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Oxidative DNA Damage Preventive Activity and Antioxidant Potential of *Stevia rebaudiana* (Bertoni) Bertoni, a Natural Sweetener

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At 0.1 mg/mL, the ethyl acetate extract (EAE) of the crude 85% methanolic extract (CAE) of *Stevia rebaudiana* leaves exhibited preventive activity against DNA strand scission by $^{\bullet}OH$ generated in Fenton's reaction on pBluescript II SK (–) DNA. Its efficacy is better than that of quercetin. The radical scavenging capacity of CAE was evaluated by the DPPH test ($IC_{50} = 47.66 \pm 1.04$ μg/mL). EAE was derived from CAE scavenged DPPH ($IC_{50} = 9.26 \pm 0.04$ μg/mL), $ABTS^{+}$ ($IC_{50} = 3.04 \pm 0.22$ μg/mL) and $^{\bullet}OH$ ($IC_{50} = 3.08 \pm 0.19$ μg/mL). Additionally, inhibition of lipid peroxidation induced with 25 mM $FeSO_4$ on rat liver homogenate as a lipid source was noted with CAE ($IC_{50} = 2.1 \pm 1.07$ mg/mL). The total polyphenols and total flavonoids of EAE were 0.86 mg gallic acid equivalents/mg and 0.83 mg of quercetin equivalents/mg, respectively. Flavonoids, isolated from EAE, were characterized as quercetin-3-O-arabinoside, quercitrin, apigenin, apigenin-4-O-glucoside, luteolin, and kaempferol-3-O-rhamnoside by LC-MS and NMR analysis. These results indicate that *Stevia rebaudiana* may be useful as a potential source of natural antioxidants.

KEYWORDS: ***Stevia rebaudiana*; oxidative DNA damage; antioxidant potential; flavonoids**

INTRODUCTION

Stevia, commonly known as sweet leaf or sugarleaf, is a genus of about 150 species of herbs and shrubs in the sunflower family (Asteraceae), native to subtropical and tropical South America and Central America. For centuries, the Guarani tribes of Paraguay and Brazil used *Stevia* species, primarily *S. rebaudiana*, which they called **ka'a he'ê** ("sweet herb"), as a sweetener in yerba mate and medicinal teas for treating heartburn and other ailments. Presently, this herb has become well-known for its high (about 4–20%) sweet diterpene content in the dried mass of the leaf, which includes stevioside, rebaudiosides A–F, and dulcoside A. Stevioside is about 300 times sweeter than common sugar (*1*). It has particular advantages for those suffering from obesity, diabetes mellitus, heart disease, and dental caries (*2*). The leaves of this small green plant, which is particularly very popular in Japan where it has been widely used as a sweetener for over 35 years, have a delicious and refreshing taste that can be 30 times sweeter than sugar. In 2006, the World Health Organization (WHO) performed a thorough evaluation of recent experimental studies of stevioside and steviols conducted on animals and humans, and concluded that "stevioside and rebaudioside A are not genotoxic in vitro or in vivo and that the genotoxicity of steviol and some of its oxidative derivatives in vitro is not expressed in vivo" (*3*).

Recently, much attention has been focused on dietary natural antioxidants capable of inhibiting reactive oxygen radical mediated oxidative stress, which is involved in several pathological diseases, such as cancer, atherosclerosis, diabetes, inflammation, and aging (*4*, *5*). Exogenous dietary antioxidants, called nutraceuticals, are capable of scavenging free radicals and have shown promise in preventing certain disease conditions (*6*). Dietary antioxidants can increase cellular defense and help prevent oxidation damage to cellular components (*7*). Among natural antioxidants, plant polyphenols play a very important role (*8*). Flavonoids are a class of compounds that has been and still is an area of tremendous interest from a scientific as well as therapeutic point of view, out of which the antioxidant activity of the flavonoids has attracted the maximum attention. This group of polyphenolic compounds is widely and ubiquitously found in fruits, vegetables, grains, nuts, seeds, spices, medicinal plants, and in some commonly consumed beverages such as tea, red wine, and beer. Flavonols such as quercetin, myricetin, isorhamnetin, and kaempferol (**Figure 1**) and the corresponding flavones, apigenin and luteolin (**Figure 2**), have been well-established as potent antioxidants that prevent oxidation of low-density lipoprotein and that inhibit lipid peroxidation (*9*). The flavones apigenin and luteolin are predominant in cereal grains and aromatic herbs (*10*). On the other hand, the flavonols quercetin and kaempferol are common in fruits and vegetables. Most of the beneficial health effects of flavonoids are attributed to their antioxidant and chelating abilities; the protective effects

