



Original/Alimentos funcionales

Antioxidant capacity of leaf extracts from two *Stevia rebaudiana* Bertoni varieties adapted to cultivation in Mexico

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Abstract

The recent introduction of the cultivation of *Stevia rebaudiana* Bertoni in Mexico has gained interest for its potential use as a non-caloric sweetener, but some other properties of this plant require studies. Extracts from two varieties of *S. rebaudiana* Bertoni adapted to cultivation in Mexico were screened for their content of some phytochemicals and antioxidant properties. Total pigments, total phenolic and flavonoids contents of the extracts ranged between 17.7-24.3 mg/g, 28.7-28.4 mg/g, and 39.3-36.7 mg/g, respectively. The variety "Criolla" exhibited higher contents of pigments and flavonoids. Trolox equivalent antioxidant capacity ranged between 618.5-623.7 mM/mg and DPPH decolorization assay ranged between 86.4-84.3%, no significant differences were observed between varieties. Inhibition of β -carotene bleaching ranged between 62.3-77.9%, with higher activity in the variety "Criolla". Reducing power ranged between 85.2-86% and the chelating activity ranged between 57.3-59.4% for Cu^{2+} and between 52.2-54.4% for Fe^{2+} , no significant differences were observed between varieties. In conclusion, the results of this study showed that polar compounds obtained during the extraction like chlorophylls, carotenoids, phenolic compounds and flavonoids contribute to the antioxidative activity measured. The leaves of *S. rebaudiana* Bertoni could be used not only as a source of non-caloric sweeteners but also naturally occurring antioxidants.

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Key words: *Stevia rebaudiana* Bertoni. Leaves extracts. Phytochemicals. Antioxidant properties.

CAPACIDAD ANTIOXIDANTE DE EXTRACTOS FOLIARES DE DOS VARIEDADES DE *STEVIA REBAUDIANA* BERTONI ADAPTADAS AL CULTIVO EN MÉXICO

Resumen

La reciente introducción del cultivo de *Stevia rebaudiana* Bertoni en México ha ganado interés debido a su potencial uso como fuente de edulcorantes no calóricos, pero otras propiedades de esta planta aun requieren de estudios. Extractos de hojas de dos variedades de *S. rebaudiana* Bertoni adaptadas al cultivo en México fueron evaluados en cuanto a su contenido de algunos fitoquímicos y sus propiedades antioxidantes. El contenido de pigmentos, fenoles totales y flavonoides en los extractos, osciló entre 17.7-24.3 mg/g, 28.7-28.4 mg/g, y 39.3-36.7 mg/g, respectivamente. La variedad "Criolla" exhibió los mayores contenidos de pigmentos y flavonoides. La capacidad antioxidante equivalente de Trolox osciló entre 618.5-623.7 mM/mg y el ensayo de decoloración del radical DPPH osciló entre 86.4-84.3%, no observándose diferencias significativas entre ambas variedades. La inhibición de la decoloración del β -caroteno osciló entre 62.3-77.9%, siendo mayor en la variedad "Criolla". El poder reductor osciló entre 85.2-86%, las capacidades quelantes de cobre y hierro oscilaron entre 57.3-59.4% y 52.2-54.4%, respectivamente, no observándose diferencias significativas entre ambas variedades. En conclusión, los resultados de este estudio demuestran que los compuestos de naturaleza polar obtenidos durante la extracción, tales como pigmentos clorofílicos, carotenoides, compuestos fenólicos y flavonoides contribuyen a la actividad antioxidante. Las hojas de *S. rebaudiana* Bertoni podrían ser empleadas no solo como fuente de edulcorantes no calóricos, sino también como fuente de antioxidantes de origen natural.

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Palabras clave: *Stevia rebaudiana* Bertoni. Extractos foliares. Fitoquímicos. Propiedades antioxidantes.

