



## The antioxidant activity and the bioactive compound content of *Stevia rebaudiana* water extracts

Il-Suk Kim<sup>a</sup>, Mira Yang<sup>a</sup>, Ok-Hwan Lee<sup>b</sup>, Suk-Nam Kang<sup>a,\*</sup>

<sup>a</sup>Department of Animal Resources Technology, Gyeongnam National University of Science and Technology, Gyeongnam, South Korea

<sup>b</sup>Department of Food Science and Biotechnology, Kangwon National University, Chuncheon 200-701, South Korea

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### ABSTRACT

*Stevia rebaudiana* (SR), a chrysanthemum herb, has been used as a vegetable-based sweetening additive in health drinks and in other foods. This study was conducted to investigate the antioxidant activity and the bioactive compounds found in water extracts taken from SR leaves and calli. Analysis of vitamins in the leaves showed that folic acid (52.18 mg/100 g) was a major component, followed by vitamin C. The total phenolic and flavonoid contents were found to be 130.76 µg catechin and 15.64 µg quercetin for leaves and 43.99 µg catechin and 1.57 µg quercetin for callus at mg of water extracts, respectively. Pyrogallol was the major material among the phenolic compounds in both leaf and callus extracts. Furthermore, our results showed that the leaf extracts contained higher amounts of free radicals, hydroxyl radicals and superoxide anion radical scavenging activities than those of the callus extracts.

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### 1. Introduction

The reactive oxygen species (ROS) that induce autoxidation and the thermal oxidation of lipids are associated with aging and membrane damage in living organisms. The production of ROS includes the superoxide anion ( $O_2^{\cdot-}$ ), hydrogen peroxide ( $H_2O_2$ ), hydroxyl radicals ( $HO\cdot$ ) and singlet oxygen ( $^1O_2$ ) which causes degenerative human diseases such as cancer, heart disease, and cerebrovascular disease through multiple mechanisms (Huang, Ou, & Priop, 2005; Kang & Hamasaki, 2003).

Recently, natural foods and food-derived antioxidants, such as vitamins and phenolic phytochemicals, have received growing attention because they are known to function as chemopreventive agents against oxidative damage (Carrasco-Pancorbo et al., 2005; Perez-Bonilla et al., 2006; Valavanidis et al., 2004). Plants constitute an important source of active natural products that differ widely in terms of their structure and their biological properties. In recent years, the prevention of cancer and cardiovascular diseases has been associated with the ingestion of fresh fruits, vegetables or teas that are rich in natural antioxidants (Virgili, Scaccini, Packer, & Rimbach, 2001). The protective effects of plant products are due to the presence of several components that have distinct mechanisms of action; some are enzymes and proteins, while others are low molecular weight compounds such as vitamins, carotenoids,

flavonoids, anthocyanins and other phenolic compounds (Amro, Aburjai, & Al-Khalil, 2002; Park, Lee, Park, Ahn, & Han, 2005).

Stevioside is a diterpene glycoside ( $C_{38}H_{60}O_{18}$ ) present in *Stevia rebaudiana* (SR), and it is an abundant component of the leaf. Stevioside has become well known for its intense sweetness (250–300 times sweeter than sucrose), and it is used as a non-caloric sweetener in several countries (Chatsudthipong & Muanprasat, 2009). In addition, the *Stevia* plant and stevioside have been used as substitutes for sucrose in the treatment of diabetes mellitus (Chen et al., 2006; Jeppesen, Gregersen, Poulsen, & Hermansen, 2000), obesity, hypertension (Hsieh et al., 2003), inflammation and cancer (Sehar, Kaul, Bani, Pal, & Saxena, 2008). However, the compositions of phenolics and vitamins and antioxidant activities in the extract of SR are not well known (Komissarenko, Derkach, Kovalyov, & Bublik, 1994; Tadhani, Patela, & Subhasha, 2007). In this study, we investigated the total phenolic compounds, the vitamin content and the antioxidant activity of SR water extracts taken from the leaves and the calli.