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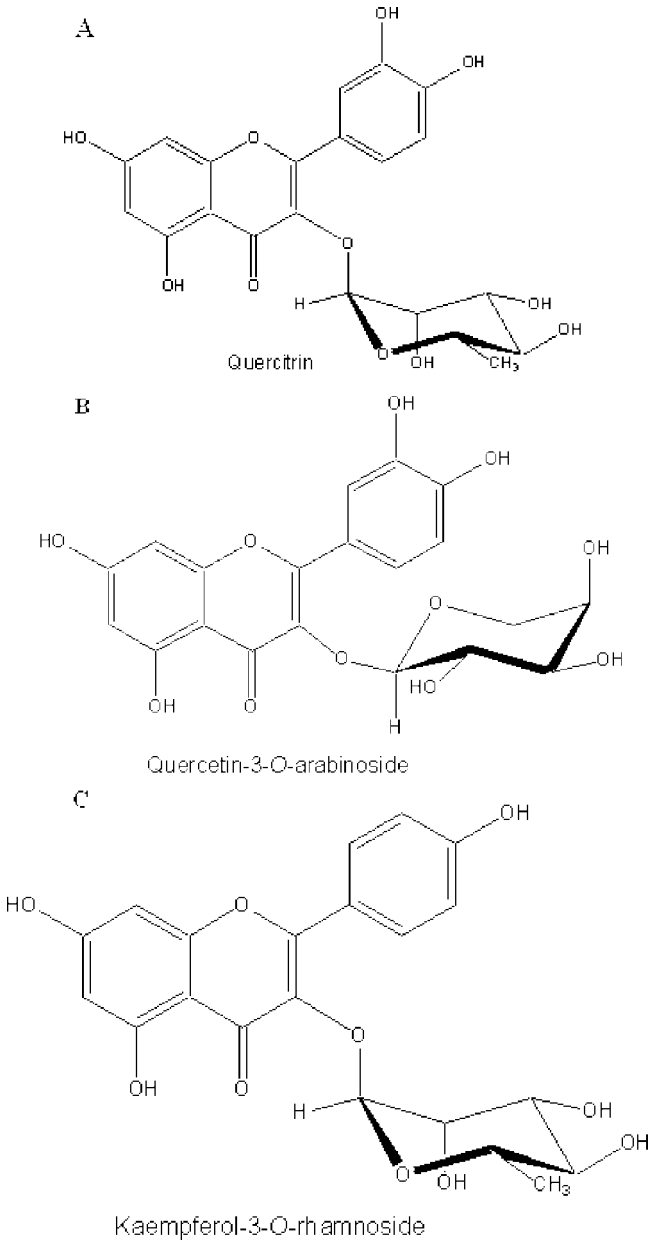

Figure 1. Flavonols from *Stevia rebaudiana* (Bertoni) Bertoni.

can be ascribed to their capacity to transfer electron free radicals, chelate metal catalysts, activate antioxidant enzymes, reduce α-tocopherol radicals, and inhibit oxidases (*11*).

To date, the sweet diterpenes are the major area of research as far as *S. rebaudiana* is concerned (*12*). The antioxidant activity of this herb has been evaluated but not in detail (*13, 14*). In this study, we evaluated the oxidative DNA damage preventive activity and antioxidant potential of the crude methanolic extract (CAE) and flavonoid-rich ethyl acetate extract (EAE) of *S. rebaudiana* leaves. We found that the crude extract of *S. rebaudiana* could scavenge reactive oxygen species (ROS) and prevent DNA strand scission by $^{\bullet}$OH generated in the Fenton reaction on pBluescript II SK (–) DNA. We have isolated six known flavonoid glycosides from EAE (*15*) and have identified the prospective active compounds responsible for oxidative DNA damage preventive activity and antioxidant potential. This research also provides information on *Stevia* and/or their extracts as a food additive and its health-promoting potential.

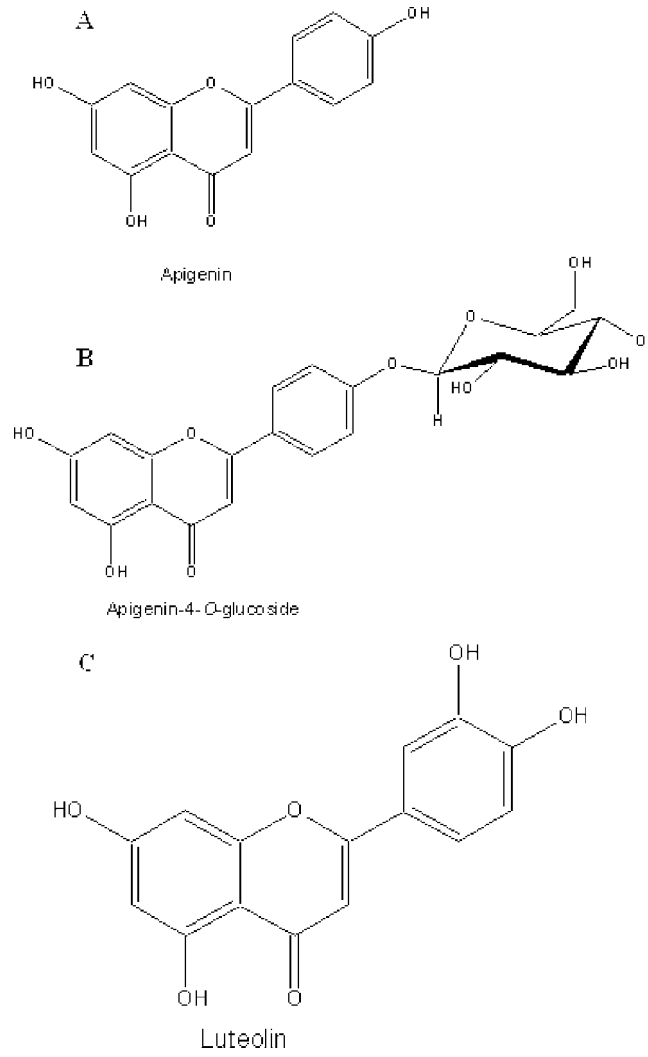

Figure 2. Flavones from *Stevia rebaudiana* (Bertoni) Bertoni.

