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Application of a degreasing process and sequential ultrasound-assisted extraction to obtain phenolic compounds and elucidate of the potential antioxidant of *Siparuna guianensis* Aublet

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This study investigated appropriate means to determine the total phenolic content and antioxidant activity of the leaves of *Siparuna guianensis*. Evaluation by thin layer chromatography (TLC) revealed phenolic compounds in all extracts. The results were confirmed by high-performance liquid chromatography (HPLC), with the identification and quantification of catechin, epigallocatechin gallate and rutin. The methanolic extract had the highest content of phenolic compounds, total tannins and condensed tannins (221.16 ± 3.39 mg GAE/g, 183.72 ± 1.85 mg GAE/g and 135.92 ± 1.00 µg/mL CE/g, respectively) and the strongest radical scavenging activity (IC_{50} = 37.54 ± 0.41 and 73.13 ± 2.09 µg/mL for DPPH and ABTS, respectively). The ethanolic and hydroethanolic extracts had the highest flavonoid (111.28 ± 4.59 and 103.27 ± 3.00 mg RE/g, respectively) and flavonol content (99.73 ± 0.64 and 77.31 ± 0.33 mg RE/g, respectively). In the correlation analysis, it was verified that tannins are the main phenolic compounds and antioxidants of *S. guianensis* leaves, and their flavonoid content consists mainly of flavonols. The data obtained establish *S. guianensis* as a new alternative source of antioxidant compounds, implying its potential application as a food preservative or in the production of nutraceutical and pharmacological products.

Key words: Flavonoid, polyphenol, condensed tannins, flavonol, plant extract, free radical scavenging.

INTRODUCTION

The current century is marked by the incidence of a number of lifestyle-related diseases, aggravated mainly by excess free radicals in the body. Interestingly, free radicals are byproducts of aerobic metabolism, and the human body's defenses against these harmful agents are limited, involving only the enzymes superoxide dismutase, catalase and glutathione peroxidase (Peterson, 2001). Several factors, such as environmental pollution, increased exposure to ultraviolet radiation, smoking, stress, etc., have led to an increase in the rate of these chemical species in the human body (Dal and Sigrist, 2016), contributing significantly to the appearance of various diseases associated with the oxidation of biomolecules such as cancer, diabetes, hepatic disorders, hypertension, atherosclerosis, Alzheimer's disease and arthritis among others (Kala et al., 2016). It is evident, then, that the search for products capable of neutralizing free radicals constitutes an emergency for humanity.

With regard to the development of products capable of combating free radicals, plants are currently the most promising sources, since this ability is intrinsic to them, being one of the factors that guarantee them survival in the environment (Agati et al., 2013). Moreover, many plants already have a long history of use by traditional populations; this contributes to the development of safe products. Research with plant bioactive compounds aimed at this purpose demonstrates that there is potential for both the development of antioxidant drugs and functional foods and natural preservatives (Rashid et al., 2016).

Siparuna guianensis Aublet, popularly known as negramina, is a plant that shows potential for obtaining drugs because it has a broad ethnobotanical indication, including the treatment of stomach disorders, malaria, hypertension, migraines, rheumatism, snake bites, as a postpartum bath and an insect repellent (Valentini et al., 2010; Aguiar et al., 2015). It is an important medicinal species in several countries of Latin America, distributed from southern Mexico to northern Paraguay, and from the Pacific to southeastern Brazil (Renner and Hausner, 2005).

The leaves of *S. guianensis* have already been evaluated for the potential elimination of free radicals (Andrade et al., 2013). However, because it is an aromatic plant, the evaluation was restricted to its essential oil and provided unsatisfactory results regarding this biological role. On the other hand, studies of *S. guianensis* chemical composition reported the presence of phenolic compounds in its leaves (Leitao et al., 2005; Negri et al., 2012). This class of compounds is nowadays the most promising for natural antioxidant products (Kala et al., 2016). As no work that determined the total phenolic content of this plant was found, it was inferred that research aimed at extracting this class of substances could evidence the antioxidant potential of *S. guianensis* leaves and further validate the use of the herb by popular medicine.

Thus, ultrasound-assisted extractions were performed, this being considered one of the most efficient and economical means of recovering polyphenols and other valuable compounds (Zhu et al., 2016). These extractions were preceded by a degreasing process, which is a fundamental step in the success of the extraction of phenolic compounds since the presence of interfering substances such as waxes, fats, terpenes and chlorophylls is an aspect that directly affects the efficiency of the extractive process of this class of compounds (Naczk and Shahidi, 2004).

This work aimed to establish adequate means to evaluate and quantify antioxidant compounds in leaves of *S. guianensis*. In view of this, the objective of this work was to determine the phenolic composition and *in vitro* antioxidant activity of leaf extracts of *S. guianensis*.

