BJPS

Brazilian Journal of
Pharmaceutical Sciences

http://dx.doi.org/10.1590/s2175-97902022e19723

Article

Physicochemical characterization and cosmetic applications of *Passiflora nitida* Kunth leaf extract

Priscilla Tobias Ribeiro[1], Tatiana do Nascimento Pedrosa[2], Francisco Celio Maia Chaves[3], Lucindo José Quintans-Júnior[4], Adriano Antunes de Souza Araújo[4], Marne Carvalho de Vasconcellos[1], Silvya Stuchi Maria-Engler[2], Cláudia Cândida Silva[5], Felipe Moura Araújo da Silva[6], Héctor Henrique Ferreira Koolen[5], Emerson Silva Lima[1*], Ádley Antonini Neves de Lima[7]

[1]Faculty of Pharmaceutical Sciences, Federal University of Amazonas, Manaus, AM, Brazil, [2]Faculty of Pharmaceutical Sciences, University of São Paulo, São Paulo, SP, Brazil, [3]Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA), Manaus, AM, Brazil, [4]Department of Pharmaceutical Sciences, Federal University of Sergipe, São Cristóvão, SE, Brazil, [5]Superior School of Technology, Amazonas State University, Manaus, AM, Brazil, [6]Metabolomics and Mass Spectrometry Research Group, Superior School of Healthy Sciences, Amazonas State University, Manaus, AM, Brazil, [7]Department of Pharmaceutical Sciences, Federal University of Rio Grande do Norte, Natal, RN, Brazil

Passiflora nitida Kunth, an Amazonian *Passiflora* species, is little studied, although the specie's high biological potential. Herein the plant's pharmacognostic characterization, extract production, antioxidant potential evaluation, and application of this extract in cosmetic products is reported. The physical chemical parameters analyzed were particle size by sieve analysis, loss through drying, extractive yield, total ash content, laser granulometry, specific surface area and pore diameter (SBET), differential scanning calorimetry, thermogravimetry (TG), and wave dispersive X-Ray fluorescence (WDXRF). Total phenol/flavonoid content, LC-MS/MS analysis, DPPH and ABTS antioxidant radical assays, cytotoxicity, melanin, and tyrosinase inhibition in melanocytes test provided evidence to determine the content of the major constituent. *P. nitida* dry extract provided a fine powder with mesopores determined by SBET, with the TG curve showing five stages of mass loss. The antioxidant potential ranged between 23.5-31.5 $mg{\cdot}mL^{-1}$ and tyrosinase inhibition between 400–654 $\mu g{\cdot}mL^{-1}$. The species presented an antimelanogenic effect and an inhibitory activity of cellular tyrosinase (26.6%) at 25 µg/mL. The LC-MS/MS analysis of the spray-dried extract displayed the main and minor phenolic compounds constituting this sample. The results indicate that *P. nitida* extract has promising features for the development of cosmetic formulations.

Keywords: Antioxidant. Cosmetic. *Passiflora nitida* Kunth. Tyrosinase inhibitor.

INTRODUCTION

The Amazon Rainforest has an enormous diversity of plants that remains primarily underused and which has a great potential for industry and research. The search for new assets for cosmetic use has aroused great interest in products of natural origin (Burlando, Cornara, 2017). *Passiflora nitida* Kunth, which is found in Amazonian flora, is popularly known as "maracujá do mato" and "bell apple" and grows spontaneously in secondary vegetation, riverbanks, and highways (Carvalho *et al.*, 2010).

Few studies on the pharmacological and phytochemical constituents of *P. nitida* have been published. The plant extract's effect on blood coagulation has been recently demonstrated, and platelet aggregation activities are attributed to the presence of flavonoids and

***Correspondence:** E.S. Lima. Faculdade de Ciências Farmacêuticas. Universidade Federal do Amazonas. Av. Gen. Rodrigo Otavio, 6200. CEP: 69010-300 Manaus, Brasil. E-mail: eslima@ufam.edu.br. Orcid: https://orcid.org/0000-0002-9367-2812

coumarins in the methanolic extract (Carvalho *et al.*, 2010). Another study demonstrated the antioxidant and antimicrobial activity of *P. nitida*, which seems to have a relationship with the phenolic content (Bendini *et al.*, 2006). Furthermore, the plant extract is used in traditional medicine to treat gastrointestinal disorders (Carvalho *et al.*, 2010).