MATERIAL AND METHODS

Plant Material. *Stevia rebaudiana* (Bertoni) Bertoni was collected from Jhargram area of West Bengal and was taxonomically identified in the Botanical Survey of India (BSI), Shibpur, Howrah, West Bengal, India. The voucher specimen (vide No. SR 51, dated June 22nd, 2007) has been submitted to BSI, Howrah, West Bengal.

Chemicals. Silica gel (60–120 mesh, SRL, India) and thin-layer chromatography (TLC) plate (Kieselgel 60 F254 5715, Merck) were used for column chromatography and analytical/preparative TLC, respectively. 2,2-Diphenyl-1-picrylhydrazyl (DPPH), thiobarbituric acid (TBA), trichloroacetic acid (TCA), Folin–Ciocalteu's phenol reagent, quercetin dihydrate, butylated hydroxytoluene, agarose, and ethidium bromide were purchased from Sigma-Aldrich, USA. 2,2′-Azinobis- (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS), potassium persulphate, aluminum chloride, iron (III) chloride, and iron (II) sulfate were obtained from ICN Biomedicals, Germany. 2-Deoxy-D-ribose and ascorbic acid were procured from Himedia Laboratories Pvt. Ltd., Mumbai, India. The QIAprep Spin Miniprep Kit was purchased from Qiagen. All other chemicals and reagents used were of analytical grade. Stevioside was obtained as a gift from Prof. Lin Pen-Yuan, Taipei Medical University.

Extraction. Leaves (120 g, fresh weight) of *Stevia rebaudiana* (Bertoni) were washed in cold tap water, shade-dried, ground and extracted with 3.6 L of 85% methanol (3 × 1 day). The pooled methanol portion was then evaporated under reduced pressure in a rotary vacuum evaporator. The rest (aqueous portion, approximately 400 mL) was partitioned with hexane (1 L), chloroform (2 L), and ethyl acetate (2 L). The extracts were concentrated at room temperature resulting in hexane (2 g), chloroform (3.5 g), and ethyl acetate (2.5 g) extracts using

a rotary vacuum evaporator. The EAE was used for further analysis. The remaining aqueous layer was lyophilized and was used for the DPPH test.

Chromatographic Separation of EAE and Characterization of Flavonoids. The EAE (2.5 g) was separated into fractions by SiO_2 column chromatography using a mixed solvent of ethyl acetate/ethyl methyl ketone/methanol/water (5:3:3:1). Six fractions were sequentially obtained from this column, which are the mixtures of flavonoids. These six fractions were further separated by preparative TLC using the same solvent system with the following yields: quercitrin (112 mg), quercetin-3-*O*-arabinoside (88 mg), apigenin (11 mg), apigenin-4-O-glucoside (10 mg), luteolin (15 mg), and kaempferol-3-O-rhamnoside (12 mg), which were in close agreement with the previous report (*15*). Each compound was characterized by LC-MS/MS and ^{1}HNMR.

Structural Elucidation of Isolated Compounds. *Quercetin-3-O-arabinoside.* ^{1}H NMR (DMSO-d_6, 300 MHz, ppm): δ 3.21 (1 H, *dd*, J = 11.5 and 2.5, H-5a″), 3.51 (1 H, *dd*, J = 7.0 and 3.5, H-3″), 3.59 (1 H, *dd*, J = 11.5 and 5.5, H-5b″), 3.64 (1 H, *m*, H-4″), 3.75 (1 H, *dd*, J = 8.3 and 5.0, H-2″), 5.27 (1 H, *d*, J = 5.0, H-1″), 6.19 (1 H, *d*, J = 2.0, H-6), 6.39 (1 H, *d*, J = 2.0, H-8), 6.84 (1 H, *d*, J = 8.5, H-5′), 7.50 (1 H, *d*, J = 2.5, H-2′), 7.66 (1 H, *dd*, J = 8.5 and 2.5, H-6′). LC-MS (positive ion) *m/z*: 457 $[M + Na]^+$.

Quercitrin. ^{1}H NMR (CD_3OD, 300 MHz, ppm): δ 7.33 (1 H, *s*), 7.27 (1 H, *d*, J = 7.5), 6.90 (1 H, *d*, J = 6.5),6.13(1 H, *s*), 5.34 (1 H, *s*), 4.22 (1 H, *s*), 3.76 (1 H, *bdr*, J = 7.5), 3.43 (1 H, *m*), 3.31 (1 H, *dd*, J = 3.5, 2), 0.94 (3 H, *d*, J = 6). LC-MS (positive ion) *m/z*: 487 $[M + Na]^+$.