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Introduction

The role of Reactive Oxygen Species (ROS) has been determined in many human degenerative diseases, including ageing, cancer, arthritis, and Parkinson's disease¹. For example, Hydrogen Peroxide (H₂O₂), a prominent ROS, caused lipid peroxidation and DNA damage in cells². The antioxidant action of some natural compounds, such as vitamins, minerals, polyphenols and other non-nutrient compounds of plants, inhibit the generation of reactive oxygen species or the scavenging of free radicals, was believed to be beneficial for human health³. Indeed, natural antioxidants have displayed a wide range of pharmacological activity, such as anticancer, anti-inflammatory and anti-ageing actions⁴. To counteract this threat to their integrity, cells have evolved a variety of defense systems based on both water-soluble and lipid-soluble antioxidant species, and on antioxidant enzymes. A high proportion of the antioxidant systems of the human body are dependent on dietary constituents⁵. Synthetic antioxidants such as butylhydroxyanisole (BHA) or butylhydroxytoluene (BHT) are used to decelerate these processes. However, due to their unstable and highly volatile nature, they have frequently brought some questions about their safety and efficiency ever since their first introduction to the food industry⁶. Consequently, the need to identify alternative natural and safe sources of antioxidant, and the search for safe and natural antioxidants, especially of plant origin, has notably increased in recent years⁷.

The genus *Stevia* belongs to the Asteraceae family, a tribe of the Eupatoriae, and comprises 240 species, growing mostly at the altitude of 500-3000 m in semidry mountainous terrain. Different species of *Stevia* contain several potential sweetening compounds, with *Stevia rebaudiana* (Bertoni) being the sweetest of all⁸. *Stevia* (*Stevia rebaudiana*, Bertoni) is a perennial shrub indigenous to Paraguay and Brazil. Nowadays the extraction of sweeteners from stevia leaves is a growing industrial and commercial worldwide sector; more than 750 tons of stevia leaves are used as crude extract for consumption and extraction of glycosides per year⁹. The sweetening property is associated with their contents of several glycosides, stevioside, steviobioside, rebaudiosides A to F, dulcoside A and steviol. These glycosides and their derivatives are known to account for 4-20% of the dry weight of stevia leaves¹⁰.

Stevia and its extract have been studied widely from the sweetener point of view however, a search through literature shows no information on the non-sweetening components, which make up 80- 90% dry weight of this plant. The non-sweet constituents identified in the *S. rebaudiana* leaves are diterpenes, triterpenes, sterols, flavonoids, volatile oil constituents, pigments and inorganic matters¹¹. *S. rebaudiana* leaves can be a potential source of natural antioxidants. The bioactivity of any plant products greatly varies with the change

of geographical conditions such as soil, water cultivation process etc. So far, the antioxidant capacity of *Stevia rebaudiana* cultivated in Yucatan, Mexico has not been evaluated.

The objective of the present study was to evaluate the content of chlorophyll and carotenoids pigments, phytochemical compounds and antioxidant capacities of the leaves' extracts of *Stevia rebaudiana* varieties from Yucatan, Mexico.

Materials

Raw material and chemicals

Stevia rebaudiana Bertoni variety Morita II and criolla were obtained from plots in the Yucatan State in México. The crop was managed according to the production technology established parameters for Mexico¹². Samples were obtained from the first cut of the plot at an age of three months; samples consisted on branches of leaves. All chemicals were reagent grade or better and purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Methods

Drying treatment and extracts preparation

Stevia rebaudiana leaves were subjected to convection drying at 60 °C for 24 h. The vegetal material was distributed in aluminum trays and drying was stop until there was no significant weight loss; this is until vegetal material reached equilibrium in moist content. The leaves were milled in a Wiley equipment to obtain particles of 1.0 mm in size. Samples were stored at room temperature in polyethylene bags until extract preparation. The uniform particle-sized powder (50 g) was packed in thimble and extracted in Soxhlet apparatus with methanol (200 mL) till the solution in the thimble becomes clear⁸. The extract so obtained was then filtered through Whatman filter paper no. 40. All the filtrate together was concentrated with rotary vacuum evaporator at bath temperature not exceeding 40 °C and then freeze dried. Finally the extract was resuspended in 5 mL of distilled water and stored at 10 °C for further use.

