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Antioxidant activity of different pitanga (*Eugenia uniflora* L.) fruit fractions

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Abstract

The antioxidant activity of different fractions of *Eugenia uniflora* fruits were investigated by employing DPPH• scavenging. The more active fraction was subjected to further isolation of compounds with antioxidant activity properties. The fraction 3 exhibited higher total antioxidant capacity (74.59 ± 5.30%) when compared with others. Two compounds were isolated from fraction 3, which exhibited radical scavenging activity with values of 87.2% for compound 1 at 50 ppm and the lowest EC_{50} (26 µg/mL), very similar to some stardards, and 62% for compound 2 at 1000 ppm. Both compounds were analyzed by Q-TOF-MS/MS technique. In conclusion, the simple technique of separation step applied to the crude pitanga extract gives rise to a fraction (named fraction 3) concentrated in components, which presented antioxidant potential.

Key words: *Eugenia uniflora*, antioxidant activity, fractionation, phenolic compounds, Brazilian tropical fruit.

Introduction

Originally from Brazil, the pitanga tree (*Eugenia uniflora* L.), from the family Myrtaceae, grows in the tropical and subtropical regions where it is prized for its fruit, the pitanga, which is distinguished by its peculiar red color, flavor and fragrance. It is marketed mainly in the form of pulp, which is widely used by the Brazilian industry for juice production [1]. The pitanga tree leaf is known for containing substances with biological effects that are beneficial for human health, which favors its use as a medicinal plant [2-5]. In addition, the pitanga pulp stands out as a source of carotenoids and phenolic compounds, mainly flavonoids with high antioxidant activities [6-8]. Antioxidant activities are directly related to the slowing of aging [9] and prevention of diseases such as cancer [10] and cardiovascular and circulatory diseases [11].

Synthetic antioxidants used by the food industry, although very effective and stable, have raised concerns regarding the safety of the doses and toxicity [12]. Thus, there is great interest in finding new antioxidants from natural sources [13] that show the same effects as the synthetic antioxidants. Natural antioxidants are safe, readily acceptable by consumers and identical to the food components that people have consumed for over a hundred years [14]. Pitanga is a promising fruit for obtaining natural antioxidants because it is a significant source of phenolic compounds. Therefore, the aim of this study was to identify fractions from the crude extract of pitanga that are concentrated in compounds with antioxidant properties.

Materials and Methods

Chemicals and reagents: Ethanol, ethyl acetate, methanol and sodium carbonate were purchased from Synth (Diadema, SP, Brazil). The Folin-Ciocalteu phenol reagent and gallic acid were from Dinamica (SP, Brazil) and Vetec (Duque de Caxias, RJ, Brazil), respectively. Aluminium-coated silica gel TLC plates and silica gel were from Merck (Darmstadt, Germany), and 1.1 diphenyl-2-picrylhydrazyl (DPPH•), butylated hydroxytoluene (BHT), ((+/-)- 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (trolox) were purchased from Sigma Chemical Co. (Steinheim, Germany). Authentic standards of the phenolic acids and flavonoids were purchased from Extrasynthese Co. (Genay, France). The solvents used for chromatography were of HPLC grade.

Raw material: Fresh pitanga was produced in the São Paulo State, in Southeastern Brazil, wet tropical climate, 22°32´S and 46°37´W and harvested in August 2008. The fruits were washed with running water and, after removing the seeds, they were crushed in a blender to obtain a homogeneous pulp, which was frozen and lyophilized. The freeze-dried pitanga pulp was stored in a freezer at -20°C in sealed packaging until the time of analysis.

Fractionation and isolation

Extraction of pitanga pulp: A representative amount of lyophilized pitanga pulp (386 g) extracted with 80% ethanol at a

pulp/solvent ratio of 1:10 (w/v). The extraction was performed by sonication for 30 min at room temperature. Then, the material containing the pulp and solvent was centrifuged at 5000 x *g* at 23°C for 15 min and filtered through qualitative filter paper. The hydroalcoholic extract (80%) was evaporated at 40°C under low pressure, and the resulting aqueous extract was frozen and lyophilized.