MATERIALS AND METHODS

Plant material

The leaves of *S. guianensis* were harvested in Gurupi City, Tocantins, Brazil (GPS coordinates: 11° 43' 45" latitude S, 49° 04' 07" longitude W) in August 2014. Access to the genetic patrimony for the purposes of research was authorized by the National Council for Scientific and Technological Development (CNPq) under registry no. 010580/2013–1. The plant was identified in the Herbarium of the Federal University of Tocantins and a voucher specimen was deposited (no. 10.496). The leaves were dried in a drying oven at 50°C and powdered using a knife mill. The powder obtained was stored in glass bottles and kept at room temperature sheltered from light and moisture.

Plant extract preparation

The plant material (45 g of leaf powder) was initially defatted with petroleum ether using Soxhlet apparatus (50°C for 24 h). Then, it was extracted successively by ultrasound with methanol, ethanol, 70% aqueous ethanol and distilled water. In sonication extraction, the sample was mixed with 350 mL of extracting solvent in a beaker (1000 mL). The sample flask was immersed in an ultrasound cleaning bath (USC1600, ULTRASONIC CLEANER, UNIQUE, São Paulo, Brazil) which has a frequency of 40 kHz (135 W), for a 1 h cycle at room temperature. For each solvent extraction, the process was repeated five times combining the supernatants. Each time, before extracting with the next solvent, the powdered material was air-dried at room temperature. After extraction, the resultant slurries were filtered under vacuum. The respective extracts had the

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solvents removed on a rotary evaporator at -600 mm Hg and 45°C, and were then lyophilized and stored in an amber bottle and kept in a desiccator until analysis.

Content of phenolic compounds

Total phenolic and total tannin content

Total phenolic and total tannin content was quantified using the Folin-Ciocalteu method coupled with the casein precipitation method as described by Amorim et al. (2008), with slight modification. To quantify the total phenolic content, methanolic solutions (0.2 mL) of *S. guianensis* extracts (0.25 mg/mL, w/v) or the standard used (gallic acid, 3-100 µg/mL, w/v) were mixed with Folin-Ciocalteu reagent (0.5 mL of 10%, v/v), sodium carbonate (1 mL of 75%, w/v) and purified and deionized water in a Millipore® Milli-Q purifier gradient system (Milli-Q water) (8.3 mL). The mixture was gently agitated and kept for 30 min in the dark. The absorbance was measured at 760 nm using a UV-visible spectrophotometer (Biospectro® SP-220, SP, Brazil). Total phenolic content was determined by interpolation of the absorbance of the samples against a calibration curve constructed with different concentrations of gallic acid in methanol (y = 1.3631x + 0.0213, adjusted r^2 = 0.962). The result was expressed as mg gallic acid equivalents (GAE) per gram of *S. guianensis* extract (mg GAE/g). To quantify the tannin content, the extract (1 mL, 1 mg/mL) was mixed with casein (0.1 g) and distilled water (5 mL) followed by vigorous shaking until homogenized. This solution was allowed to stand for 3 h and then centrifuged at 1358 × *g* for 10 min at 10°C. In the supernatant, the non-tannin phenolic constituents were determined in a way similar to the total phenolic content. The amount of tannins was calculated as the difference between total phenolic and non-tannin phenolic content in the extract. The total tannin content was expressed as milligrams of GAE per gram of *S. guianensis* extract (mg GAE/g).

Total condensed tannins

Condensed tannins were determined according to the method of Burns (1971) with slight modification, by using catechin as a reference compound. A volume of 400 µL of diluted extract in methanol (1 mg/mL) was added to 0.5 mL of a solution of hydrochloric acid (10% in methanol). The mixture was allowed to stand for 10 min. After that, 0.5 mL of vanillin (1% in methanol) was added and the mixture incubated for 10 min in a water bath at 60°C. Afterward, the absorbance was measured at 500 nm against methanol as a blank. Total condensed tannins were determined by interpolation of the absorbance of the samples against a calibration curve constructed with different concentrations of catechin in methanol (y = 2.5218x + 0.0072, adjusted r^2 = 0.9878). The amount of total condensed tannins is expressed as mg catechin equivalents per gram of *S. guianensis* extract (mg CE/g).

Total flavonoids

Total flavonoid content was determined by using a method described by Soares et al. (2014) with some modifications. Briefly, reactions were performed in triplicate by mixing 0.5 mL of methanolic solutions of the extract (0.25 mg/mL w/v) or standard (8–400 µg/mL w/v) with an aqueous solution of 0.5 mL of 60% acetic acid, 2 mL methanolic solution of 20% pyridine (v/v), 1 mL of 5% aluminum chloride (w/v) and 6 mL of Milli-Q water. The blank was made up of all of the reaction components with the extract or standard and replacing the aluminum chloride with methanol. This reaction mixture was gently stirred, kept for 30 min in the dark and then its absorbance was measured at 420 nm in a spectrophotometer. Total flavonoid content was determined by interpolating the absorbance of the samples against a calibration curve (y = 1.3324x + 0.0074, adjusted r^2 = 0.999) constructed with different concentrations of rutin standard and expressed as milligrams of rutin equivalents (RE) per gram of dry extract (mg RE/g). All experiments were performed in triplicate.