Determination of chlorophylls (a and b), carotenoids and total pigments contents

The weighed samples, having been put separately in 90% acetone (1 ml for each 50 mg), were homogenized at 1000 rpm for one minute. The homogenate was filtered and centrifuged at 2500 rpm for ten min. The supernatant was separated and the absorbances were read at 665, 645, 630 and 444 nm. The amount of chlo-

rophylls and carotenoids were calculated according to the formulas¹³:

$$\text{Chlorophyll A (mg/g)} = 11.6 * A_{665} - 1.31 * A_{645} - 0.14 * A_{630}$$

$$\text{Chlorophyll B (mg/g)} = -4.34 * A_{664} + 20.7 * A_{645} - 4.42 * A_{630}$$

$$\text{Total carotenoids (mg/g)} = 0.0051 * A_{444} + 0.00003$$

Determination of total phenolic content

The phenolic compounds were determined using the Folin-Ciocalteu method, based on the reduction of phosphor-wolframate-phosphomolybdate complex by phenolics to a blue reaction product¹⁴. One g of sample was suspended in 40 mL of 80% methanol (v/v) in a beaker. The dispersion was stirred on a magnetic plate at room temperature for 3 h. After the extraction the dispersion was centrifuged at 2500 rpm for 20 min at 10 °C. The supernatant was filtered, refrigerated at 4 °C and protected from light until analysis. A volume of 0.2 mL from the methanol extract was placed in a plastic cell for spectrophotometer and 0.2 mL of Folin-Ciocalteu reagent were added and homogenized. Then 2 mL of distilled water were added and the cell was kept in dark at room temperature for 1 h. Absorbance was measured at 765 nm. The data was calculated by comparison between a standard curve (0 - 500 µg/mL of gallic acid) and the absorbance of each sample. The analysis was performed on three replicates. The total amount of phenolic compounds was determined in micrograms of gallic acid equivalents/mg of sample.

Determination of total flavonoids content

The flavonoids content was determined using the aluminum chloride method¹⁵. Briefly, 0.5 mL from the ethanol extract was placed in a plastic cell for spectrophotometer, then 1.5 mL of ethanol 95%, 0.1 mL AlCl₃ 10%, 0.1 mL of potassium acetate 1.0 M and 2.8 mL of distilled water were added and the cell was kept in dark at room temperature for 30 min. Absorbance was measured at 415 nm. The data was calculated by comparison between a standard curve (0 - 100 µg/mL of quercetin) and the absorbance of each sample. The analysis was performed on three replicates. The total amount of flavonoid compounds was determined in micrograms of quercetin equivalents/mg of sample.

Trolox equivalent antioxidant capacity

The ABTS^{•+} radical cation was produced by reacting 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid (ABTS) with potassium persulfate¹⁶. To prepare the stock solution, ABTS was dissolved at a 2 mM concentration in 50 mL phosphate-buffered saline. The ABTS radical cation was produced by reacting 10 mL

ABTS stock solution with 40 µL K₂S₄O₈ 70 mM solution and allowing the mixture to stand in darkness at room temperature for 16-17 h before use. The radical was stable in this form for more than 2 days when stored in darkness at room temperature. Antioxidant compound content in the stevia extracts was analyzed by diluting the ABTS^{•+} solution with PBS to an absorbance of 0.800 ± 0.030 AU at 734 nm. After adding 990 µL diluted ABTS^{•+} solution (A 734 nm= 0.800 ± 0.030) to 10 µL of 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (TROLOX) standard (final concentration 0.5-3.5 mM) in PBS, absorbance was read at room temperature exactly 6 min after initial mixing. All analyses were run in triplicate. The percentage decrease in absorbance at 734 nm was calculated and plotted as a function of the antioxidant concentration of Trolox for the standard reference data. To calculate the Trolox equivalent antioxidant capacity (TEAC), the slope of the absorbance inhibition percentage vs. antioxidant concentration plot was divided by the slope of the Trolox plot. This produces the TEAC at a specific point in time.