Apigenin. ^{1}H NMR (CD_3OD, 300 MHz, ppm): δ 7.88 (2 H, *d*, J = 9 2′, 6′ H), 6.90 (2 H, *d*, J = 9 3′-, 5′-H), 6.83 (1 H, *s*, 3-H), 6.65 (1 H, *s*), 6.50 (1 H, *s*), 5.08 (1 H, *d*, J = 7.2, 1″ H), 3.94 (1 H, *dd*, J = 12.5, 2.4, H_A-6″), 3.72 (1 H, *dd*, J = 12.5, 5.4, H_B-6″), 3.4–3.6 (several protons). LC-MS (positive ion) *m/z:* 293 $[M + Na]^+$.

Apigenin-4-O-glucoside. ^{1}H NMR (CD_3OD, 300 MHz, ppm): δ 7.91 (2 H, *d*, J = 8.7, 2′-, 6′-H), 6.95 (2 H, *d*, J = 8.7, 3′-, 5′-H), 6.85 (1 H, *s*, 3-H), 6.63 (1 H, *s*), 6.53 (1 H, *s*). LC-MS (positive ion) *m/z:* 455 $[M + Na]^+$.

Luteolin. ^{1}H NMR (DMSO-d_6, 300 MHz, ppm): δ 7.42 (2 H, dJ = 7, 2′-, 6′-H), 7.4 (1 H, *s*), 6.92 (2 H, *d*, J = 7, 3′-, 5′-H), 6.80 (2 H, *d*, J = 2), 6.73 (1 H, *s*), 6.46 (2 H, *d*, J = 2). LC-MS (positive ion) *m/z:* 309 $[M + Na]^+$.

Kaempferol-3-O-rhamnoside. ^{1}H NMR (CD_3OD, 300 MHz, ppm): δ 7.78 (2 H, *d*, J = 8.4, 2′-, 6′-H), 6.95 (2 H, *d*, J = 8.4, 3′-,5′-H), 6.19 (1 H, *s*), 6.36 (1 H, *s*), 5.38 (1 H, *s*,1″-H), 0.94 (3 H, *d*, J = 4, 6″-H_3). LC-MS (positive ion) *m/z*: 455 $[M + Na]^+$.

Determination of Total Polyphenol and Total Flavonoid. Total polyphenols were estimated, and the results were expressed as gallic acid equivalents after modifications (*16*). Thus, 2 mL of 2% Na_2CO_3 was added to 100 μl of test samples containing different concentrations of EAE, and the samples were incubated at 25 °C for 2 min. Then, 100 μl of 50% Folin–Ciocalteu's phenol reagent was added to the mixture, and the contents were mixed thoroughly. Absorbance of the samples was taken after 30 min at 720 nm with a Shimadzu UV–vis spectrophotometer.

Total flavonoid content was estimated, and the results were expressed as quercetin equivalents (*17*). Different concentrations of EAE and quercetin were diluted to 2.5 mL with distilled water, and 150 μl of 5% $NaNO_2$ was added to it. The resulting mixture was incubated at 25 °C for 5 min. Then, 1.5 mL of 10% $AlCl_3$ was added to the mixture, and the solution was incubated for 6 min. The contents were mixed properly after the addition of 1 mL of 1N NaOH, and the absorbance was noted at 510 nm. Because EAE is rich in flavonoids, quercetin was used to construct the calibration curve.

Determination of Radical Scavenging Activity by DPPH. The free-radical scavenging activity of CAE and four extracts was evaluated using DPPH. Stock solutions of CAE and four extracts at 10 mg/mL and a freshly prepared DPPH solution (100 mM) were used as previously described (*18*). The control solution did not contain any test sample. Quercetin was used as a standard. The percent radical scavenging activity (% RSC) was calculated using the formula shown below.

$$\%\ \mathrm{RSC} = [(A_{\mathrm{Control}} - A_{\mathrm{Sample}})/A_{\mathrm{Control}}] \times 100\%$$

Determination of Radical Scavenging Activity by ABTS$^+$. ABTS radical scavenging activity was performed with CAE as well as with EAE (*19*). The ABTS stock solution was prepared by reacting ABTS (7 mM) and potassium persulphate (2.45 mM) and allowing the mixture to stand for at least 16 h to generate $ABTS^+$ free radicals. The working solution was prepared by diluting the stock solution with methanol such that its absorbance reached 0.7 ± 0.02 at 734 nm (A_{Control}). The reaction was performed in a 1 mL volume containing different concentrations of the extracts in 10 μl volumes and 990 μl of ABTS working solution. Their absorbance (A_{Sample}) was noted at 734 nm exactly 6 min after the reaction mixture was prepared. Quercetin was used as a standard. The % RSC value was calculated as previously described (*18*).