Total flavonols

The total flavonol content was measured based on the reported method of Miliauskas et al. (2004). Briefly, 0.5 mL of extracts (1 mg/mL) was mixed with 0.5 mL of aluminum chloride (20 mg/mL), followed by 1.5 mL of sodium acetate (50 mg/mL). The mixture was incubated at room temperature for 2.5 h. The absorbance was measured by a spectrophotometer at 440 nm. Total flavonol content was determined by interpolation of the absorbance of the samples against a calibration curve (y = 6.2744x + 0.0016, adjusted r^2 = 0.999) constructed with different concentrations of rutin in methanol (2-400 µg/mL, w/v). The result was expressed as mg RE per gram of *S. guianensis* extract (mg RE/g).

Evaluation of antioxidant activity

DPPH radical scavenging assay

The quantification of antioxidant activity performed by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) (Sigma-Aldrich) assay followed the description from Peixoto Sobrinho et al. (2011). Briefly, six dilutions (varying between 10 and 250 µg/mL in methanol) of samples or positive control substances (rutin and ascorbic acid (Sigma-Aldrich)) were prepared. An aliquot (0.5 mL) of each dilution was mixed with DPPH (3 mL of 40 µg/mL in methanol). For contrast of the extractive solution color with the assay reaction, a blank was prepared, replacing the DPPH solution with methanol. After 30 min the absorbance was measured at 517 nm. The percentage scavenging values were calculated from the absorbance using the following equation:

$$AA\ (\%) = [(A_0 - (A_S - A_{blank}))/A_0] \times 100$$

Where AA is the antioxidant activity, A_S is the absorbance of the sample, A_{blank} is the absorbance of the blank and A_0 is the absorbance of the 40 µg/mL DPPH (without sample). The IC_{50} value was also calculated, denoting the concentration of a sample required to decrease the absorbance at 517 nm by 50%. The IC_{50} was expressed in µg/mL.

ABTS radical scavenging assay

The ABTS assay was performed according to the method described by Re et al. (1999), modified by Liu et al. (2014). Initially, a radical cation ($ABTS^{+\bullet}$) was prepared with potassium persulfate solution (5 mL of 2.45 mM) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) aqueous solution (2.5 mL of 7 mM). This mixture was kept in the dark at room temperature for 16 h before use. The final solution was diluted with ethanol to an absorbance of 0.70 ± 0.05 at 734 nm. A 200 µL sample in methanol at different concentrations (1.25-500.00 µg/mL) was mixed with 3.0 mL $ABTS^{+\bullet}$ solution. After 6 min the absorbance was measured at 734 nm. Rutin and ascorbic acid served as positive controls. The percentage inhibition was calculated by the following formula:

$$\text{ABTS radical scavenging activity}\ (\%) = [1 - (A_S/A_c)] \times 100$$

Where Ac is the absorbance of the blank (without sample) and A_s is

the absorbance of the sample. The IC_{50} value was also calculated to measure the concentration of a sample which was required to decrease the absorbance at 734 nm by 50%.

Profiling of phenolic compounds

TLC analysis

Qualitative analysis of investigated extracts was evaluated by using the TLC method described by Wagner and Bladt (1996). The test was performed on a 0.20 mm Silica gel 60 Alugram® Sil G plate (Macherey-Nagel GmbH & Co., Düren, Germany) using benzene-ethyl acetate-formic acid-methanol (60/30/10/5, v/v/v/v) as the mobile phase. The developed plates were dried with a hairdryer, then sprayed with NEU/PEG reagent (spray with a 1% solution of 2-aminoethyl diphenylborinate in methanol and spray with a 0.5% ethanolic solution of polyethylene glycol). After a new drying, the plates were visualized under UV light at 365 nm using a darkroom viewing cabinet (SOLAB®, model SL 204) equipped with 365 and 254 nm lamps and then photographed using a Samsung WB100 digital camera. The retention factor (Rf) values were calculated and compared with reference standards rutin, quercetin, catechin and gallic acid.

HPLC analysis

The *S. guianensis* extracts were analyzed by high-performance liquid chromatography (HPLC) using a Shimadzu® chromatograph (LC-10 Avp series, Kyoto, Japan) equipped with an LC-10AD pump, DGU-14A degasser, UV-vis SPD-10A detector, CTO-10A column oven, Rheodyne manual injector (loop 20 µL) and a CLASS LC-10A integrator. The separation was carried out using a 250 × 4.6 mm C18 reverse-phase Phenomenex Luna column of 5 µm particle size. The mobile phase consisted of water/acetic acid (100:1, v/v) (A) and acetonitrile (B). The linear gradient program was: 10-24% B from 0-10 min, 24-25% B from 10-12 min, 25% B from 12-23 min, 25-28% B from 23-25 min, 28-30% B from 25-26 min, 30% B from 26-35 min, 30-100% B from 35-40 min and 100% B from 40-50 min. The flow rate was 0.87 mL/min, column oven temperature 30°C and UV detection at 280 nm. Identification was based on retention time by comparison with commercial standards, such as catechin, epigallocatechin gallate and rutin (Sigma®). To confirm the identification of the compounds in the extracts, co-chromatography was performed. The quantities of the compounds were determined by correlating the area of the analyte with the calibration curves of the standards. The calibration curves for each substance were: catechin, $y = 24246x + 20852$ ($r^2 = 1.0000$); epigallocatechin gallate, $y = 35453x - 72831$ ($r^2 = 0.9879$) and rutin, $y = 62021x + 8132.5$ ($r^2 = 0.9942$). The results were then expressed in micrograms per milligram of extract (µg/mg). Before being injected into the equipment, the samples (1 mg/mL) and reference substances (5-300 µg/mL) were dissolved in methanol-acetonitrile solution (80:20 v/v) and filtered through a 0.45 µm membrane filter (Millipore).