DPPH free radical-scavenging assay

The scavenging effect on α,α-diphenyl-β-picrylhydrazyl (DPPH) free radical was measured at 517¹⁷. With some modifications, a sample solution (1.5 mL) with 20 mg was added to 1.5 mL of 0.1 mM DPPH in 95% ethanol. The mixture was shaken and left for 30 min at room temperature. Absorbance was measured at 517 nm. Measurement was performed at least in triplicate. The percentage of the DPPH scavenging effect was calculated using the following equation:

$$\text{DPPH scavenging effect (\%)} = (A_0 - A_1) / A_0 \times 100.$$

Where A₀ was the absorbance of the control and A₁ was the absorbance in the presence of the *Stevia rebaudiana*. The IC₅₀ (concentration providing 50% inhibition) values were calculated using the dose inhibition curve in linear range by plotting the extract concentration versus the corresponding scavenging effect. Samples were tested in a range of concentrations from 0.5 to 3.0 mg/mL.

β-carotene bleaching assay

This method evaluates the capacity of the leaf extracts to reduce the oxidative loss of β-carotene in the presence of CuSO₄¹⁸. β-Carotene (10 mg) was dissolved in 1 mL of chloroform (CHCl₃) and 1 ml of Tween 80. The chloroform was removed using nitrogen and resuspended in phosphate buffer (100 mM, pH 7). The absorbance was immediately measured at 470 nm. Distilled water was used as the positive control. In the negative control, the distilled water was substituted with an equal volume of CuSO₄ (50 µM). The antioxidant activity (%) of the leaf extracts was evaluated in

terms of the bleaching of the β -carotene using the following formula: % inhibition = [(At-Ct)/CO] \times 100.

Where, At and Ct are the absorbances measured for the leaf extract and positive control, respectively, after reaction, and CO is the absorbance values for the negative control measured.

Ferric reducing power assay

This method is based on the reduction of Potassium Ferricyanide (Fe^{+3}) to (Fe^{2+}) in the presence of an antioxidant, KFeIII forming the blue complex $[\text{FeI}(\text{CN})_6]$, which absorbing a 700 nm¹⁹. First, 200 μL of sample (containing 1 mg of protein), 500 μL of phosphate buffer (0.2 M, pH 6.6), and 500 μL of potassium ferricyanide (1%) were mixed in a test tube. The test tube was then incubated at 50 °C for 20 min. Subsequently, 500 μL of trichloroacetic acid (10%) were added, and the tube was centrifuged at 3000 g for 10 min. An aliquot of 500 μL of the supernatant, which is dissolved in an equal amount of distilled water, immediately 500 μL of ferric chloride (0.1%) were added. Absorbance was determined at 700 nm. Samples were tested in a range of concentrations from 200 to 1000 mg/mL. Butylated hydroxytoluene was used as control in the same range of concentrations.

Chelating of metal ions Cu^{2+} and Fe^{2+}

Cu^{2+} -chelating activity was determined using the pyrocatechol violet reagent²⁰. Briefly, 1.0 mL of sodium acetate buffer (100 mM, pH 4.9), 100 mL of Cu (II) standard solution (1.0 mg/mL), and 100 mL of sample (containing 200 mg) were mixed in a test tube. The mixture was allowed to react for 5 min at room temperature and 25 mL of a pyrocatechol violet solution (4.0 mM) was then added. Absorbance was determined at 632 nm. Copper chelating activity was calculated as follows: Chelating activity (%) = (1 - sample absorbance/control absorbance) \times 100.

