America to sweeten soft drinks, soju, soy sauce, yogurt and other foods, whereas in the United States it is used as dietary supplements. The leaf extract of *S. rebaudiana* has been used traditionally in the treatment of diabetes. The main sweet component in the leaves of *S. rebaudiana* is stevioside (Geuns, 2000).

Stevia sweetener extractives have been suggested to exert beneficial effects on human health, including antihypertensive (Chan et al., 2000; Lee et al., 2001), antihyperglycemic non-cariogenic, antihuman rota virus activities, glucose metabolism (Suanarunsawat and Chaiyabutr, 1997) and renal function (Jutabha and Chatsudthipong, 2000). Aqueous extract of *S. rebaudiana* dried leaves induce systemic and renal vasodilation, causing hypotension, diuresis and natriuresis in rats (Melis, 1995).

However, no reports are available on the antioxidant activity of *S. rebaudiana* leaves, therefore, present investigation was undertaken to examine the total phenolic content and antioxidant activities of ethanolic leaf extract of *S. rebaudiana* (ELES) through various *in vitro* models. Possible relationship between phenolic content and antioxidant activity was also seen.

2. Materials and methods

2.1. Chemicals

Chemical reagents nitroblue tetrazolium (NBT), 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma Germany, gallic acid (standard solution) (Loba Chemie, Mumbai), sodium carbonate (S.D-Fine Chemicals, Mumbai) and sodium nitroprusside (10 mM) solution and trichloro acetic acid (TCA) (S.D-Fine Chemicals, Mumbai). All other reagents used were of analytical grade.

2.2. Plant material

The leaves of *S. rebaudiana* were collected in March 2006 from Sagar District, Madhya Pradesh, India. Further taxonomic identification was conducted by Dr. Pradeep Tiwari, Harberium incharge, Department of Botany, Dr. H.S. Gour University, Sagar, MP, India. A voucher specimen was deposited in the herbarium of our laboratory under the number (Bot/H/3352).

2.3. Preparation of the extract

The air-dried leaves of *S. rebaudiana* (50 g) were powdered and then extracted with 500 ml of ethanol by using soxhlet apparatus. The crude extract was filtered and evaporated under reduced pressure to give a viscous dark mass with a percentage yield of 4.5% (w/w). This crude extract was dissolved in water or solvent and used for the assessment of antioxidant activity.

2.4. Determination of total phenolic content

Total soluble phenolics in the leaf extract of *S. rebaudiana* were determined with Folin–Ciocalteau reagent according to the method using gallic acid as a standard phenolic compound (Slinkard and Singleton, 1977). About 1.0 ml of extract solution containing 1.0 g extract in a volumetric flask was diluted with 46 ml of distilled water. About 1.0 ml of Folin–Ciocalteau reagent was added and mixed thoroughly. Three minutes later 3.0 ml of 2% sodium carbonate was added and the mixture was allowed to stand for 3 h with intermittent shaking. The absorbance of the blue color that developed was measured at 760 nm. The concentration of total phenols was expressed as mg/g of dry extract (Kim et al., 2003). The concentration of total phenolic compounds in the extract was determined as μg of gallic acid equivalent using an equation obtained from the standard gallic acid graph:

$$\text{Absorbance} = 0.0008 \times \text{gallic acid } (\mu g).$$

2.5. Determination of DPPH (1-1-diphenyl 2-picryl hydrazyl) radical scavenging activity

The free radical scavenging activity of ethanolic leaf extract of *S. rebaudiana* was measured in terms of hydrogen donating or radical scavenging ability using the stable radical DPPH (Blios, 1958). About 0.1 mM solution of DPPH in ethanol was prepared and 1.0 ml of this solution was added to 3.0 ml of extract solution in water at different concentrations (20–200 μg/ml). Thirty minutes later, the absorbance was measured at 517 nm. Ascorbic acid was used as the reference compound. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. Radical scavenging activity was expressed as the inhibition percentage of free radical by the sample and was calculated using the following formula:

$$\% \text{ inhibition} = ((A_0 - A_t)/A_0 \times 100)$$

where A_0 was the absorbance of the control (blank, without extract) and A_t was the absorbance in the presence of the extract. All the tests were performed in triplicate and the graph was plotted with the mean values.