Deoxyribose Assay to Assess $^{\bullet}$OH Scavenging Activity. The assay involves the formation of a chromogen (malondialdehyde-like compound) by the degradation of deoxyribose in the presence of a low concentration of iron salt and reacting with thiobarbituric acid (*20*). The reaction mixture consisted of different concentrations of CAE and EAE (15–40 μg/mL and 2–10 μg/mL, respectively), 3.6 mM deoxyribose, 0.1 mM EDTA, 0.1 mM L-ascorbic acid, 1 mM H_2O_2 and 0.1 mM $FeCl_3 \cdot 6H_2O$, and the volume was increased to 500 μl with 25 mM phosphate buffer at pH 7.4. After incubating for 1 h at 37 °C, 500 μl of 1% TBA and 500 μl of 1% TCA were added to this mixture, which was heated in a boiling water-bath for 15 min and then cooled. The absorbance was noted at 532 nm. The control reaction was devoid of test sample, and quercetin (20 μg/mL) was used as a standard. % RSC was calculated as described above.

Determination of Preventive Activity against Oxidative Damage of pBS Plasmid DNA. The determination of the oxidative DNA damage preventive activity of CAE and EAE was performed as described (*21*) with some minor modifications. Plasmid DNA was isolated using a QIAprep Spin Miniprep Kit, according to manufacturer's instructions. In brief, 250 ng of plasmid pBluescript II SK (–) was treated with $FeSO_4$, H_2O_2, and phosphate buffer (pH 7.4) to final concentrations of 0.5 mM, 25 mM, and 50 mM, respectively, and test samples at different concentrations. The total reaction volume of 12 μl was incubated for 1 h at 37 °C. After the incubation, the extent of DNA damage and the preventive effect of the test samples were analyzed on 1% agarose gel at 70 V at room temperature. Quercetin (1 mM) was used as a standard.

Densitometric Analysis of Treated and Control pBS Plasmid DNA. Gel was scanned on a Gel documentation system (GelDoc-XR, Bio-Rad, Hercules, CA, USA). Bands on the gels were quantified using discovery series Quantity One 1-D analysis software (Bio-Rad).

Antilipoperoxidant Activity of Crude Extract by TBA Reactive Substances Assay. *Preparation of Rat Liver Homogenate, 10% w/v.* For this assay, liver homogenate was prepared as previously described (*18*). In brief, livers of adult male Wistar rats were excised, perfused, and homogenized in 50 mM phosphate buffer, pH-7.4, containing 120 mM KCl in a ratio of 1:10 w/v. The supernatant was collected after centrifuging the homogenate at 700*g* for 10 min at 4 °C and was stored at −20 °C until use.

TBA Reactive Substances (TBARS) Assay. The TBARS assay determines the extent of lipid peroxidation, wherein the decrease in absorbance of the test samples as compared to the control evaluates the extent of protection. The reaction mixture was prepared by adding $FeSO_4$ (final concentration of 250 μM) and different concentrations of the extract to 200 μl of liver homogenate, and the volume was increased to 250 μl with deionized water. The control sample was devoid of any test samples. This mixture was incubated at 37 °C for three hours. A 0.67% TBA solution was added to 75 μl of this incubated reaction mixture to a final volume of 2 mL; the mixture was heated in a boiling water-bath for 20 min, cooled on ice, and centrifuged at 5000 rpm for 10 min at room temperature. The absorbance of the supernatant was noted at 532 nm. Butylated hydroxytoluene (BHT) was used as a standard. The % antioxidant index (%AI) was calculated using the equation shown below.

$$\%\mathrm{AI} = [(A_{\mathrm{Control}} - A_{\mathrm{Sample}})/A_{\mathrm{Control}}] \times 100\%$$

Statistical Analysis. All in vitro tests were performed in three replicates, and all values were expressed as mean ± standard deviation

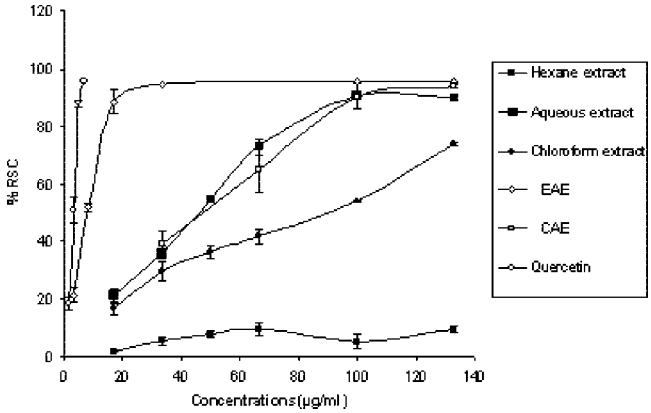

Figure 3. The concentration-dependent (5–130 μg/mL) DPPH° scavenging activity of CAE and different extracts derived from it (mean ± SD, $n = 3$).