Fe^{2+} -chelating activity was determined by measuring the formation of the Fe^{2+} -ferrozine complex²¹. Briefly,

1.0 mL of sodium acetate buffer (100 mM, pH 4.9), 100 mL of Fe(II) standard solution (1.0 mg/mL), and 100 mL of sample (containing 200 mg) were mixed in a test tube. The mixture was allowed to react for 5 min at room temperature and 50 mL of a ferrozine solution (40 mM) was then added. Absorbance was determined at 562 nm. Iron chelating activity was calculated as follows: Chelating activity (%) = (1 - sample absorbance/ control absorbance) \times 100.

Statistical analysis

All results were analyzed using descriptive statistics with a central tendency and dispersion measures. One-way ANOVAs were run to evaluate proximate composition and *in vitro* antioxidant activities. The Least Significant Difference (LSD) multiple range test was used to determine differences among drying treatments. All analyses were done according to Montgomery²² and processed with the Statgraphics Plus version 5.1 software.

Results and Discussion

Determination of chlorophylls (a and b), carotenoids and total pigments contents

Extracts from two varieties of Stevia were analyzed for chlorophyll (A and B), carotenoids and total pigments content (table 1). It was found that Criolla extract showed higher contents of carotenoids and total pigments than the Morita II extract. Abou-Arab et al.²³, quantified the content of chlorophylls (A and B), carotenoids and total pigments in fresh leaves of Stevia reporting values of 10.1, 6.6, 3.9 and 20.1 g/g of chlorophylls A, B, carotenoids and total pigments, respectively.

The same authors reported reductions of 47.4, 41.3, 74.8, and 50.9% with final values of 4.7, 2.7, 0.76 and 7.5 g/g of chlorophylls A, B, carotenoids and total pigments, respectively after sun-drying them and using the extraction with methanol-water at room tempera-

Table I
Chlorophylls, carotenoids, and phytochemicals of *Stevia rebaudiana* Bertoni leaf extracts

Parameter	Criolla	Morita II
Chlorophyll a (mg/g)	4.0 ^a	3.0 ^a
Chlorophyll b (mg/g)	4.0 ^a	4.0 ^a
Carotenoids (mg/g)	16.3 ^a	10.7 ^b
Total pigments	24.3 ^a	17.7 ^b
Total phenolic compounds (mg gallic acid equivalents/g)	28.7 ^a	28.4 ^a
Total flavonoids compounds (mg quercetin equivalents/g)	39.3 ^a	36.7 ^b

Data are presented as means (n = 3). Different letters indicate significant differences (P < 0.05)

ture. In the present study the leaves were dried at 60 °C by convection and the leaves extracts were obtained with methanol in a Soxhlet system also at 60 °C. Despite this the contents of chlorophylls, carotenoids and total pigments were higher in this study (Table I).

This may be due to the drying and extraction treatments not caused an excessive degradation of pigments. Although chlorophylls are not considered dietary antioxidants, they are widely distributed among green fruits and vegetables with chlorophyll A and B derivatives predominate in higher plants and possess antimutagenic activity and antioxidant activity by breaking the radical chain reaction caused by autoxidation via a hydrogen donating mechanism²⁴. Meanwhile, carotenoids often occur along with chlorophylls during the chloroplasts, but are also present in other chromoplasts²⁵. There are three possible mechanisms that could account for the reaction of carotenoids with radical species, especially singlet oxygen (O₂) and peroxy radicals (ROO•), including electron transfer, hydrogen abstraction, and addition of a radical species²⁶. Whilst drying and extraction processes affect the content of chlorophylls and carotenoids, the remaining contents of both pigments could partially confer the antioxidant activity of the *Stevia rebaudiana* extracts.