Statistical analysis

All tests were performed in triplicate. Results were expressed as the means ± SD (n = 3). Results showing a probability of occurrence of the nullity hypothesis of less than 5% ($p < 0.05$) were considered statistically different applying analysis of variance (ANOVA), followed by multiple comparisons by the Tukey test in ASSISTAT 7.0 software. Pearson's correlation coefficient was determined between results of the different assays: Total phenolic content, total tannin content, total condensed tannins, total flavonoids, total flavonol, IC_{50} DPPH and IC_{50} ABTS.

RESULTS AND DISCUSSION

Several studies report that *S. guianensis* leaves constitute a material rich in terpenes, which are extracted for various purposes (Valentini et al., 2010; Portella et al., 2014). However, terpene presence is not a feature favorable to phenolic compound extraction in plants because these two classes of metabolites act in a competitive way for some solvents in extraction (Naczk and Shahidi, 2004), this perhaps justifies the scarcity of quantitative data regarding the total phenolic content of the *S. guianensis* species. In the present work, the degreasing process seems to have eliminated this interference, favoring phenolic compound extraction. This aspect has also been observed by Zhu et al. (2010) under study with *Portulaca oleracea* L.

The sequential extraction carried out on the defatted powder of *S. guianensis* leaves provided 9.584 ± 0.294 g (21.299 ± 0.007%) of methanolic extract, 1.947 ± 0.173 g (4.327 ± 0.004%) of ethanolic extract, 2.262 ± 0.182 g (5.028 ± 0.004%) of hydroethanolic extract and 1.123 ± 0.107 g (2.495 ± 0.002%) of aqueous extract. This result suggests that absolute methanol is a better solvent for the extraction of the degreased powder of *S. guianensis* leaves.

The sequence in which the solvents were used in the extraction proved to be adequate, mainly because it showed some selectivity for specific groups of polyphenols in each extract. Based on the results presented in Table 1, flavonoids and flavonols predominated in the ethanol and hydroethanolic extracts. The lowest content of phenolic compounds was found in the aqueous extract, which was constituted mainly by flavonoids. The solvent selectivity was also evident in the observation that in the hydroethanolic extract, obtained at intermediate polarity (70% ethanol), the tannin content was equivalent to that of flavonoids. Alothman et al. (2009), in a study of tropical fruits from Malaysia, also found that the recovery of phenolic compounds is dependent on the solvent used and its polarity.

The methanolic extract had the highest concentration of phenolic compounds and tannins in comparison with those obtained by other solvents. There is no information in the literature investigating quantification of this class of compounds in *S. guianensis* leaves. However, methanolic extraction in the aerial parts of other plants has been described (Wijekoon et al., 2011). The results of the *S. guianensis* leaf tannin quantification are in accordance with those obtained by Naima et al. (2015), who compared the impact of different extraction methods of tannins from *Acacia mollissima* bark and reported that the highest polyphenol content was obtained using methanol.

In light of the expressive concentrations of phenolic compounds obtained in this study, it can be said that the

Table 1. Total phenolic content, total tannin content, total condensed tannins, total flavonoids content and total flavonols of leaf extracts of *S. guianensis* obtained by degreasing process and sequential ultrasound-assisted extraction.

Extract	Total phenolic (mg GAE/g)	Total tannins (mg GAE/g)	Cond. tannins (mg CE/g)	Flavonoids (mg RE/g)	Flavonols (mg RE/g)
Methanolic	221.16±3.39[a]	183.72±1.85[a]	135.92±1.00[a]	79.25±3.00[b]	46.28±0.84[c]
Ethanolic	126.28±1.69[c]	65.37±1.12[c]	8.88±0.91[c]	111.28±4.59[a]	99.73±0.64[a]
Hydroethanolic	155.63±1.69[b]	103.76±0.73[b]	23.66±1.00[b]	103.27±3.00[a]	77.31±0.33[b]
Aqueous	47.05±1.69[d]	10.34±0.42[d]	7.29±0.23[c]	22.11±2.29[c]	6.12±0.83[d]

Means followed by the same letter in column do not significantly differ (p >0.05) by Tukey test.