Other studies carried out with the Passiflora genus demonstrated their potential in cosmetic use, presenting antioxidant activities, sun protection ability, skin whitening by inhibition of the tyrosinase enzyme, and collagen synthesis (Lourith, Kanlayavattanakul, 2013; Maruki-Uchida *et al.*, 2013). Despite the relevant evidence of the use of Passiflora in skin abnormalities, few studies on this use were found for *P. nitida* in the literature. To our knowledge, no studies have been done on characterizing the plant's raw material, nor on obtaining the dry extract.

This work aimed at characterizing the species leaves' raw material, dry extract production, the study of the antioxidant potential, and application of this extract for cosmetic formulations.

MATERIAL AND METHODS

Material

P. nitida Kunth leaves were collected from EMBRAPA (Empresa Brasileira de Pesquisa Agropecuária). The voucher specimen was deposited in the INPA (National Institute of Amazonian Research) herbarium under No. 209547. Access to botanical material was registered in the SISGEN (National System of Management of Genetic Heritage and Associated Traditional Knowledge) under the number A3965C3. All solvents and chemical reagents used in the experiments were of analytical purity.

Granulometric analysis through sieving

Approximately 25.0 g of dry leaves were crushed using a knife mill, weighed and passed through sieves with mesh openings of 2.00, 1.00, 0.850, 0.710, 0.600, 0.500, 0.425, 0.355, 0.250 mm and a collector. The sifting was performed through sieves at 60 vibrations per second for 15 min, following the methodology described in the Brazilian Pharmacopoeia 5th edition (Anvisa, 2010). The fractions retained in the sieves and collector were weighed and analyzed through a graphical method. Histogram distribution was constructed in order to obtain the distribution of the particles. By means of passage and retention curves, the average particle size was calculated. The results represent the mean of triplicates (Figueiredo *et al.*, 2014).

Determination of loss through drying

This was determined following gravimetric method described in the Brazilian Pharmacopoeia 5th edition (Anvisa, 2010). The assay was performed in triplicate.

Extractive content

About 1.0 g of ground dry leaves was transferred to an Erlenmeyer flask with 100 mL of distilled water. The mixture was weighed and subjected to extraction by decoction for 10 minutes. After cooling, it was weighed again and reconstituted to the initial mass with distilled water and filtered discarding the first 20 mL of filtrate. About 20 g of the filtrate was weighed and placed on weight filter that had been previously weighed, and evaporated to dryness in a water bath, with occasional stirring. After evaporation, the flat-bottomed dish containing the sample was placed in an oven at 105 ºC ± 2 ºC until reaching a constant weight, and the solids were then calculated. The result was expressed in triplicate and the extractive yield was calculated according to the equation below (Bundesvereinigung, 1986):

$$EC = \frac{(g \times FD \times 100)}{m - Ld} \times 100 \quad (1)$$

where: EC = extractive content (%, m/m); g = mass (g) of dry residue, FD = constant, equal to 5; m = mass of the initial sample (g); Ld = Loss through drying of the sample (m/m).

Total ashes analysis

About 3 g of sample, accurately weighed, were transferred and distributed evenly in porcelain crucibles that were previously tared. The samples were incinerated in a muffle furnace at 500 ºC until all the organic matter was eliminated, then the crucibles were cooled in a desiccator cabinet and weighed, following the methodology described in the Brazilian Pharmacopoeia 5th edition (Anvisa, 2010). We calculated the percentage of ash in relation to the dried drug. The analysis was performed in triplicate.