Determination of total phenolic content

The total phenolic content of *Stevia rebaudiana* extracts was determined using Folin-Ciocalteu method and reported as gallic acid equivalents by reference to standard curve (table 1). According to Jahan et al.²⁷ the total phenolic content in ethanolic extracts of stevia leaf ranged between 25.3 - 65.2 mg/g gallic acid equivalents. In the present study the phenolic content of the extracts will be considered as included in this range. Interest on natural food additives has been increasing and plant extracts possessing natural food antioxidants were shown to influence human health when consumed daily²⁸. The phenolic compounds present in the herbs and spices have been reported to show natural antioxidant activity and are applied as food preservatives²⁹. The predominant mode of antioxidant activity of phenolic compounds is believed to be a radical scavenging via hydrogen donation. Other radical quenching mechanisms are through electron donation and singlet oxygen quenching. Substituents on the aromatic ring affect the stabilization and therefore affect the radical-quenching ability of these compounds³⁰. As antioxidants, dietary polyphenols may protect cell constituents against oxidative damage and therefore, limit the risk of various degenerative diseases associated to oxidative stress. Numerous studies on animal models have shown that, when added to the diet, they limit the development of cancers, cardiovascular diseases, neurodegenerative diseases, diabetes, and osteoporosis³¹.

Determination of total flavonoids content

The total flavonoids content of *Stevia rebaudiana* extracts was determined using the aluminum chloride method, and the total amount of flavonoid compounds was determined in mg of quercetin equivalents/g (table 1). Mandal and Madan³², evaluated the content of flavonoids in varieties of *Stevia rebaudiana* from different geographical sources of India, and reported the total flavonoids content in methanol extracts of stevia leaf ranged between 39.8 - 62.2 mg/g quercetin equivalents. In the present study the flavonoids content of the extracts would be considered as included in this range. Flavonoids are a broad class of low molecular weight, secondary plant phenolics characterized by the flavan nucleus. In plants, these compounds afford protection against ultraviolet radiation, pathogens, and herbivores³³. The protective effects of flavonoids in biological systems are ascribed to their capacity to transfer electrons free radicals, chelate metal catalysts, activate antioxidant enzymes, reduce alpha-tocopherol radicals and inhibit oxidases. It is now widely accepted that dietary flavonoids may play an important role in protecting the body against chronic diseases, such as cancer, cardiovascular diseases and diabetes mellitus³⁴.

Trolox equivalent antioxidant capacity

The Trolox equivalent antioxidant capacity (TEAC) method uses a spectrophotometer to measure the loss of color when an antioxidant is added to the blue-green chromophore ABTS•+, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid). The antioxidant reduces ABTS•+ to ABTS, decolorizing it. ABTS•+ is a stable radical. The *Stevia rebaudiana* extracts efficiently scavenged ABTS radicals generated by the reaction between 2,2'-azinobis (3-ethylbenzothiazolin-6-sulphonic acid) (ABTS) and potassium persulfate (figure 1).

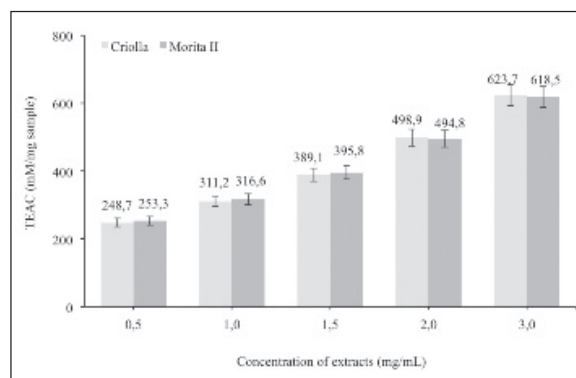


Fig. 1.—Trolox equivalent antioxidant capacity (TEAC) in extracts of *Stevia rebaudiana* Bertoni. Data are presented as means (n = 3).