Fractionation and isolation of compounds with antioxidant activity: The freeze-dried pitanga extract was fractionated with an open dry column chromatography on normal phase silica gel (G60 Merck 70-230 mesh, particle size of 0.063-0.02 mm). The elution was performed with a mixture of ethyl acetate:methanol:water (77:13:10), and seven fractions were obtained. The fractions were monitored by thin layer chromatography (TLC) using the anisaldehyde reagent (4-methoxy-benzaldehyde:acetic acid:sulfuric acid - 1.0:48.5:0.5), followed by incubation at 100°C for 5 min. Fluorescent substances were visualized under ultraviolet (UV) light at wavelengths of 254 nm and 366 nm [15, 16]. The most bioactive fraction was purified by semi-preparative reverse-phase HPLC [Shimadzu PREP-ODS (H) 250 x 20 mm column eluted with gradient starting with acetonitrile: H_2O (15:85) to acetonitrile: H_2O (95:5) during 23 min, flow rate of 7 mL/min].

Absorbance of UV-visible spectrum: Measurements of the absorption spectra were recorded on a Shimadzu UV mini1240 apparatus at room temperature. The region from 200 to 660 nm was used for scanning.

Determination of total phenolic content: The total phenolic content was determined by the Folin-Ciocalteu method [17]. Aliquots of 0.50 mL of extracts and purified fractions were placed in test tubes, and 2.50 mL of the Folin-Ciocalteu/water solution (10/90 v/v) was added. After incubating for 5 min, 2 mL of 4% sodium carbonate was added. The absorbance was measured using a spectrophotometer at 740 nm (UV mini 1240, Shimadzu Co.) after incubating for 2 h. Gallic acid was used as chemical standard for calibration (5 to 100 μg/mL). The total phenolic content was expressed as mg gallic acid equivalent (GAE) per 1.0 g of extract or fraction (mg GAE/g).

Analytical high performance liquid chromatography (HPLC): A HPLC system equipped with two Shimadzu LC-6AD pumps, a Shimadzu SIL 10ADvp autosampler, a SPD-M10AVp detector, an oven CTO 10ASvp and a Shimadzu ODS-A column (RP-18 column size 4.6 mm x 250 mm, particle size, 5 μm) were used. Chromatograms were recorded at 260 nm. The column was eluted by using a linear gradient of water/acetic acid (98/2, solvent A) and water/acetonitrile/acetic acid (68/30/2, solvent B), starting at 0% B and increasing to 30% B (20 min), 50% B (30 min), 70% B (50 min) and held at 100% B (55-75 min) with a solvent flow rate of 1 mL/min. The following authentic standards of the phenolic acids and flavonoids (Extrasynthese Co.) were examined: ρ-coumaric acid, ferulic acid, gallic acid, sinapic acid, caffeic acid quercetin, kaempferol, myricetin, rutin, quercetin-3-β-D-glucoside, quercetin-3-D-galactoside, isovitexin, (-)-catechin, (-)-epigallocatechin gallate, (-)-epigallocatechin and (-)-epicatechin.

Q-TOF-MS/MS analysis: The MS system used was a quadrupole time-of-flight instrument (UltrOTOF- Q, Bruker Daltonics, Billerica, MA, U.S.A.) equipped with an ESI positive and negative ion source. The analyses were performed with the mass spectrometer in the full scan mode. The following settings were applied throughout the analyses: capillary voltage of 3.900 V; dry gas temperature of 150°C; dry gas flow of 4 L h^{-1}; and nebulizer gas nitrogen. The LC eluent was split 1:3, allowing the introduction of the sample into the stainless steel capillary probe where the compounds were ionized.