Extractive solution preparation

To prepare the extraction solution, we used *P. nitida* leaves that had been previously dried in a circulating air oven and then ground in a knife mill. The extraction solution of 5%, 7.5% and 10% (w/v) was obtained using, respectively, 50 g, 75 g and 100 g, to 1000 mL of hydroethanolic solvent at 20% (v/v). The extractive solutions were macerated for 48 h under agitation and at room temperature. Subsequently, they were subjected to both regular and vacuum filtering.

Dry extract through spray drying (DEPN)

The volume of 1,000 mL of extractive solution 5 %, 7.5 % and 10 % (w/v) was subjected to a spray dryer (LM MSD 1.0, Labmaq of Brazil Ltda), under the following conditions: inlet and outlet temperature 140/100°C, 85 % flow, flow rate 1.2 mL·min^{-1} and 0.07 mm nozzle. Thus, three kinds of DEPN were prepared.

Specific surface area and pore diameter (S_{BET})

Specific surface area and mean pore diameter of *P. nitida* extracts were determined through the nitrogen adsorption technique by applying the model proposed by Brunauer, Emmett and Teller [BET], following the methodology described by (Maximiano, Costa, Souza, 2010) and USP 30th edition (USP, 2008). A sample of about 200 mg of the drug was transferred to the sample tube and was degassed for 72 hours at 120 °C to remove any material adsorbed within the pores and on the surface of the material. An ASAP 2420 accelerated surface area and porosimetry system was used, which was equipped with software for determining the surface area (SBET) and porosity.

Particle size distribution through light scattering (laser granulometry)

The sample was dispersed in a solution of surfactant Triton X-100 at 0.02 % and then subjected to ultrasound for 3 minutes. In order to analyze the particle size distribution, a Microtrac S3500 laser diffraction particle size analyzer was used, with a measurement time of 10 seconds, flow at 70% by volume, and distribution using the Fraunhofer method.

Differential scanning calorimetry (DSC)

The analysis of differential scanning calorimetry was performed using a calorimeter (DSC-50, Shimadzu, Tokyo Japan), operating at a temperature of 25–500 °C. Approximately 3 mg of the sample were placed into flat aluminum caps and then subjected to a heating rate of 10 °C·min^{-1} under a synthetic air atmosphere (50 mL·min^{-1}). The calorimeter was calibrated with the reference standards, indium and zinc. Heating-cooling-heating cycles were also performed in order to confirm the thermal phenomena (Araújo *et al.*, 2006).

Thermogravimetry analysis

The thermoanalytical characterization was performed in duplicate using a thermobalance (model TGA 50, Shimadzu, Tokyo Japan), under nitrogen atmosphere flow at 50 mL·min^{-1}, and the mass of the sample of about 5 mg (± 0.4) was heated in an aluminum crucible over a temperature range of 30-600°C, at a heating rate of 10 °C·min^{-1}. Before the tests, instrument calibration was performed using the reference standards aluminum and zinc (Araújo *et al.*, 2006).

Wave dispersive X-ray fluorescence (WDXRF) analysis

The samples were impregnated in a filter paper, suitable for analysis. Data was collected from a sample

of white filter paper for reference during the analysis process. The samples had their data collected under the same conditions as the filter paper. The water and alcohol used in the extract preparation, as well as cotton samples and filter paper used in the filtration of the extracts were also analyzed.

For all analyses, the wave dispersive x-ray fluorescence (WDXRF) method was used, using the equipment Rigaku® Supermini, with a palladium tube and 200 s of exposure time and 200 W of power. The conditions were adjusted, taking into account the sample matrix, sampler and sample holder. All elements were identified by their Kα and/or Kβ energy (Janssens, 2003).

Geological references, such as GBW 3125, 7105 and 7113, were used for equipment calibration. Through mathematical software, the emission peaks were related to the respective concentrations of a particular element. Each element found was quantified using an external standard of salts of a known purity, diluted boric acid, also with known purity of at least six predetermined concentrations, which were subjected to the same conditions as the analysis samples.