2.6. Determination of hydroxyl radical scavenging activity

The hydroxyl radical scavenging capacity was measured using modified method as described previously (Halliwell et al., 1987). Stock solutions of EDTA (1 mM), $FeCl_3$ (10 mM), ascorbic acid (1 mM), H_2O_2 (10 mM) and deoxyribose (10 mM) were prepared in distilled deionized water. The assay was performed by adding 0.1 ml EDTA, 0.01 ml of $FeCl_3$, 0.1 ml of H_2O_2, 0.36 ml of deoxyribose, 1.0 ml of extract (20–200 μg/ml) each dissolved in distilled water, 0.33 ml of phosphate buffer (50 mM, pH 7.4) and 0.1 ml of ascorbic acid in sequence. The mixture was then incubated at 37 °C for 1 h. About 1.0 ml portion of the incubated mixture was mixed with 1.0 ml of (10%) TCA and 1.0 ml of (0.5%) TBA (in 0.025 M NaOH containing 0.025 M NaOH BHA) to develop the pink chromogen measured at 532 nm. The hydroxyl radical scavenging activity of the extract was reported as the percentage of inhibition of deoxyribose degradation and was calculated according to the following equation:

$$\% \text{ inhibition} = ((A_0 - A_t)/A_0 \times 100)$$

where A_0 was the absorbance of the control (blank, without extract) and A_t was the absorbance in the presence of the sample of the extract. All the tests were performed in triplicate and the graph was plotted with the mean values. Ascorbic acid was used as a positive control.

2.7. Determination of nitric oxide radical scavenging activity

Nitric oxide was generated from sodium nitroprusside and measured by the Greiss reaction. Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide (Marcocci et al., 1994) which interacts with oxygen to produce nitric ions that can be estimated by using Greiss reagent. Scavengers of nitric oxide compete with oxygen leading to reduce production of nitric oxide. Sodium nitroprusside (5 mM) in phosphate buffer saline (PBS) was mixed with 3.0 ml of different concentrations (20–200 μg/ml) of the ELES and incubated at 25 °C for 150 min. The samples were added to Greiss reagent (1% sulphanilamide, 2% H_3PO_4 and 0.1% napthylethylenediamine dihydrochloride). The absorbance of the chromaphore formed during the diazotization of nitrite with sulphanilamide and subsequent coupling with napthylethylenediamine was measured at 546 nm and referred to the absorbance of standard solutions of ascorbic acid treated in the same way with Greiss reagent as a positive control. The percentage of inhibition was measured by the following formula:

$$\% \text{ inhibition} = ((A_0 - A_t)/A_0 \times 100)$$

where A_0 was the absorbance of the control (blank, without extract) and A_t was the absorbance in the presence of the extract. All the tests were performed in triplicate and the graph was plotted with the mean values.

2.8. Determination of superoxide radical scavenging activity

This activity was measured using NBT (nitroblue tetrazolium reagent) method as described by Sabu and Ramadasan (2002). The method is based on generation of superoxide radical (O_2^-) by auto-oxidation of hydroxylamine hydrochloride in presence of NBT, which gets reduced to nitrite. Nitrite in presence of EDTA gives a color that was measured at 560 nm. Test solutions of extract (20–200 μg/ml) were taken in a test tube. To this, reaction mixture consisting of 1 ml of (50 mM) sodium carbonate, 0.4 ml of (24 mM) NBT and 0.2 ml of 0.1 mM EDTA solutions were added to the test tube and immediate reading was taken at 560 nm. About 0.4 ml of (1 mM) of hydroxylamine hydrochloride was added to initiate the reaction then

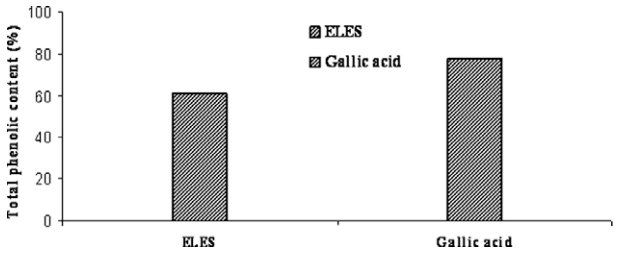

Fig. 1. Presence of total phenolic content in the ethanolic leaf extract of *Stevia rebaudiana*. *ELES: Ethanolic leaf extract of *S. rebaudiana*.