Antioxidant activity: DPPH radical (DPPH•) scavenging: The measurement of the DPPH free radical scavenging activity was determined as described by Moraes de Souza *et al.* [18]. The reaction medium consisted of 0.5 mL of the extract or purified fractions at a concentration of 1000 μg/mL, 3.0 mL of P.A. ethanol and 0.3 mL of 0.5 mM DPPH• solution in ethanol. The mixture was incubated in the absence of light at room temperature for 45 minutes. Subsequently, the absorbance was measured using a spectrophotometer (UV mini 1240, Shimadzu Co.) at 517 nm. The standards used were α-tocopherol, BHT and trolox, tested at a concentration of 50 μg/mL. The anti-radical activity was calculated using the following formula:

$$\text{Antioxidant activity (\%)} = 100 - ((Aa - Ab) * 100/Ac)$$

where Aa = absorbance of the sample; Ab = absorbance of the blank (prepared by replacing the volume of the DPPH• solution with an equal volume of P.A. ethanol); Ac = absorbance of the control (prepared by replacing the volume of the extract with an equal volume of P.A. ethanol).

To determine the antioxidant activity of the isolated compounds, the analysis of the DPPH scavenging activity was performed in a 96-well polystyrene microplate. In each microplate cavity, 170 μL of the solution, 60 μL of the DPPH radical in ethanol and 50 μL of the isolated compounds were added. After incubating for 45 min at room temperature, the absorbance was measured at 517 nm with a spectrophotometer. The antioxidant activity was determined as previously described.

The concentrations of the samples (extract, purified fractions and isolated compounds) responsible for the 50% decrease in the DPPH free radical initial activity (EC_{50} μg/mL) were calculated by linear regression of the antioxidant activity, measured at various concentrations of each sample.

Results and Discussion

Fractionation of pitanga pulp and isolation of antioxidant compounds: In this study, the antioxidant activities of the crude extract, purified fractions and isolated compounds obtained from the pitanga, a typical Brazilian fruit, were investigated. The activity-directed purification of the crude extract using several chromatographic separations resulted in the isolation of two compounds with high antioxidant activities (Fig. 1). The tests for the detection of the antioxidant activity were monitored throughout every phase of the fractionation and isolation steps.

Fractionation by the cellulose column produced seven fractions that displayed different colors and chemical profiles through the TLC technique (Fig. 2). The yields obtained from the freeze-dried pitanga pulp were 1.53, 3.64, 3.90, 5.46, 18.98, 70.2 and 156 g (w/w) for fractions 1 - 7, respectively (Fig. 1).

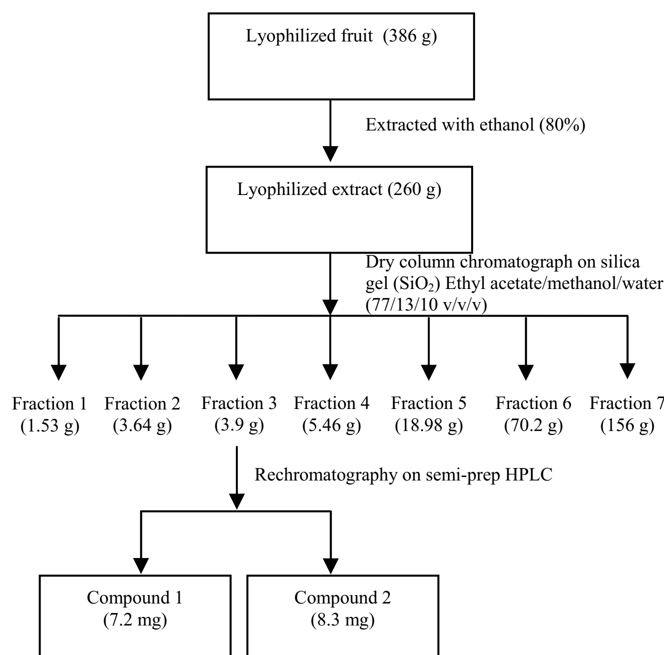

Figure 1. Procedure for antioxidant activity-guided fractionation and the isolation of compounds from the pitanga fruit.