Table 1. Scavenging Activity (IC_{50}) of CAE and different extracts derived from CAE against DPPH°

CAE	47.66 ± 1.04
hexane extract	327.17 ± 13.84
chloroform extract	76.74 ± 2.57
EAE	9.26 ± 0.04
aqueous extract	45.32 ± 0.41
quercetin	3.35 ± 0.07

(SD). The 50% inhibitory concentration (IC_{50}) values were calculated by regression analysis. Standard differences were considered significant at $P < 0.05$.

RESULTS

DPPH Radical Scavenging Activity of CAE and the Four Extracts Derived from CAE. The DPPH radical scavenging activity of CAE and the four extracts derived from CAE was performed, and their relative antioxidant activity, in a concentration-dependent manner, is shown in **Figure 3**. Preliminary screening was performed using this assay to find out the extract having the best antioxidant activity. The IC_{50} values of CAE and the four extracts derived from CAE were calculated (**Table 1**). Because EAE had the highest radical scavenging activity and because it has been reported to be rich in flavonoids (*15*), further bioactivity studies were carried out with this.

Analysis of Total Phenols and Total Flavonoids. The average contents of total polyphenols and total flavonoids in EAE were 0.86 mg gallic acid equivalents/mg of dry weight and 0.83 mg of quercetin equivalents/mg of dry weight, respectively.

Effect of $ABTS^+$ Scavenging Activity of Crude Extracts and the Bioactive Extract. The free-radical scavenging potentials of CAE and EAE were determined by $ABTS^+$ scavenging activity. The comparative profile of the different extracts is shown (**Figure 4**). The IC_{50} values of CAE and EAE were 28.6 ± 0.64 and 3.04 ± 0.22 μg/mL, respectively, as compared to that of 1.3 ± 0.06 μg/mL for quercetin (**Table 2**).

$^{\bullet}$OH Scavenging Activity of CAE and EAE by Deoxyribose Assay. The $^{\bullet}$OH scavenging potentials manifested by CAE and EAE were also evaluated by the decrease in formation of the chromogen by the Fenton reaction. The % RSA of the bioactive extract EAE is shown in **Figure 5**. The IC_{50} value of CAE was 33.9 ± 9.58 μg/mL, and that of EAE was 3.08 ± 0.19 μg/mL. The $^{\bullet}$OH scavenging activity of EAE correlates with its DNA damage protecting activity.

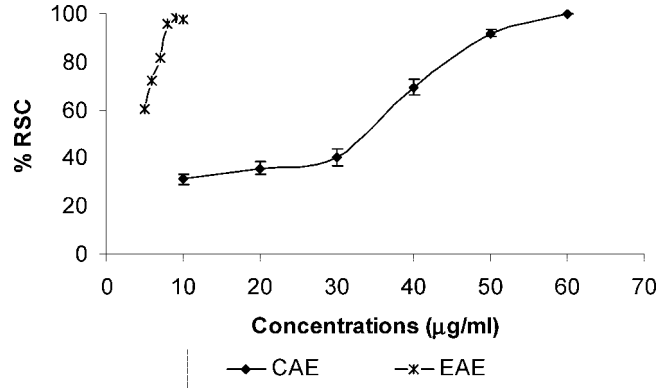

Figure 4. $ABTS^+$ scavenging activity in a concentration-dependent manner of CAE (10–60 μg/mL) and EAE (5–10 μg/mL) of *Stevia rebaudiana* (mean ± SD, $n = 3$).

Table 2. Comparative Study of IC_{50}

	IC_{50} (μg/mL)		
assay	CAE	EAE	quercetin
DPPH scavenging activity	47.66 ± 1.04	9.26 ± 0.04	3.35 ± 0.07
$ABTS^+$ scavenging activity	28.6 ± 0.64	3.04 ± 0.22	1.3 ± 0.06
$^{\bullet}$OH scavenging activity	33.9 ± 9.58	3.08 ± 0.19	7.45 ± 0.43

Oxidative DNA Damage Preventive Activity of CAE and EAE. The protective effects of CAE and EAE were checked on Fenton reaction-induced damage of pBluescript II SK (−) supercoiled DNA maintained in *E.coli*, XL-1 strain. Control pBS DNA showed two bands, one of open circular that was hardly visible and the other of supercoiled. Treatement with $FeSO_4$ and H_2O_2 in the absence of any extract led to the formation of open circular DNA by the strand scission of supercoiled DNA, whereas the presence of CAE or EAE prevented this strand scission to a considerable extent in comparison to quercetin (**Figure 6**). Stevioside, the principal sweetening agent in *Stevia*, was also used to check its activity against the prevention of DNA strand scission. However, no such activity was noted, as reflected in **Figure 6**. Densitometric analysis confirmed the experimental data (**Figure 7**).

Effect of CAE on Lipid Peroxidation by the TBARS Assay. The inhibition of lipid peroxidation induced by $FeSO_4$ in rat liver homogenate was assayed by measuring the lipid oxidation products such as TBARS. CAE exerted its antilipoperoxidant activity in a concentration-dependent manner (**Figure 8**). At 1 mg/mL, it was able to inhibit TBARS formation by 32% (AI%), as compared to standard BHT activity (AI% 87.7% at 0.5 mg/mL); the IC_{50} value was 2.1 ± 1.07 mg/mL. However, the antilipoperoxidant activity study of EAE was not carried out because of the low activity of CAE.