reaction mixture was incubated at 25 °C for 15 min and reduction of NBT was measured at 560 nm. Ascorbic acid was used as the reference compound. Decreased absorbance of the reaction mixture indicates increased superoxide anion scavenging activity. All the extracts of ELES were treated in the similar manner, absorbance was recorded and the percentage of inhibition was calculated according to the following equation:

$$\% \text{ inhibition} = ((A_0 - A_t)/A_0 \times 100)$$

where A_0 was the absorbance of the control (blank, without extract) and A_t was the absorbance in the presence of the samples of the extract. All the tests were performed in triplicate and the graph was plotted with the mean values.

3. Results and discussion

3.1. Total phenolic content

The total amount of phenolic content present in ELES is shown in Fig. 1. In 1 g of ELES, 61.50 mg gallic acid equivalent of phenols was detected. These results suggest that the higher levels of antioxidant activity were due to the presence of phenolic components. The same relationship was also observed between phenolics and antioxidant activity in rosehip extracts (Gao et al., 2000). Phenols are very important plant constituents because of their scavenging ability due to their hydroxyl groups (Hatano et al., 1989). The phenolic compounds may contribute directly to antioxidative action (Duh et al., 1999). It is known that polyphenolic compounds have inhibitory effects on mutagenesis and carcinogenesis in humans when ingested up to 1 g daily from a diet rich in fruits and vegetables (Tanaka et al., 1998). Phenolic compounds from plants are known to be good natural antioxidants. However, the activity of synthetic antioxidants was often observed to be higher than that of natural antioxidants (Ningappa et al., 2007). Phenolic compounds, at certain concentrations, markedly slowed down the rate of conjugated diene formation. The interests of phenolics are increasing in the food industry because they retard oxidative degradation of lipids and thereby improve the quality and nutritional value of food (Aneta et al., 2007).

3.2. Inhibition of DPPH radical

The DPPH radical is considered to be a model for a lipophilic radical. A chain in lipophilic radicals is initiated by the lipid auto-oxidation (Ingold et al., 1993). DPPH is a stable free radical at room temperature and accepts an electron or hydrogen radical to become a stable diamagnetic molecule (Soares et al., 1997). The reduction capability of DPPH was determined by the decrease in its absorbance at 517 nm, which is induced by antioxidants. Positive DPPH test suggests that the samples were free radical scavengers. The scavenging effect of ELES and ascorbic acid on DPPH radical was compared. On the DPPH radical, ELES had significant scavenging effects with increasing concentration in the range of 20–200 μg/ml when compared with that of ascorbic acid, the scavenging effect of ELES was lower. A 200 μg/ml of ELES and ascorbic acid exhibited 68.76% and 82.58% inhibition, respectively and the IC_{50} values were found to be 93.46 and 26.75 μg/ml for ELES and ascorbic acid, respectively (Table 1). The different concentrations of ELES (20, 40, 50, 100 and 200 μg/ml) showed antioxidant activities in a dose dependent manner (36.93%, 41.74%, 48.94%, 62.76% and 68.76% inhibition, respectively) on the DPPH radical scavenging assay (Fig. 2). A higher DPPH radical scavenging activity is associated with a lower IC_{50} value.