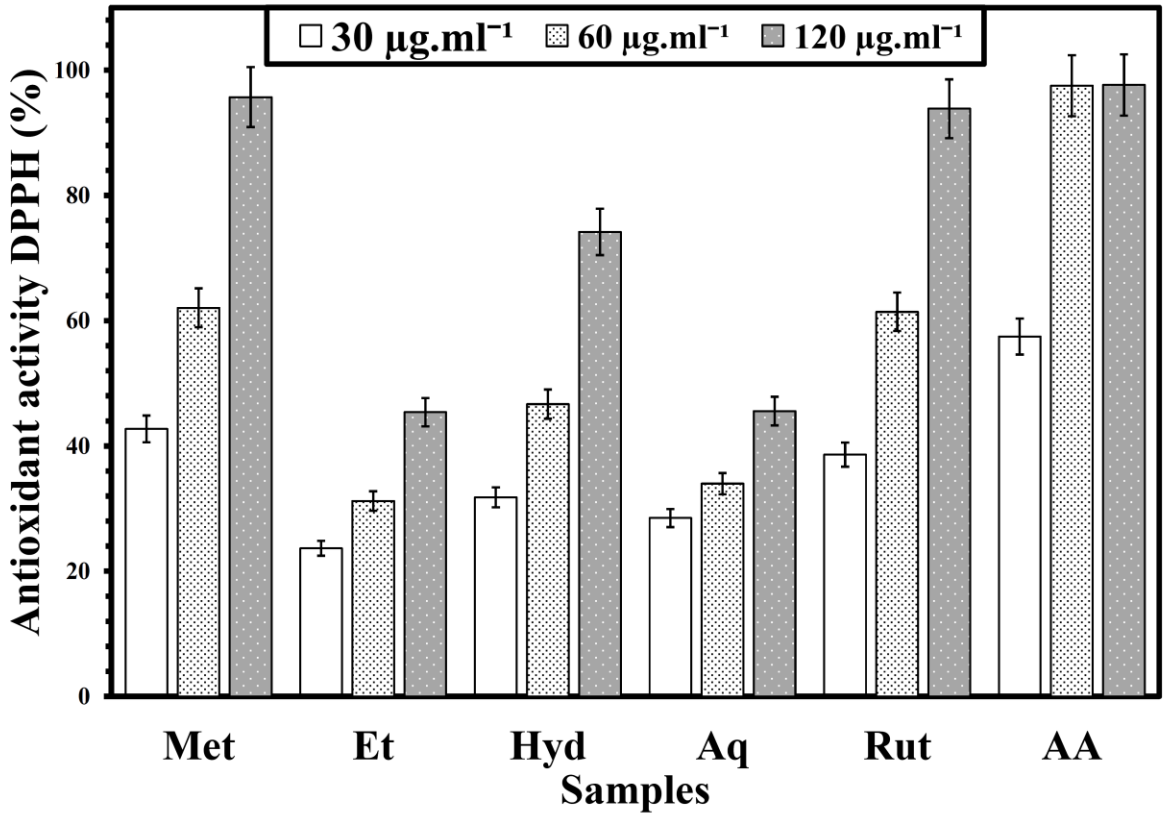

Figure 1. Antioxidant potential of the extracts, measured in the DPPH assay in three different concentrations. Values represent percentage radical scavenging. Met: methanolic extract; Et: ethanolic extract; Hyd: hydroethanolic extract; Aq: aqueous extract; Rut: rutin; AA: ascorbic acid. The bars indicate the standard errors.

broad ethnobotanical indication of *S. guianensis* may have a strong relationship with this class of compounds, since they have diverse biological roles. Valentini et al. (2010) reported that this plant is used for therapeutic purposes by different populations of Latin America.

Among the non-enzymatic molecules produced by plants, phenolic compounds, especially flavonoids and phenolic acids, are the most effective in free radical neutralization; as a consequence of this they are also the most investigated to obtain natural antioxidants (Gülçin, 2012; Shebis et al., 2013). The data for total phenolic content quantification and evaluation of the antioxidant activity in *S. guianensis* leaves allowed us to infer that this plant is efficient in this biological role, and could be used in the development of pharmacological products capable of acting on disease-related oxidative damage.

The total antioxidant activity evaluated by the DPPH method in the methanolic extract, ethanolic extract, hydroethanolic extract, aqueous extract and of reference substances, rutin and ascorbic acid, at concentrations of 30, 60 and 120 µg mL are shown in Figure 1. These data make it clear that free radicals were eliminated by the

Table 2. IC_{50} of DPPH and ABTS radical scavenging activity of leaf extracts of *S. guianensis* obtained by degreasing process and sequential ultrasound-assisted extraction.

Sample	DPPH (IC_{50}) (µg/mL)	ABTS (IC_{50}) (µg/mL)
Methanolic extract	37.54±0.41[e]	73.13±2.09[e]
Ethanolic extract	137.04±2.08[b]	213.15±1.66[b]
Hydroethanolic extract	68.98±1.60[c]	114.46±1.52[c]
Aqueous extract	150.06±0.35[a]	218.044±1.73[a]
Rutin	44.74±0.38[d]	78.029±0.26[d]
Ascorbic acid	25.13±0.04[f]	27.35±1.61[f]

Means followed by the same letter in column do not significantly differ ($p > 0.05$) by Tukey test.