The TEAC activity shows a direct correlation in a dose-dependent manner with the concentration of the extracts. This behavior was similar to that reported by Leong and Shui³⁵ for the extracts of different parts of *Stevia rebaudiana* (root, stem, leaf, and flower). TEAC and DPPH assays are excellent tools for determining the antioxidant activity of hydrogen-donating antioxidants and of chain-breaking antioxidants.

DPPH free radical-scavenging assay

The DPPH free radical-scavenging assay measures by spectrophotometer, the reduction effect that the antioxidants have on the radical 2,2-diphenylpicrylhydrazyl (DPPH). This radical has the advantage of being unaffected by certain side reactions, such as metal-ion chelation and enzyme inhibition, brought about by various additives. It is also important to note that the DPPH test only recognizes free-radical scavenging effects and not pro-oxidant activity. Free radical scavenging capacities of the *Stevia rebaudiana* leaves extracts measured by DPPH assay are shown in figure 2.

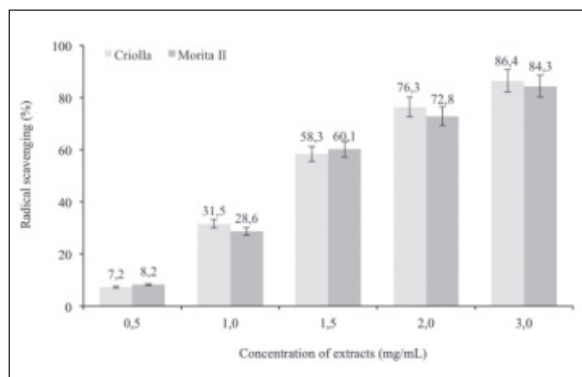


Fig. 2.—DPPH radical scavenging activity in extracts of *Stevia rebaudiana* Bertonii. Data are presented as means (n = 3).

The antioxidant activity showed a direct correlation in a dose-dependent manner with the concentration of the extracts. Singh et al.⁷ evaluated the antioxidant activity of methanolic extracts of different parts of *Stevia rebaudiana* (root, stem, leaf, and flower) and reported radical scavenging activities (%) ranged between 47.1 - 82.4% for leaf and flower, respectively. In the present study both varieties of *Stevia rebaudiana* exhibited higher values of scavenging activity that indicate that the leaf extracts of *Stevia rebaudiana* act like hydrogen-donating and of chain-breaking antioxidants.

β -carotene bleaching assay

The β -carotene bleaching assay evaluates the principle of antioxidant activity based on the availability of

electrons to neutralize free radicals. β -Carotene usually undergoes rapid discoloration in the absence of an antioxidant; this is because the oxidation of β -carotene in the presence of CuSO_4 generates free radicals that attack the highly unsaturated β -carotene molecule. Hence β -carotene is oxidized losing its orange color which is then monitored spectrophotometrically. The results obtained from this assay are shown in figure 3.

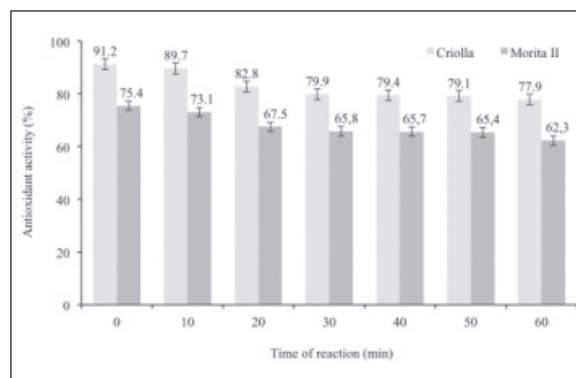


Fig. 3.—Antioxidant activity of extracts of *Stevia rebaudiana* Bertonii. Determined by β -carotene bleaching test. Data are presented as means (n = 3).

Although, both extracts prevented the bleaching of β -carotene during the test (0-60 min), the Criolla extract had a slightly higher activity. When the Criolla extract interacted with β -carotene it lost only 14.5% of the β -carotene compared with the loss of 17.4% in presence of the Morita II extract. These results are also consistent with the results obtained from TEAC and DPPH test.