3.3. Hydroxyl radical scavenging

The hydroxyl radical is an extremely reactive free radical formed in biological systems and has been implicated as a highly damaging species in free radical pathology, capable of damaging almost every molecule found in living cells (Hochestein and Atallah, 1988). This radical has the capacity to join nucleotides in DNA and cause strand breakage which contributes to carcinogenesis, mutagenesis and cytotoxicity (Manian et al., 2008). Hydroxyl radical scavenging capacity of an extract is directly related to its antioxidant activity (Babu et al., 2001). The highly reactive hydroxyl radicals can cause oxidative damage to DNA, lipids and proteins (Trease and Evans, 1983). The effect of ELES on the inhibition of free radical-mediated deoxyribose damage was assessed by means of the iron(II)-dependent DNA damage assay. The Fentone reaction generates hydroxyl radicals (OH) which degrade DNA deoxyribose, using Fe^{2+} salts as an important catalytic component. Oxygen radicals may attack DNA either at the sugar or the base, giving rise to a large number of products. ELES was also capable of reducing DNA damage at all concentrations used. Ascorbic acid was highly effective in inhibiting the oxidative DNA damage. The % inhibition of ELES (20–200 μg/ml) on hydroxyl radical scavenging was found to be 38.53%, 46.17%, 51.10%, 66.97% and 74.61%, respectively. All results showed antioxidant activity in dose dependent manner. A 200 μg/ml of ELES and ascorbic acid exhibited 74.61% and 72.47% inhibition, respectively (Fig. 3) and their respective IC_{50} values were found to be 81.08 and 71.41 μg/ml (Table 1). The ability of the above mentioned extracts to quench hydroxyl radicals seems to be directly related to the prevention of propagation of the process of lipid peroxidation and seems to be good scavenger of active oxygen species, thus reducing the rate of the chain reaction. Ascorbic acid was used as reference standard. Yen and Hsieh (1995) reported that xylose and lysine maillard reaction products had scavenging activity on hydroxyl radical that depends on dose response manner and which might have been attributed to the combined effects of reducing power, donation of hydrogen atoms and scavenging of active oxygen. Hagerman et al. (1998) have also explained that high molecular weight and the proximity of many aromatic rings and hydroxyl groups are more important for the free

Table 1
Effect of ethanolic leaf extract of *Stevia rebaudiana*, on different radical scavenging activities.

IC_{50} value of ELES							
DPPH radical scavenging activity	AA	Hydroxyl radical scavenging activity	AA	Nitric oxide radical scavenging activity	AA	Superoxide radical scavenging activity	AA
93.46	2675	81.08	71.41	132.05	66.01	109.01	36.69

ELES: Ethanolic leaf extract of *S. rebaudiana* (values in μg/ml).
AA: Ascorbic acid.

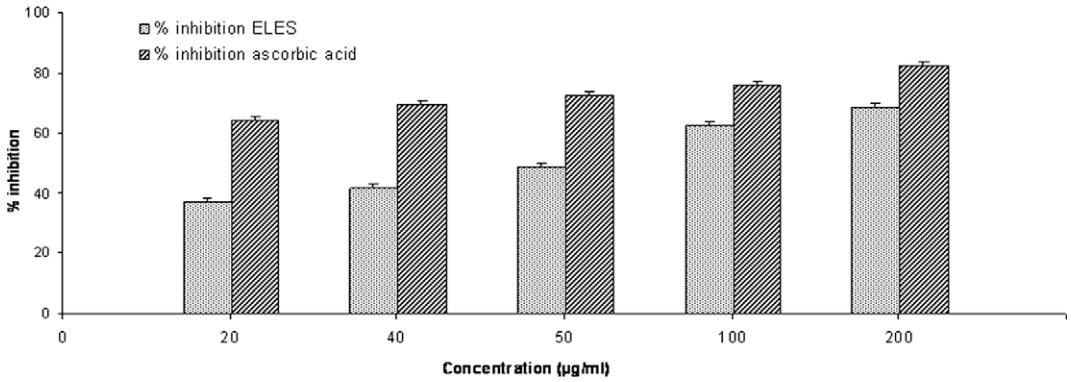

Fig. 2. DPPH radical scavenging activity of the ethanolic leaf extract of *Stevia rebaudiana*. ELES: Ethanolic leaf extract of *S rebaudiana*.