### 2. Materials and methods

#### 2.1. Preparation of water extract from SR

The *Stevia rebaudiana* plants used in this study were supplied by the Gochang farm (Chunbuk, Korea). After harvest, the leaves and calli of the SR plants were removed, washed with distilled water and dried at  $40 \pm 5$  °C for 12 h. The dried leaf and callus samples

\* Corresponding author. Tel.: +82 31 8041 1835; fax: +82 31 8041 0629.

E-mail address: [whitenight@hanmail.net](mailto:whitenight@hanmail.net) (S.-N. Kang).

were ground to 20–30 mesh using a grinder (IKA M 20, IKA, Staufen, Germany). The sample powder (5 kg) was refluxed with 10 volumes (v/w) of distilled water at 100 °C for 3 h, and the extraction was repeated in this manner three times. The extracts were filtered with filter paper (Whatman No. 2) and then filtered through a Millipore membrane (0.45 µm) prior to analysis of further study.

## 2.2. Analysis of the vitamin content

For the analysis of the vitamin content, a reverse phase SunFire C-18 3.5 µm 4.6 × 150 mm Column (Waters Co., Milford, MA, USA), a JASCO HPLC (LC-2000, Japan) system consisting of a column oven (JASCO, CO-2060, Japan), a UV–Vis diode array detector (JASCO, MD-2010DAD, Japan) set at 220 nm, a liquid chromatography pump (JASCO, PU-980, Japan), and a ChromNAV software program (JASCO, Japan) were used. The column temperature was 35 °C. The prepared extracts were introduced onto the column through an autosampler (JASCO, AS-2059-SF, Japan) equipped with a sample loop (20 µL). A binary gradient from mobile phase A consisting of 1% (v/v) acetic acid in distilled water (millipore) to mobile phase B consisting of 60% (v/v) methanol in distilled water (millipore) was used with the following elution program: a linear gradient from 10% to 30% (A) for 5 min and from 30% to 100% (B) for 30 min. Vitamin C, vitamin B2, niacin, vitamin B6, thiamine, and folic acid were used as the standards (Sigma–Aldrich, Louis, USA). Identification and quantification of vitamins in samples were performed compared to chromatographic retention times and areas of external standards.

## 2.3. Analysis of the phenolic compounds

For the analysis of phenolic acids, Nova pack C<sub>18</sub> UG120 (150 × 4.6 mm I. D., 5 µm, shiseido co., Ltd., Tokyo, Japan) equipped with a Guard column (10 × 4.0 mm I.d), a JASCO HPLC (LC-2000, Japan) system consisting of a column oven (JASCO, CO-2060, Japan), a UV–Vis diode array detector (JASCO, MD-2010DAD, Japan) set at 280 nm, a liquid chromatography pump (JASCO, PU-980, Japan), and a ChromNAV software program (JASCO, Japan) were used. The column temperature was 40 °C. The prepared extracts were introduced onto the column through an autosampler (JASCO, AS-2059-SF, Japan) equipped with a sample loop (20 µL). A binary gradient from a mobile phase A consisting of 50 mM Sodium phosphate monomer in water: Methanol (93:7) and a mobile phase B consisting 70% methanol in water was used with the following elution program: 0–15 min linear from 100% to 70% A; 15–45 min linear from 70% to 65% A; 45–65 min linear from 65% to 60% A; 65–70 min linear from 60% to 50% A; 70–95 min linear from 50% to 0% A; 95–100 min linear from 0% to 100% A; 100–105 min isocratic at 100% A. Pyrogallol, methoxybenzoic acid, p-coumaric acid, 4-methylcatechol, sinapic acid, cinnamic acid, and salicylic acid were used as the standards (Sigma–Aldrich, Louis, USA). Identification and quantification of phenolic acids in samples were performed compared to chromatographic retention times and areas of external standards.

## 2.4. Determination of total flavonoid contents

The total flavonoid content was determined using aluminum chloride colorimetric method (Chang, Yang, Wen, & Chern, 2002) using quercetin as a standard and expressing the results as mg quercetin equivalents to the extract.