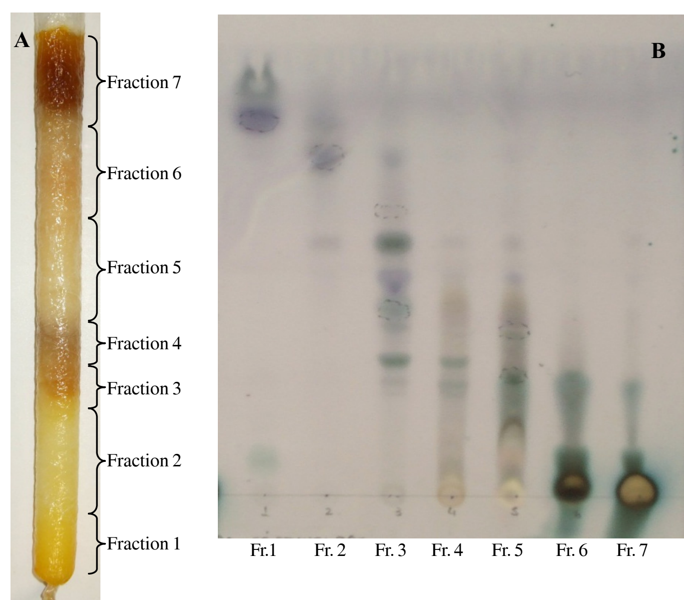

Figure 2. Color fractions eluted by dry column chromatography (A) and TLC fractionation process (B).

According to Fig. 3, fraction 3 showed the highest absorption peak in the 220 to 350 nm range, a region of light absorption of the phenolic compounds. Moreover, fraction 3 showed the highest level of total phenolic compounds (35.11 mg GAE/g) and the highest antioxidant activity among all fractions and the lyophilized crude extract (Table 1). The levels of total phenolic compounds were highly correlated ($R^2 = 0.92$) with the antioxidant activity, thus indicating that the phenolic compounds were responsible for the expression of the antioxidant activity. In fact, there are several authors who found strong correlations between antioxidant activity and the content of phenolic compounds [19-22].

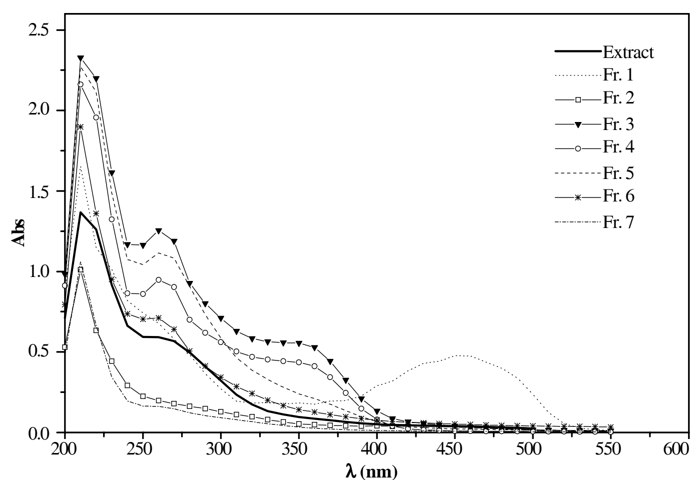

Figure 3. UV/visible absorption spectra of the crude extract and fractions from the pitanga fruit.

Table 1. Content of total phenolic compounds and antioxidant activity by DPPH of the pitanga extract and fractions.