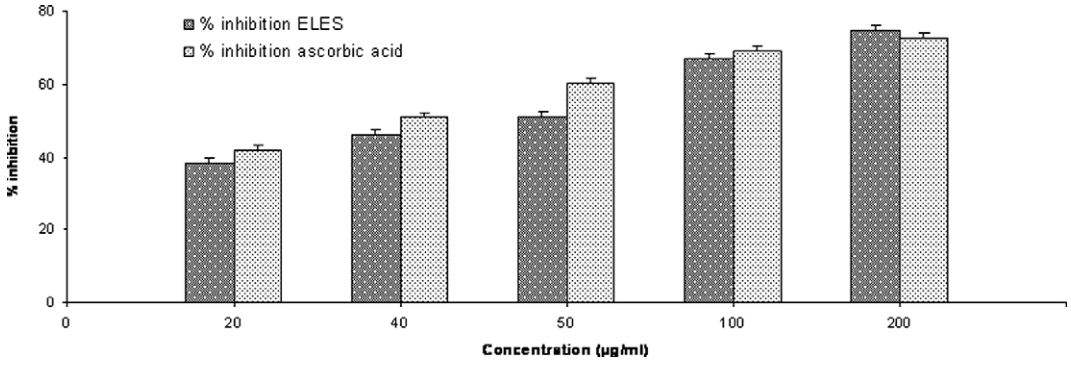

Fig. 3. Hydroxyl radical scavenging activity of the ethanolic leaf extract of *Stevia rebaudiana*. ELES: Ethanolic leaf extract of *S. rebaudiana*.

radical scavenging activity by phenolics than their specific functional groups.

3.4. Nitric oxide radical scavenging

Nitric oxide plays an important role in various types of inflammatory processes in the animal body. In this study, the ethanolic leaf extract of *S. rebaudiana* (ELES) was checked for its inhibitory effect on nitric oxide production. Nitric oxide radical generated from sodium nitroprusside at physiological pH was found to be inhibited by ELES. The various concentrations of ELES (20–200 µg/ml) showed 35.42%, 39.01%, 41.10%, 42.29% and 58.29% inhibition, respectively. Results showed the percentage of inhibition in a dose dependent manner (Fig. 4). A 200 µg/ml of ELES and ascorbic acid exhibited 58.29% and 71.52% inhibition, respectively. The concentration of ELES needed for 50% inhibition (IC_{50}) was found to be 132.05 µg/ml, whereas 66.01 µg/ml was needed for ascorbic acid (Table 1). These results were found to be statistically significant ($P < 0.05$).

3.5. Superoxide radical scavenging

Superoxide is a reactive oxygen species, which can cause damage to the cells and DNA leading to various diseases. It was therefore proposed to measure the comparative interceptive ability of the antioxidant extracts to scavenge the superoxide radical. Several *in vitro* methods are available for generation of superoxide radicals (Vani et al., 1997). In our study, superoxide radicals were generated by auto-oxidation of hydroxylamine in presence of NBT (nitroblue tetrazolium). The reduction of NBT in presence of antioxidants was measured. The decrease of absorbance at 560 nm with antioxidants thus indicates the consumption of superoxide

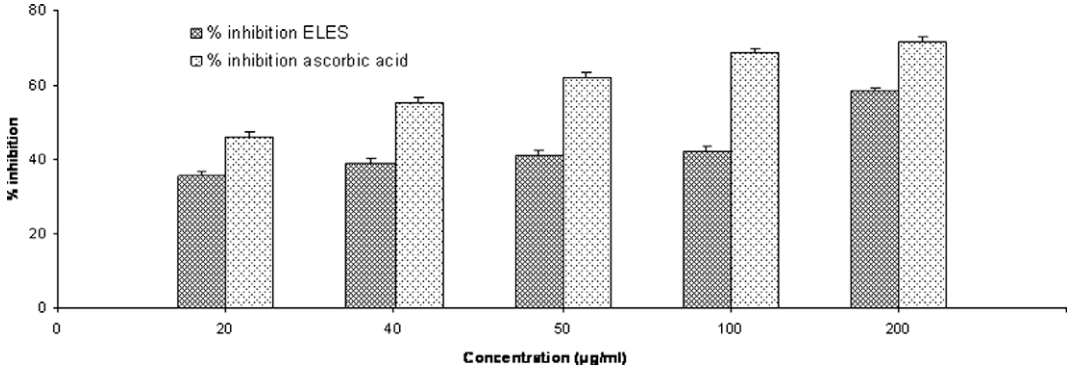

Fig. 4. Nitric oxide radical scavenging activity of the ethanolic leaf extract of *Stevia rebaudiana*. ELES: Ethanolic leaf extract of *S. rebaudiana*.