## 2.5. Determination of total phenolic contents

The total phenolic contents of the extracts were determined spectrophotometrically according to the Folin–Ciocalteu

colorimetric method (Singleton & Rossi, 1965), calibrating against the catechin standards and expressing the results as mg catechin equivalents to the (CAE)/g extract.

## 2.6. Determination of DPPH radical scavenging activity

The DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity of the samples (0, 10, 100, 200, and 1000 µg/ml) was measured using the method of Brand-Williams, Cuvelier, and Berset (1995).

## 2.7. Determination of hydroxyl radical scavenging activity

The scavenging activity of samples toward the hydroxyl radical (OH•) was measured using the deoxyribose method (Halliwell, Gutteridge, & Arurma, 1987).

## 2.8. Determination of superoxide anion radical scavenging activity

Superoxide radicals were generated using a modification of the method developed by Liu et al. (Liu, Ooi, & Chang, 1997).

## 2.9. Statistical analysis

All of the measurements were repeated three times. The results are shown as the mean values with the standard deviation. The results were statistically analyzed using ANOVA and Duncan's multiple range tests. Statistical significance was accepted at a level of  $p < 0.05$  and  $p < 0.01$  (SAS Institute, 1988). Correlation analyses were performed using the Pearson's correlation coefficient (R).

# 3. Results and discussion

## 3.1. Contents of water-soluble vitamins

The protective effects of plant products are due to the presence of several components that have distinct mechanisms of action; some are enzymes and proteins, and others are low molecular weight compounds such as vitamins (Halliwell et al., 1987). It has been reported that the levels of plasma antioxidant vitamins and minerals such as vitamin C, E, folic acid, and zinc declined and oxidative damage increased in stressed animals (Sahin, Kucuk, Sahin, & Sari, 2002). The amounts of water-soluble vitamins in the SR leaf and callus extracts are shown in Table 1. The contents of folic acid, vitamin C and vitamin B2 in the leaf extracts were significantly higher than those of callus extract ( $p < 0.01$ ). The folic acid (52.18 mg/100 g) was found to be the major compound, followed by vitamin C (14.97 mg/100 g) in leaf extract. In callus extract, the vitamin C (1.64 mg/100 g) was the major compound, followed by vitamin B (0.23 mg/100 g). The content of folic acid in water extracted from the leaves was 3.5-fold higher than the vitamin C content from the calli.

**Table 1**

Water-soluble vitamins of *Stevia rebaudian* leaf and callus extracts (mg/100 g dry base of extract).

	Leaf	Callus
Vitamin C	14.98 ± 0.07 <sup>b</sup>	1.64 ± 0.02 <sup>a</sup>
Vitamin B2	0.43 ± 0.02 <sup>c</sup>	0.23 ± 0.02 <sup>b</sup>
Vitamin B6	0.00 ± 0.00 <sup>d</sup>	0.00 ± 0.00 <sup>e</sup>
Folic acid	52.18 ± 0.21 <sup>a</sup>	0.09 ± 0.01 <sup>c</sup>
Niacin	0.00 ± 0.00 <sup>d</sup>	0.00 ± 0.00 <sup>d</sup>
Thiamin	0.00 ± 0.00 <sup>d</sup>	0.00 ± 0.00 <sup>d</sup>

Values are mean of triplicate determinations ( $n = 6$ ) ± standard deviation.

<sup>a–e</sup>Mean ± S.D. was significantly different within the same column ( $p < 0.01$ ).

### 3.2. Compounds of phenolic acids

Amounts of phenolic acids in the SR leaf and callus extracts are shown in Table 2. The content of phenolics acid in leaf is higher than in callus of SR water extract. The pyrogallol was found to be the major compound in SR leaf and callus extracts. In leaf extract, the pyrogallol (951.27 mg/100 g) was the major compound, followed by 4-methoxybenzoic acid (33.80 mg/100 g), p-coumaric acid (30.47 mg/100 g), 4-methylcatechol (25.61 mg/100 g), sinapic acid (9.03 mg/100 g), and cinnamic acid (2.42 mg/100 g) ( $p < 0.01$ ). In callus extract, the pyrogallol (40.72 mg/100 g) was the major compound, followed by 4-methylcatechol or salicylic acid (range from 3.22 to 3.27 mg/100 g).