Ferric reducing power assay

Reducing power was measured by direct electron donation in the reduction of $\text{Fe}^{3+}(\text{CN})_6^- \rightarrow \text{Fe}^{2+}(\text{CN})_6^-$. The product was visualized by forming the intense Prussian blue color complex; a higher absorbance value that indicates a stronger reducing power of the samples. table 2 presents the reductive capabilities of the methanolic extracts of *Stevia rebaudiana*.

In the concentration range investigated (0.5 - 3.0 mg/mL), the extracts demonstrated reducing power that increased linearly with concentration. The results of this assay indicate that both extracts are able to donate electrons to reactive radicals, reducing them into more stable and unreactive species.

Chelating of metal ions Cu^{2+} and Fe^{2+}

Metal ion chelating activity of an antioxidant molecule prevents oxyradical generation and the consequent oxidative damage. Metal ion chelating capaci-

Table II
Ferric reducing power and chelating capacity of extracts of Stevia rebaudiana Bertoni

Parameter (%)	<i>S. rebaudiana</i> <i>Bertoni</i>	Concentration of extract (mg/mL)				
		0.5	1.0	1.5	2.0	3.0
Reducing power	Criolla	54.8 ^a	72.1 ^a	76.9 ^a	82.3 ^a	86.0 ^a
	Morita	58.4 ^a	73.7 ^a	78.8 ^a	84.4 ^a	85.2 ^a
Cu ²⁺ chelating capacity	Criolla	26.6 ^a	29.2 ^a	39.5 ^a	48.3 ^a	57.3 ^a
	Morita	24.7 ^a	34.7 ^a	41.0 ^a	52.8 ^a	59.4 ^a
Fe ²⁺ chelating capacity	Criolla	13.8 ^a	17.2 ^a	22.0 ^a	38.2 ^a	52.2 ^a
	Morita	13.5 ^a	18.9 ^a	30.1 ^a	41.5 ^a	54.4 ^a

Data are presented as means (n = 3)
 Different letters indicate significant differences (P < 0.05)

ty plays a significant role in antioxidant mechanisms since it reduces the concentration of transition metal ions that are potent catalysts, and capable of initiating processes in lipid peroxidation, especially in cellular membranes. Chelating of metal ions capacities of the *Stevia rebaudiana* leaves extracts are shown in Table 2. The chelating activity showed a direct correlation in a dose dependent manner with the concentration of the extracts. Although, both extracts exhibited chelating activity, the Morita II extract had a slightly higher activity. The main strategy to avoid reactive oxygen species generation that is associated with redox active metal catalysis involves chelating of the metal ions. Both *Stevia rebaudiana* methanolic extracts interfered with the formation of ferrous and cupric complex, suggesting that these have chelating activity and capturing metallic ions.

Conclusion

The ethanolic extracts of varieties of *Stevia rebaudiana* Bertoni adapted to cultivation in southern Mexico were found to be effective radical scavengers, possessing a good reducing power and chelating activity. Earlier reports on the antioxidant activity of varieties of *Stevia rebaudiana* Bertoni adapted to cultivation in southern Mexico are very rare in the literature. The high antioxidant activity of *Stevia rebaudiana* Bertoni enhanced the potential interest in this natural source of sweeteners to improve the efficacy of different products as nutraceutical and pharmacological agents. The consumption of the *Stevia rebaudiana* Bertoni may play a role in preventing human diseases in which free radicals are involved such as cancer, cardiovascular disease, and aging. The results of this study indicate that methanolic leaf extracts of *Stevia rebaudiana* Bertoni attenuated oxidative activity via its antioxidant properties in *in vitro* assays. However, further investigations on their *in vivo* antioxidant activity, the different antioxidant mechanism, and identification of the different phytochemicals involved are warranted.

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