DISCUSSION

The antioxidant potential of CAE from *S. rebaudiana* (Bertoni) leaf was evaluated by DPPH, $ABTS^+$, and $^{\bullet}$OH scavenging activity. **Table 1** shows the comparative study of the IC_{50} of the radical scavenging activity of CAE and further extracts derived from it. The concentrations of CAE required to scavenge 50% of DPPH, $ABTS^+$, and $^{\bullet}$OH was 47.66 ± 1.04, 28.6 ± 0.64, and 33.9 ± 9.58 μg/mL, respectively (**Table 2**). The corresponding values for EAE were 9.26 ± 0.04, 3.04 ± 0.22, and 3.08 ± 0.19 μg/mL, respectively. The IC_{50} values were calculated from regression analysis. It was observed that the flavonoid-rich sample EAE had the highest potential in terms

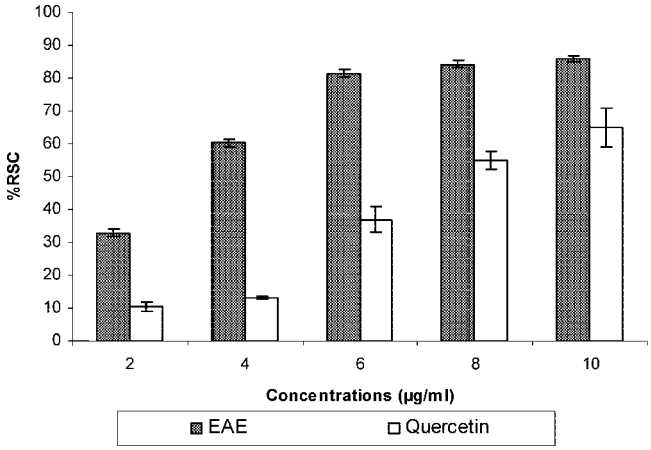

Figure 5. Effect of •OH scavenging activity of EAE (2–10 μg/mL) by deoxyribose assay (mean ± SD, $n = 3$).

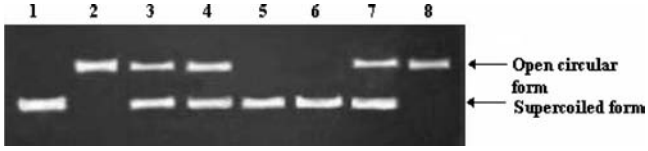

Figure 6. Electrophoretic pattern of pBluescript II SK (–) DNA breaks by •OH generated from the Fenton reaction and prevented by CAE and EAE. Lane 1: untreated control DNA (250 ng), lane 2: $FeSO_4$ (0.5 mM) + H_2O_2 (25 mM) + DNA (250 ng), lane 3: only H_2O_2 (25 mM) + DNA (250 ng), lane 4: only $FeSO_4$ (0.5 mM) + DNA (250 ng), lanes 5–8: $FeSO_4$ (0.5 mM) + H_2O_2 (25 mM) + DNA (250 ng) in the presence of EAE (1 μg), CAE (10 μg), Quercetin (1 mM), and Stevioside (100 μg), respectively ($n = 3$).

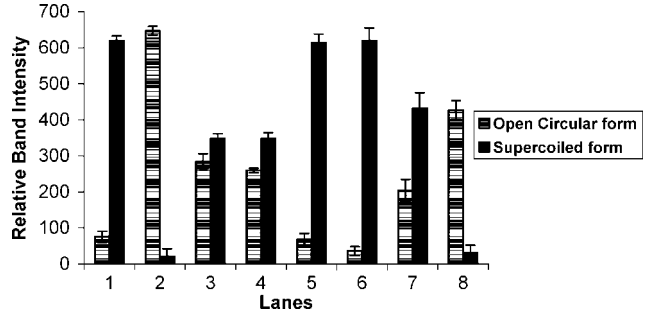

Figure 7. Densitometric analysis of open circular and supercoiled DNA damage induced by •OH generated from the Fenton reaction in the presence or absence of CAE and EAE (mean ± SD, $n = 3$).

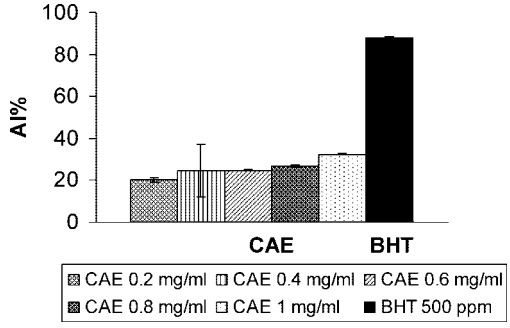

Figure 8. Inhibition of lipid peroxidation in a concentration-dependent manner (0.2–1 mg/mL) by CAE, and the standard antioxidant BHT (mean ± SD, $n = 3$).

of radical scavenging capacity. The DPPH model provides a method for evaluating antioxidant activity in a relatively shorter time as compared to other methods. The disappearance of DPPH is directly proportional to the amount of antioxidant present in the reaction mixture.