### 3.3. Total phenolic and flavonoid contents

The present study was carried out to evaluate the total phenolic and flavonoid contents of the SR leaf and callus extracts, and the data is shown in Table 3. The total phenolic contents of the 1-mg extracts from the SR leaves and callus were 130.67  $\mu\text{g}$  catechin and 43.99  $\mu\text{g}$  catechin, respectively. And the flavonoid contents of the 1-mg extracts from the SR leaves and callus were 15.64  $\mu\text{g}$  quercetin and 1.57  $\mu\text{g}$  quercetin, respectively.

The total phenolic and flavonoid contents of the leaves was about 44 and 28-fold higher, respectively, than the content of calli in the same concentration ( $p < 0.01$ ). These results suggest that the higher levels of antioxidant activity were due to the presence of phenolic and flavonoid components. Phenols are very important plant constituents because of their scavenging ability, which is attributed to their hydroxyl ( $-\text{OH}$ ) groups (Hatano, Edamatsu, & Mori, 1989) and the methoxy ( $-\text{OCH}_3$ ) substituent in the molecules (Cai, Sun, Xing, Luo, & Corke, 2006). The phenolic compounds may contribute directly to the antioxidative action (Duh, Tu, & Yen, 1999), and they are suggested to play a preventive role in the development of cancer and heart disease (Kahkonen et al., 1999). The interest in phenolics is increasing in the food industry because these compounds retard the oxidative degradation of lipids, thereby improving the quality and the nutritional value of the food (Aneta, Jan, & Renata, 2007).

### 3.4. DPPH radical scavenging assay

The DPPH assay provides basic information on the antiradical activity of the extracts. The actual reaction that is taking place between the DPPH stable radical and the antioxidant (AH) is  $\text{DPPH}^\bullet + (\text{AH})_n \rightarrow \text{DPPH-H} + (\text{A}^\bullet)_n$ . The radical that is formed ( $\text{A}^\bullet$ ) in general is less reactive, depending on the structure of the molecule, or it can follow a radical radical interaction to create a stable molecule (Briante, Febbraio, & Nucci, 2003; Fang, Yang, & Wu, 2002; Huang et al., 2005). The DPPH radical scavenging activities of the water extracts obtained from the SR leaves and calli, along with the

**Table 2**

The phenolic acid of *Stevia rebaudian* leaf and callus extracts (mg/100 g dry base of extract).

	Leaf	Callus
Pyrogallol	951.27 $\pm$ 1.12 <sup>a</sup>	40.72 $\pm$ 0.44 <sup>a</sup>
4-Methoxybenzoic acid	33.80 $\pm$ 0.65 <sup>b</sup>	1.44 $\pm$ 0.31 <sup>c</sup>
p-Coumaric acid	30.47 $\pm$ 0.36 <sup>c</sup>	1.81 $\pm$ 0.41 <sup>cd</sup>
4-Methylcatechol	25.61 $\pm$ 0.26 <sup>d</sup>	3.27 $\pm$ 0.13 <sup>b</sup>
Sinapic acid	9.03 $\pm$ 0.17 <sup>e</sup>	0.39 $\pm$ 0.53 <sup>e</sup>
Cinnamic acid	2.42 $\pm$ 0.03 <sup>f</sup>	0.76 $\pm$ 0.59 <sup>e</sup>
Salicylic acid	–	3.22 $\pm$ 0.06 <sup>b</sup>

Values are mean of triplicate determinations ( $n = 6$ )  $\pm$  standard deviation.

<sup>a–f</sup>Mean  $\pm$  S.D. was significantly different within the same column ( $p < 0.01$ ).

**Table 3**

Total phenolic and flavonoid content of *Stevia rebaudian* leaves and callus water extracts.

	Total phenolic content <sup>c</sup> ( $\mu\text{g}$ catechin equivalents/mg)	Total flavonoid content <sup>d</sup> ( $\mu\text{g}$ quercetin equivalents/mg)
Leaf	130.67 $\pm$ 2.23 <sup>a</sup>	15.64 $\pm$ 0.25 <sup>a</sup>
Callus	43.99 $\pm$ 0.85 <sup>b</sup>	1.57 $\pm$ 0.05 <sup>b</sup>

Values are mean of triplicate determinations ( $n = 6$ )  $\pm$  standard deviation.