Table 3. Pearson correlation test between the inhibitory concentrations (IC_{50}) and Phenolic content.

	ABTS IC_{50}	DPPH IC_{50}	Total phenolic	Total flavonols	Total flavonoids	Total cond. tannins
DPPH IC_{50}	0.998*	–				
Total phenolic	-0.890	-0.919	–			
Total flavonols	-0.076	-0.140	0.409	–		
Total flavonoids	-0.310	-0.371	0.601	0.971*	–	
Total cond. tannins	-0.825	-0.825	0.825	-0.137	0.065	–
Total tannins	-0.929	-0.948*	0.987*	0.256	0.465	0.898

*Significant at $p \leq 0.05$; DPPH = DPPH scavenging activity; ABTS = ABTS scavenging activity.

different extracts and the positive controls in a concentration-dependent manner.

The methanolic and hydroethanolic extracts were the most active. When comparing the efficiency of the extracts with reference substances, the antioxidant potential of *S. guianensis* became more evident since, at concentrations of 30 and 120 µg/mL, the percentage of total antioxidant activity of the methanolic extract was statistically higher that the rutin positive control.

The minimum amount of sample or reference substance (rutin and ascorbic acid) which was able to inhibit oxidation by 50% (IC_{50}), evaluated by the DPPH and ABTS methods, also showed significant effects of the extracts of *S. guianensis* leaves on the elimination of free radicals. As shown in Table 2, the effect of eliminating ABTS radicals from the different extracts showed a similar trend to the DPPH radical scavenging activity. The IC_{50} value revealed that the extract obtained with methanol had a higher free radical scavenging activity in comparison to other extracts and was more efficient than the rutin positive control. These results are consistent with previous research reporting this biological role for tannins (Zhao et al., 2011), especially condensed tannins (Ujwala et al., 2012). According to Sandhar et al. (2011), the free radical scavenging ability of phenolic compounds is attributed mainly to the high reactivity of the substituent hydroxyls that readily interact with free radicals.

Results of correlation analysis between different pairs of assays are shown in Table 3. A strong correlation was observed between the DPPH and ABTS methods ($p < 0.0001$). The DPPH assay of antioxidant activity correlated significantly ($p = 0.0522$) with the total tannins. Moreover, significant correlations were observed between total phenolics and total tannins ($p = 0.0131$), indicating that tannins are the main constituents of this plant's leaves.

Another important observation led by the correlation analysis is the fact that *S. guianensis* leaf flavonoids are constituted mainly by flavonols ($p = 0.0286$). That suggests *S. guianensis* leaves are a good source of bioactive compounds since flavonoids have a wide pharmacological spectrum, such as antibacterial, antiviral, anti-inflammatory and antioxidant properties (Zhou et al., 2015), among these, flavonols are effective compounds against human pathogenic microorganisms (Sandhar et al., 2011).

There were no significant correlations ($p > 0.05$) between antioxidant activity and total phenolic ($p = 0.0815$ with DPPH and $p = 0.1103$ with ABTS), total flavonoid ($p = 0.1747$ with DPPH and $p = 0.1751$ with ABTS), total flavonol ($p = 0.6286$ with DPPH and $p = 0.6896$ with ABTS) and total condensed tannin content ($p = 0.1747$ with DPPH and $p = 0.1751$ with ABTS). This may be related to the presence of other compounds, especially sugars and ascorbic acid, which, according to Nur Syukriah et al. (2014), are able to interfere with quantification and antioxidant activity assays. The lack of significant correlation between total tannin content and total condensed tannins ($p = 0.1018$) indicates that other groups of the class may be present in *S. guianensis*