Decolorization of $ABTS^+$ also reflects the capacity of an antioxidant species to donate electrons or hydrogen atoms to inactivate this radical cation. In the $ABTS^+$ decolorization assay, potential activity of EAE was noted at 8.0 μg/mL, as compared to that of CAE at 50 μg/mL (**Figure 4**). CAE was effective in the hydroxyl radical scavenging assay, also in a concentration-dependent manner (data not shown). Hydroxyl radical scavenging activity of EAE was noted at 10.0 μg/mL, showing 85% inhibition (**Figure 5**), whereas the same inhibition was noted with CAE at 68.6 μg/mL. This ability of EAE to scavenge free radicals may contribute to its significant antioxidant potential. Hydroxyl radicals produced in the Fenton reaction can damage DNA (*21*). We report here that EAE exerts protective effect at 0.1 mg/ mL against free-radical induced oxidative damage of DNA. CAE also showed a protective effect but at 1 mg/ mL (**Figure 6**). Densitometric analysis by relative band-intensity further manifested the preventive activity of EAE (**Figure 7**).

Flavonoids have excellent antioxidant potential due to the fact that they have the capacity to scavenge harmful ROS and other free radicals that originate from various cellular activities and lead to oxidative stress (*22*). The mechanism of action of flavonoids is multifold; it includes the inhibition of enzymes that are involved in ROS generation, chelating of trace metals such as free iron and copper, and the ability to reduce highly oxidizing free radicals by donation of hydrogen atom, thus protecting us from various serious diseases such as heart attack, stroke, and even cancer. This study reports the antioxidant activity of the sweet woody herb *Stevia rebaudiana*. EAE from the methanol extract of the leaves had the highest antioxidant potential as compared to the other extracts. The compounds of this extract were isolated, and the findings completely agreed with the previous report (*15*), which reported six flavonoids from EAE. In addition, another flavone (apigenin) was also isolated and characterized from EAE.

In our body, the Fenton reaction is one of the major sources of •OH, which is produced near the DNA molecules in the presence of transition metal ions such as iron and copper (*23*). As previous reports suggest, polyphenol-rich diets may decrease the risk of chronic diseases by reducing oxidative stress (*24*). Flavonoids are a diverse group of compounds ranging from flavan-3-ol to flavonol, flavanone, etc. In this work, we were successful in isolating three flavones, namely, luteolin, apigenin, and apigenin-4-O-glucoside, and three flavonols, quercetin-3-O-arabinoside, kaempferol-3-O-rhamnoside, and quercitrin. It is known that flavonoids are strong antioxidants because of their structural chemistry. Three structural groups, namely, the *o*-dihydroxy (catechol) structure in the B-ring, the 2,3- double bond in conjugation with a 4-oxo function, and the 3- and 5-hydroxyl groups are important determinants for radical scavenging and/or antioxidative potential (*22*). The strong hydroxyl radical scavenging property of EAE, in comparison to CAE and other extracts, was suggestive of the presence of antioxidant flavonoids, namely quercetin, in its glycosylated form (quercetin-3-O-arabinoside), quercitrin, kaempferol-3-O-rhamnoside, apigenin, luteolin, and apigenin-4-O-glucoside. DNA damage induced by the Fenton reaction was prevented because of the presence of hydroxyl radical scavenging flavonoids (*25*). So, the probable mechanism of DNA damage prevention is the scavenging of •OH, a DNA damage causative agent, by CAE and EAE. Further, it can be stated that, in comparison to flavones, flavonols are far more potent antioxidants because of their stable resonating structure after donating hydrogen atoms to the free radicals. Quercetin is the most active flavonol in terms of antioxidant activity because it satisfies

all the three parameters of a strong antioxidant, that is, the presence of a catechol moiety in the B-ring, the presence of 3- and 5-OH group, and the presence of a 2,3 double bond in conjugation with a 4-oxo function. Glycosylation did decrease the antioxidative potential by a small degree (*26*). Antioxidant activity of quercetrin lies close to its parent compound quercetin (*26*). However, the flavonoid glycosides (which are also the main components of EAE) are more efficiently absorbed from the intestinal membrane than the aglycone residues (*27*). Therefore, the major effective antioxidant components here in EAE are quercitrin and quercetin-3-O-arabinoside. Kaempferol-3-O-rhamnoside contains a rhamnose moiety at the C-3 position of the parent compound kaempferol; it lacks the strong antioxidant nature of quercetin because of the absence of 3-OH group, but the other two characteristics provide it with antioxidant property. Other than these flavonols, the flavones present in the EAE also are responsive for it is antioxidant nature. In the case of flavones, luteolin is a stronger antioxidant than apigenin because of the presence of the catechol moiety in its B-ring (*28*). Hence, it may be concluded that the strong radical scavenging activity and oxidative DNA damage preventive activity of EAE may be correlated with its rich content of flavonoids. Thus, the use of this noncaloric sweetener will provide additional benefits in terms of oxidative DNA damage preventive activity and antioxidant potential.

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