Extract/fraction	Total phenolic compounds mg GAE/g extract or fraction*	Antioxidant activity (%)*
Extract	18.08±1.78	45.03±2.87
Fraction 1	8.78±0.06	9.46±2.73
Fraction 2	8.28±1.34	5.00±2.74
Fraction 3	35.11±0.75	74.59±5.30
Fraction 4	21.02±1.61	62.87±2.92
Fraction 5	9.68±1.24	18.22±3.73
Fraction 6	12.85±2.76	39.07±7.5
Fraction 7	8.10±1.78	16.21±4.09

* Measurements were performed in triplicate, and the standard deviations are indicated.

Thus, fraction 3 was selected for the isolation of compounds with high antioxidant activities, which allowed for the isolation of 2 compounds using the semi-preparative HPLC technique; these were named compounds 1 and 2, and their yields were 7.2 and 8.3 mg, respectively (Fig. 1).

Antioxidant activity: According to Fig. 4, compound 1 showed an activity of 87.2 (%) when measured at a concentration of 50 ppm, which was equivalent to Trolox (88.7%), a synthetic analogue of vitamin E. The crude extract and compound 2 were both evaluated at a concentration twenty times higher than that of compound 1 (1000 ppm) and showed percentages of 45.0 and 62.4, respectively; these activities were significantly lower than that of compound 1. This result demonstrates that the fractionation and isolation techniques were effective and that compound 1 is in fact largely responsible for the antioxidant activity in the pitanga fruit.

The results of the antioxidant activity, expressed as the EC_{50}, also show that compound 1 has high activity (26 µg/mL), and compound 2, fraction 3 and the crude extract displayed the lowest activity (Table 2). The EC_{50} of compound 1 was identical to that of BHA, a potent synthetic antioxidant with an EC_{50} of 25 µg/mL, as reported by Yuan *et al.*[22] and was lower to that α-tocopherol (EC_{50} of 30 µg/mL), as reported by Carpes *et al.* [23]. The EC_{50} determines the minimum amount of sample required to reduce 50% of the DPPH free radical. However, the analysis of the antioxidant activity expressed only in percentages may underestimate the real potential of the samples.

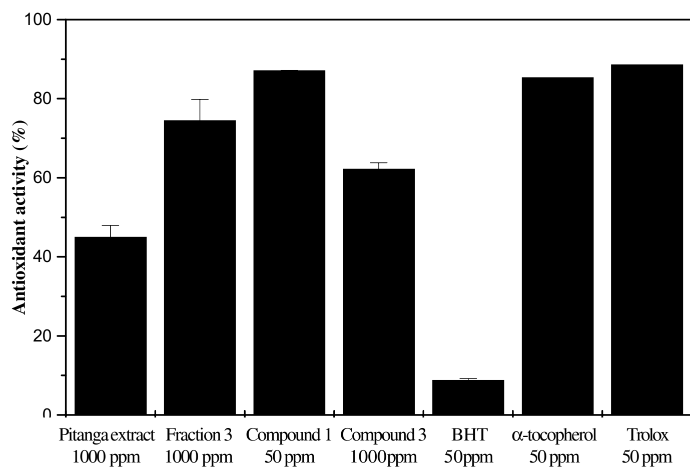

Figure 4. Antioxidant activity (%) of the pitanga extract, fractions and isolated compounds by the DPPH method.

* Measurements were performed in triplicate, and the standard deviations are indicated.

Table 2. EC_{50} values (µg / mL) for the crude extract, fraction 3 and isolated compounds from the pitanga fruit.

Sample	EC_{50} (µg/mL)
Extract	461 $\pm$ 16
Fraction 3	999 $\pm$ 39
Compound 1	26 $\pm$ 4
Compound 2	845 $\pm$ 51

* Measurements were performed in triplicate and the standard deviations are indicated.