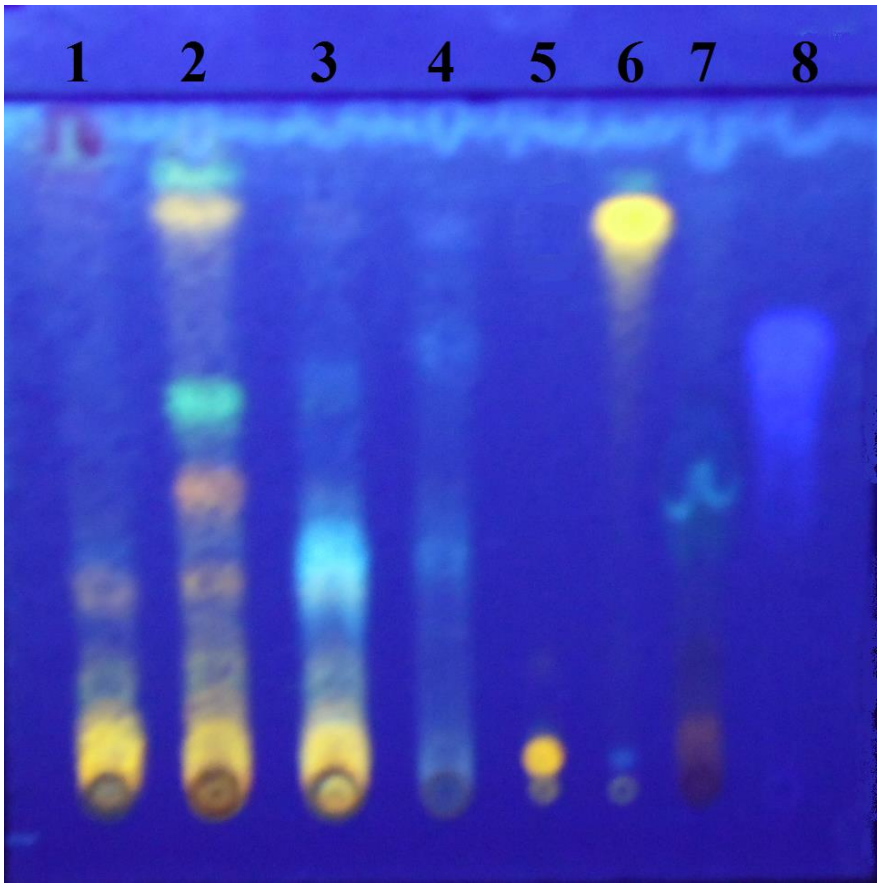

Figure 2. TLC profile of *S. guianensis* leaves extracts and reference standards, detected at under UV light at 365 nm, as described in materials and methods. 1: methanolic extract; 2: ethanolic extract; 3: hydroethanolic extract; 4: aqueous extract; 5: rutin; 6: quercetin; 7: catechin; 8: gallic acid.

leaves.

Figure 2 shows TLC chromatographic separation after chemical derivation with the NEU/PEG reagent. Following the criteria of Wagner and Bladt (1996), flavonoids were detected as zones of orange fluorescence in UV light at 365 nm and phenolic acids with cyan-blue fluorescence under the same conditions. Compounds were detected with Rf characteristics for rutin (Rf = 0.06), quercetin (Rf = 0.82), catechin (Rf = 0.43) and gallic acid reference substances (Rf = 0.62). The four extracts showed distinctive TLC chromatogram profiles, demonstrating selectivity by the solvents in the conditions used. This analysis was consistent with the HPLC analysis.

As noted in the HPLC analysis (Figure 3), most of the major peaks in the aqueous extract appeared at retention times < 18 min while in the methanolic, ethanolic and hydroethanolic extracts, peaks with retention times up to 26 min were observed. This analysis also allowed the identification of representative constituents of phenolic compounds (tannins and flavonoids), making spectrophotometric quantification for tannins, flavonoids and flavonols consistent. Moreover, it confirmed the evaluation performed by the DPPH and ABTS methods, because the identified compounds, catechin, epigallocatechin gallate and rutin, are reported in several studies as good antioxidants (Liu et al., 2015; Lins et al., 2017; Shi et al., 2017).

A comparison of the HPLC profiles of *S. guianensis* extracts and co-chromatography with reference substances showed that, among the identified compounds, epigallocatechin gallate (retention time of 12.0 min) was present in all extracts, although at different concentrations. The amounts found for this compound showed a tendency similar to the spectrophotometer measurements both for *in vitro* biologic activity and for the total phenolic content. Such an observation makes it evident that condensed tannins, besides being the predominant phenolic compounds in *S. guianensis* leaves, are also the most influential in the antioxidant activity of this organ of the plant.

The epigallocatechin gallate level was quantified to be 9.051 µg/mg in the methanolic extract, 4.753 µg/mg in the ethanolic extract, 7.254 µg/mg in the hydroethanolic extract and 4.860 µg/mg in the aqueous extract. Catechin