<sup>c,d</sup>Total phenolic and flavonoid content was determined with reference to the standard curve of catechin and quercetin content of samples, respectively.

<sup>a,b</sup>Mean  $\pm$  S.D. was significantly different within the same column ( $p < 0.01$ ).

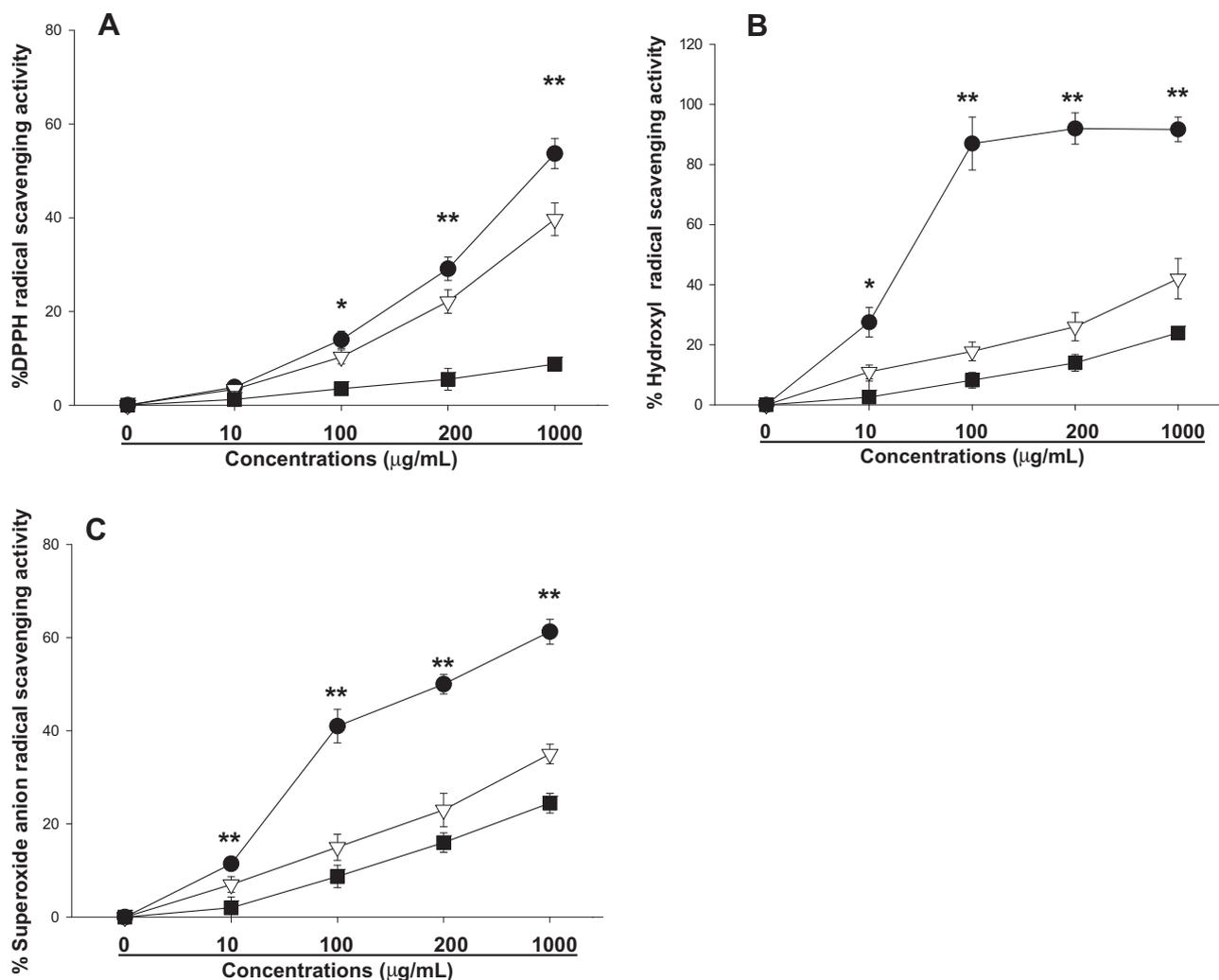
reference standards such as ascorbic acid, were determined using DPPH<sup>•</sup>, and the results are shown in Fig. 1A. The DPPH radical scavenging activity of the SR leaves at a concentration of 10 and 100  $\mu\text{g}/\text{mL}$  were 3.38% and 10.15%, respectively. And the abilities of SR leaf extract were higher than those of callus extract along the concentrations except 10  $\mu\text{g}/\text{mL}$ . Whereas, Tadhani et al. (2007) reported that the percent inhibition of DPPH radical of stevia leaf was lower than that of callus. Benavente-Garcia, Castillo, Lorente, Ortuno, and Del Rio (2000) reported that the radical scavenging activity of plant extracts depends on the amount of polyphenolic compounds in the extracts.