Chemical analyses of crude extract and isolated compounds: Previous reports on the chemical composition of pitanga have been scarce, especially those characterizing the bioactive phenolic composition. Thus, with the aim of verifying the presence of phenolic compounds that are already known, the chemical profile of the crude pitanga extract was determined through the HPLC technique. The HPLC technique has been widely used in the separation and evaluation of simple phenolic compounds, such as phenolic acids and flavonoids [24, 25]. Although several previously reported standards of flavonoids and phenolic compounds for the pitanga such as kaempferol, myricetin and quercetin have been used [8], only a few compounds were found in the crude extract. The phenolic compounds that could be confirmed were gallic acid, caffeic acid, and quercetin-3-D-galactoside; however, the peaks corresponding to the isolated compounds 1 and 2 could not be identified (Fig. 5).

In order to have an idea of the isolated compounds sizes and their chemical natures, high-resolution mass spectrometry was performed using a quadrupole time-of-flight instrument, equipped with an ESI positive and negative ion source. For compound 1, the parent ion $[M-H]^-$, obtained from the mass spectra data in the negative mode, was identified to be 459.2488 (Fig. 6A). As can be seen, an ion with 447.1381 m/z was also detected, which is a typical fragment of flavonoids, such as the derivatives of quercetin and kaempferol. However, the fragments at 285, 301 and 317 m/z that are characteristic of the flavonoids kaempferol, quercetin and myricetin, respectively, were not found, demonstrating that these compounds or their glycosylated derivatives are not involved. However, an ion with 339 m/z was observed, indicating that this compound is possibly a flavonoid with a C-glycosylation. The loss of 146 u indicates an *O*-deoxyhexoside, and the loss of 132 u indicates an *O*-pentoside. Losses associated with cross-ring saccharide cleavages, such as 120 or 90 u, are indicative of C-glycosylation [26]. Compound 2 showed the deprotonated ion $[M-H]^-$ of 621.4812 m/z and base peak of 263.1462 m/z (Fig. 6B), which cannot be identified with the flavonoid standards, their available glycosylated derivatives, or the data available in the literature.

Therefore, it can be concluded that in addition to the flavonoids quercetin, kaempferol and myricetin reported by authors such as Hoffmann-Ribani *et al.* [8], there are other phenolic compounds in the pitanga fruit with high antioxidant activity.

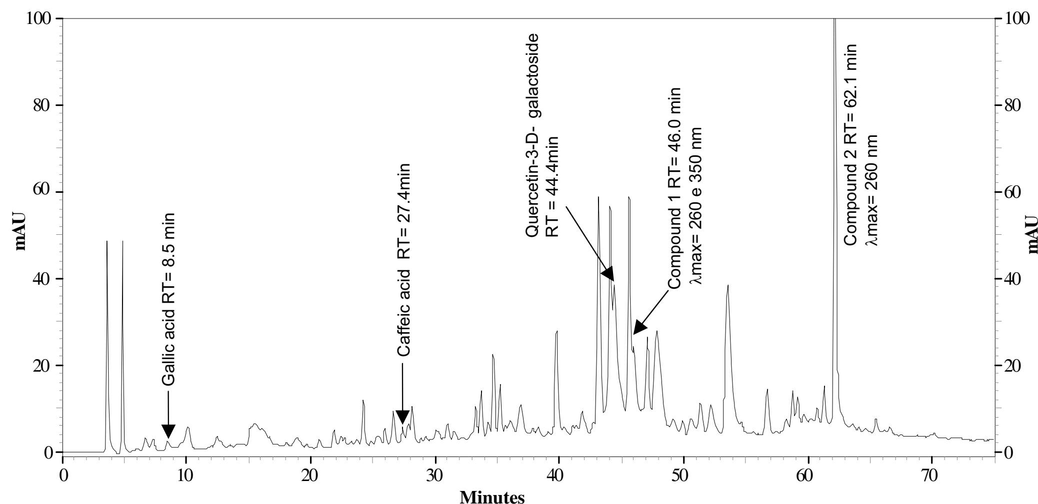

Figure 5. HPLC-DAD chemical profile of the crude extract from the pitanga fruit.