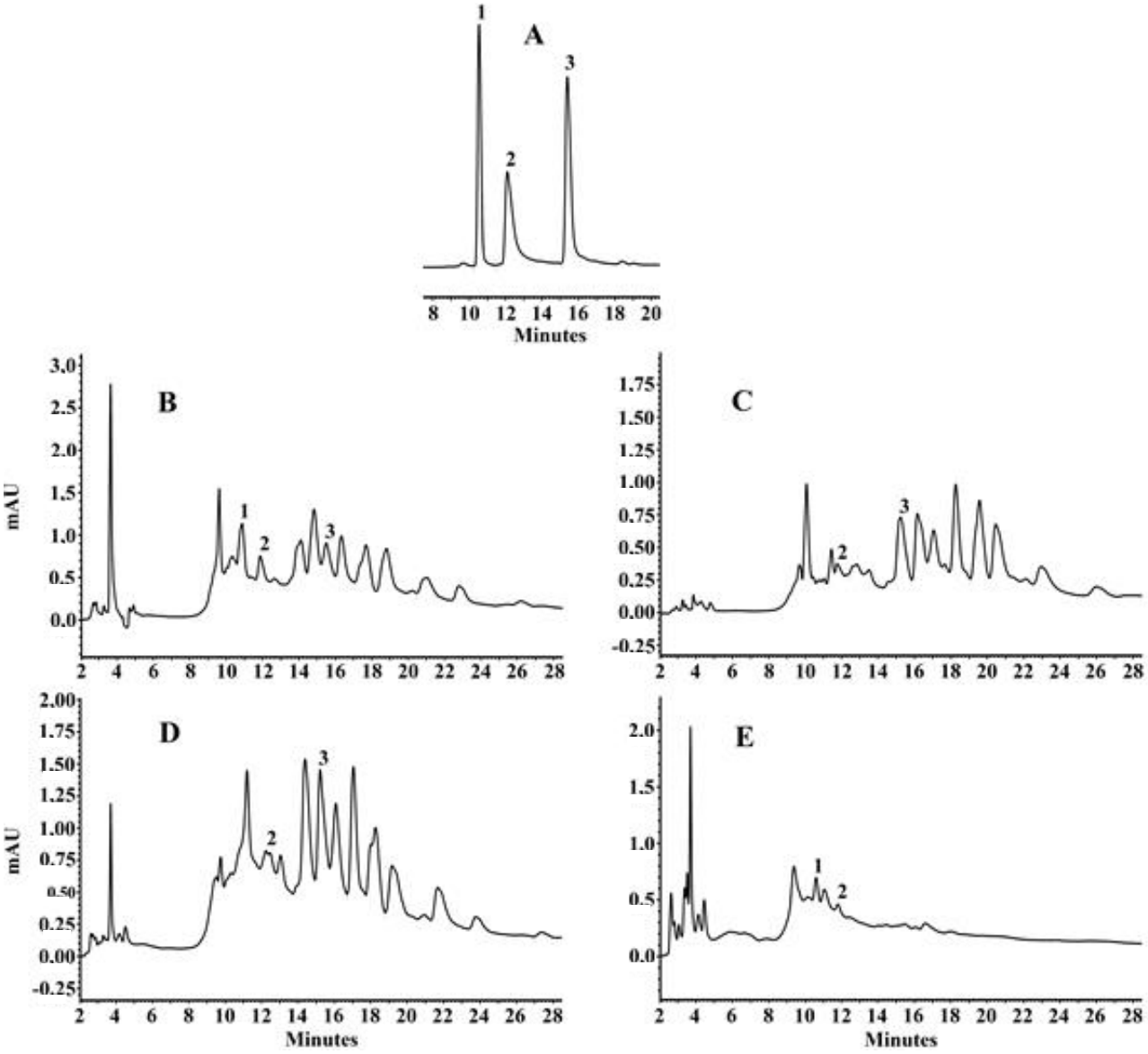

Figure 3. HPLC profile of the *S. guianensis* leaves extracts detected at 280 nm, as described in materials and methods. A: Reference substances; B: methanolic extract C: ethanolic extract; D: hydroethanolic extract; E: aqueous extract. Peak 1: catechin; peak 2: epigallocatechin gallate; peak 3: rutin.

(retention time of 10.5 min) was detected only in the methanol (11.863 µg/g) and aqueous extracts (3.910 µg/mg). Rutin (retention time of 15.3 min) was present only in the methanolic (4.928 µg/mg), ethanolic (5.037 µg/mg) and hydroethanolic extracts (12.300 µg/mg), not in the aqueous extract.

The HPLC identifications are consistent with other studies of the *S. guianensis* species. Leitao et al. (2005) and Negri et al. (2012) have also reported the presence of phenolic compounds in leaves of *S. guianensis*.

The HPLC data (Figure 3) make it evident that the obtained extracts are rich in phenolic compounds of great pharmacological importance because, beyond their antioxidant properties, catechin and epigallocatechin gallate are also regarded as antifungal (Anand and Rai, 2016), anti-inflammatory, chemopreventive and lung protective (Shahid et al., 2016). Rutin presents significant therapeutic potential against cancer, being able to decrease the adhesion and migration of cancer cells (Sghaier et al., 2016) besides having antidiabetic activity (Aitken et al., 2017).

The unidentified peaks shown in the chromatograms (Figure 3) may represent phytocomponents with biological activity different from reported potential. Like this, additional research into *S. guianensis* leaves could expand the pharmacological spectrum of that plant.

Conclusions

This study showed that degreasing with petroleum ether followed by ultrasound-assisted sequential extraction with different solvents is an efficient way to obtain antioxidants from *S. guianensis* leaves. Antioxidant activity evaluation allowed observation that *S. guianensis* leaves are rich in substances capable of scavenging free radicals, especially condensed tannins. Moreover, spectrophotometric quantification and HPLC analysis revealed that *S. guianensis* leaves are a potential source of compounds with pharmacological properties. Thus, this information set establishes *S. guianensis* as a new alternative source of antioxidant compounds, implicating its potential application as a food preservative or in nutraceutical and pharmacological products.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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