### 3.5. Hydroxyl radical scavenging activity

The hydroxyl radical is an extremely reactive free radical formed in biological systems, and it has been implicated as a highly damaging species in free radical pathology, capable of damaging almost every molecule found in living cells (Hochstein & Atallah, 1988). This radical has the capacity to join nucleotides in DNA, and it can cause strand breakage that contributes to carcinogenesis, mutagenesis and cytotoxicity (Manian, Anusuya, Siddhuraju, & Manian, 2008; Trease & Evans, 1983). The hydroxyl radical scavenging capacity of an extract is directly related to its antioxidant activity (Babu, Shylesh, & Padikkala, 2001). The hydroxyl radical scavenging activities of the SR leaf and callus extracts are shown in Fig. 1B. The inhibition percentages of the hydroxyl radical of the SR leaf extracts were significantly higher than those of callus extracts in the same concentrations ( $p < 0.05$ ). The hydroxyl radical scavenging activities of the SR leaf were inhibited by 10.40%, 16.68%, 28.00% and 42.85% at a concentration of 10, 100, 200 and 1000  $\mu\text{g}/\text{mL}$  of water extract, respectively. All of the extracts had a scavenging activity on the hydroxyl radicals in a dose-dependent manner at each concentration.

### 3.6. Superoxide anion radical scavenging activity

Generally, superoxide anions convert to oxygen and hydrogen peroxide by superoxide dismutase (SOD), or they react with nitric oxide ( $\text{NO}^\bullet$ ) to form peroxynitrite. Hydrogen peroxide can be converted into water and oxygen by catalase (Fang et al., 2002). Therefore, superoxide scavenging capacity in the human body is very important as the first line of defense against oxidative stress. The effects of the leaf and callus extracts obtained from SR on the superoxide anions generated by NBT (nitroblue tetrazolium) were determined and are shown in Fig. 1C. The superoxide radical scavenging activities of all of the plant extracts were significantly lower than those of ascorbic acid treatment ( $p < 0.05$ ). The different concentrations of SR in the leaf and callus extracts showed antioxidant activities in a dose-dependent manner in the superoxide anion scavenging assay. With the exception of the 10  $\mu\text{g}/\text{mL}$  concentration, the superoxide anion scavenging activity of the SR leaf extracts showed no significant difference compared to the SR callus extracts. At the different concentrations (10, 100, 200 and 1000  $\mu\text{g}/\text{mL}$ ),



**Fig. 1.** DPPH radical (A), hydroxyl radical (B), and superoxide anion radical (C) scavenging activities of *Stevia rebaudiana* leaves water extract (▽), callus water extract (■), and ascorbic acid (●) at different concentrations (0, 10, 100, 200, and 1000 μg/mL). Values are mean of triplicate determinations ( $n = 6$ ) ± standard deviation. Values in the same line not sharing a common letter are significantly different ( $p < 0.05$ ). Values are different significantly in the same concentration (\* $p < 0.05$ , \*\* $p < 0.01$ ).

superoxide anion scavenging activities of the SR leaf extracts accounted for an inhibition of 5.62%, 15.26%, 25.36% and 38.26%, respectively, and the activities of the SR callus extracts accounted for an inhibition of 3.35%, 8.71%, 16.85% and 25.73%, respectively.