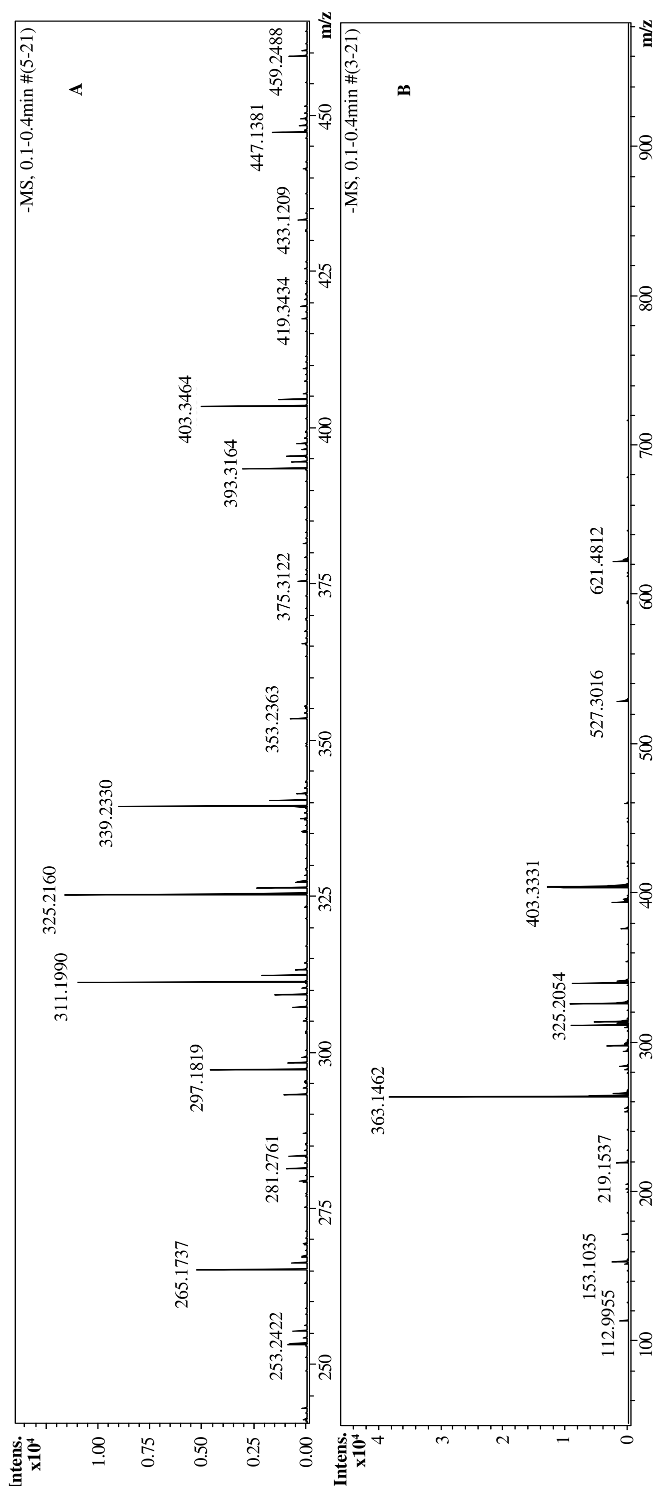

Figure 6. ESI-TOF-MS spectra in negative ion mode of compound 1 (A) and compound 2 (B).

Conclusions

The simple purification step applied to the crude *E. uniflora* extract gives rise to a fraction more concentrated in compounds, which presented high antioxidant activity by DPPH• scavenging. From this fraction it was possible to isolate two compounds. Compound 1 is a potent antioxidant and it can be a flavonoid according to the data obtained by mass spectrometry techniques

The complete identification of these compounds is necessary in future studies using UV, IR and 2D-NMR techniques. The work is important in improving the development and production of this tropical fruit, since that bioactive fraction or isolate compound could be used as mild antioxidants for food preservation and in the treatment of many human diseases, in the future.

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