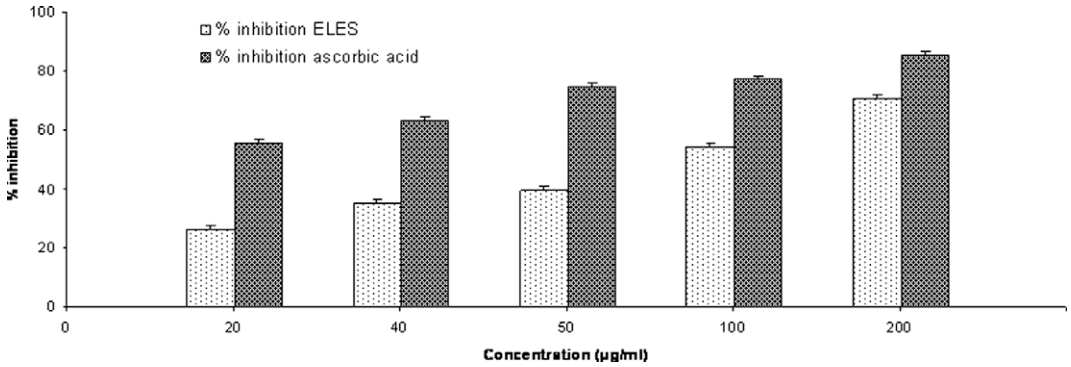

Fig. 5. Superoxide radical scavenging activity of the ethanolic leaf extract of *Stevia rebaudiana*. ELES: Ethanolic leaf extract of *S. rebaudiana*.

anion in the reaction mixture. Different concentrations of ELES (20–200 µg/ml) had strong superoxide scavenging activity (26.19%, 34.75%, 39.40%, 54.44% and 70.84% inhibition, respectively). A 200 µg/ml of ELES and ascorbic acid exhibited 70.84% and 85.42% inhibition, respectively (Fig. 5). IC_{50} value of ELES on superoxide radical scavenging activity was found to be 109.01 µg/ml, whereas the IC_{50} value of ascorbic acid was found to be 36.69 µg/ml (Table 1). All of the extracts had a scavenging activity on the superoxide radicals in a dose dependent manner (20–200 µg/ml in the reaction mixture). Nonetheless, when compared to ascorbic acid, the superoxide scavenging activity of the extract was found to be low. This could be due to the presence of reactive concentration of bioactive constituents and mixture of other nutrients in the extract. Results were found statistically significant ($P < 0.05$).

4. Conclusion

It is well known that free radicals are one of the causes of several diseases, such as Parkinson disease, Alzheimer type dementia, etc. The production of free radicals and the activity of the scavenger enzymes against those radicals such as superoxide dismutase (SOD) are correlated with the life expectancies. We have demonstrated the ethanolic extract of *S. rebaudiana* leaves contained high level of total phenolic compounds and were capable of inhibiting, quenching free radicals to terminate the radical chain reaction, and acting as reducing agents. Furthermore, phenolic compounds present in the plant kingdom are mainly responsible for the antioxidant potential of plants. Accordingly in this study, a significant and linear relationship was found between the antioxidant activity and phenolic content, indicating that phenolic compounds could be major contributors to antioxidant activity. The ethanolic extract of *S. rebaudiana* leaves showed strong antioxidant activity by inhibiting DPPH, hydroxyl radical, nitric oxide, superoxide anion scavenging and hydrogen peroxide scavenging activities when compared with standard ascorbic acid. In addition, the ELES found to contain a noticeable amount of total phenols which plays a major role in controlling antioxidants. Although the antioxidant activities found *in vitro* experiment were only indicative of the potential health benefit, these results remain important as the first step in screening antioxidant activity of *S. rebaudiana* leaves. Thus, it can be concluded that ethanolic leaf extract of *S. rebaudiana* can be used as an accessible source of natural antioxidants with consequent health benefits.

Conflict of interest statement

The authors declare that there was no conflict of interest.

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