Total phenolic and flavonoid contents have been reported to be responsible for the antioxidant activities of botanical extracts. DPPH, hydroxyl radical scavenging activity, and superoxide anion radical scavenging activity have been used to measure antioxidant activity and these results should correlate with those of total phenolic and flavonoid content. Do, Kang, Kim, Jo, and Lee (2004), Zheng and Wang (2001) demonstrated that some bioactive compounds present in medicinal plant possessed high total antioxidant activity, which was due to the presence of phenolic, carotenoids and flavonoids. A regression analysis was used to correlate the results of the five assays (Table 4). High correlation coefficients were found between the total phenolic content and DPPH and hydroxyl radical scavenging activity ( $R = 0.96$ ,  $p < 0.005$ ;  $R = 0.88$ ,  $p < 0.01$ , respectively). And the contents of flavonoid and DPPH and hydroxyl radical scavenging activity exhibited high correlation coefficient ( $R = 0.99$ ,  $p < 0.001$ ;  $R = 0.91$ ,  $p < 0.01$ , respectively). As the aluminium chloride method is specific only for flavones and flavonols, the content of total flavonoids could be underestimated by the method (Meda et al., 2005), which probably

account for a lower correlation observed between antioxidant activity and flavonoid count. Liu, Qiu, Ding, and Yoa (2008) reported a negative correlation between flavonoid content and antioxidant activity. However, a non significant correlation coefficient was found between hydroxyl and superoxide anion radical scavenging activity and vitamin contents ( $p > 0.05$ ).

**Table 4**

Correlations ( $R^a$ ) between different antioxidant capacity parameters (by DPPH, hydroxyl, and superoxide radical scavenging activity) and total phenolic and flavonoid contents or vitamin contents of *Stevia rebaudiana* extracts.

	TPC <sup>b</sup>	FLA <sup>c</sup>	VIT <sup>d</sup>	DPPH <sup>e</sup>	HRSA <sup>f</sup>	SRSA <sup>g</sup>
TPC		0.92**	1.00****	0.96***	0.88**	0.72
FLA			1.00****	0.99****	0.91**	0.63
VIT				0.95*	0.89	0.85
DPPH					0.89***	0.73*
HRSA						0.84***

Significance level at \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.005$ , \*\*\*\* $p < 0.001$ .

<sup>a</sup> R, correlation coefficient.

<sup>b</sup> TPC, total phenolic content.

<sup>c</sup> FLA, flavonoid content.

<sup>d</sup> VIT, vitamin content.

<sup>e</sup> DPPH, free radical scavenging activity.

<sup>f</sup> HRSA, hydroxyl radical scavenging activity.

<sup>g</sup> SRSA, superoxide anion radical scavenging activity.

In conclusion, it is well known that free radicals are the cause of several diseases. The production of free radicals and the activity of the scavenger enzymes against those radicals, such as superoxide dismutase (SOD), are correlated with life expectancy. We have demonstrated that the water extracted from the SR leaves contains high levels of folic acid (52.18 mg/100 g) and pyrogallol compounds (951.27 mg/100 g) at dry base of extracts. In addition, high total phenolic and flavonoid contents were detected in leaf extract (130.67 µg catechin equivalents/mg and 15.64 µg quercetin equivalents/mg respectively). These extracts are capable of inhibiting and reducing free radicals by terminating the radical chain reaction, and they thus act as reducing agents. The water extracts of the SR leaves showed relatively high antioxidant activity by inhibiting DPPH, the hydroxyl radical, and the superoxide anion scavenging activities at the different concentrations. Although the antioxidant activities found in the *in vitro* experiment were only indicative of the potential health benefits, these results remain important and are the first step in screening the antioxidant activity of the SR leaves. Thus, it can be concluded that the water leaf extracts taken from SR can be used as an accessible source of natural antioxidants with resultant health benefits.

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