Total phenol content

The determination of total phenolic content present in the DEPN was performed through spectrophotometry using the Folin-Ciocalteau reagent, according to Singleton, Rossi (1965) with modifications. First, 1 mg·mL^{-1} solutions were prepared from the dry extracts of 5%, 7.5% or 10% (w/v). In 96-well plates, 50 µL of Folin-Ciocaulteau reagent (Sigma) was added to 10 µL of extract/gallic acid/water. To eliminate the reading of the color of extracts and gallic acid, 10 µL of the extract or gallic acid was added to 50 mL of water. The microplate was incubated at 37 °C for 8 min and the first reading using a plate reader (DTX 800, Beckman Coulter) was performed at 715 nm. After this reading, 240 µL of sodium carbonate at 0.4% was added. The plate was incubated for three min and a second reading was performed. The test was performed in triplicate. The polyphenol content (PC) in mgEAG by mg of extract was calculated from the formula generated by the curve of gallic acid and converted to be expressed as a percentage using the formula below:

$$\%PC = \frac{(\frac{mgEAG}{mg\ of\ extract}) \times 100}{1\ mg\ of\ extract} \quad (2)$$

Total flavonoid content

The total flavonoids were determined according to the methodology used by Chang *et al.* (2002), with modifications. First, the extracts were diluted with ethanol at a concentration of 1 mg·mL^{-1}. Then, 30 µL of the extract and/or quercetin standard (diluted in 80% ethanol at a concentration of 1 mg·mL^{-1}) and 90 µL of ethanol were added to each well of the microplate. Afterwards, 6 µL of aluminum chloride 10% and 6 µL potassium acetate (1 mol·L^{-1}) were added to both and diluted in 168 µL of distilled water. In addition, for the blank, 30 µL of the extract and/or standard quercetin were used, as well as 270 µL of ethanol. The plate was then incubated for 30 min at room temperature and the absorbance assessment was performed at a wavelength of 405 nm in an microplate reader (DTX 800, Backman Counter). The analysis was performed in triplicate, and the results were obtained using, first, the difference between the absorbance of the sample and the standard absorbance by the respective blank. Subsequently, the percentage concentration of flavonoids was calculated:

$$TF = (\frac{Abs_{sample} - Abs_{white}}{Abs_{standard} - Abs_{white}}) \times 100 \quad (3)$$

DPPH scavenging activity

The antioxidant activity was determined by evaluating the ability of the antioxidants present in the samples to scavenge stable radical DPPH, according to the methodology by Molyneux (2003), with modifications. A total of 270 µL of DPPH (1 mg·mL^{-1}) and 30 µL of sample solution and/or standard quercetin were first added to each well of the microplate at a concentration of 100 mg·mL^{-1}, with their respective dilutions. For control, 270 µL of DPPH and 30 µL of ethanol were used. The plate was incubated for 30 min at room temperature in the dark and the reading performed at 517 nm. The calculations of the inhibition were made using the following formula:

$$\%Inhibition = 100 - (Abs_{sample} - Abs_{control}) \times 100 \quad (4)$$

ABTS radical scavenging activity

The determination of antiradical activity was carried out following the methodology described by Re *et al.* (1999) with minor modifications. The solution was initially prepared by the reaction of ABTS: 0.7 mM of ABTS radical dissolved in 5 mL of deionized water with 5 mL of 5 mM potassium persulfate. The reaction mixture was incubated at room temperature and in the dark for 12 h. The solution obtained an oxidized greenish-blue tint. Afterwards, the solution was diluted in a proportion of 1:7 solution/deionized water, which was then adjusted for an absorbance of 1.000 ± 0.1 at a wavelength of 630 nm. A 30-µL aliquot of the sample solution and 270 µL of the ABTS solution were added to microplates, in triplicate. In the control group, the same volume of diluent and ABTS was added. The samples and controls were incubated for 15 min in the dark, after reading was performed in a microplate reader. The standard used in this determination was quercetin. The calculations of the inhibition were made using the following formula:

$$\%Inhibition = 100 \times (1 - \frac{Abs_{sample} - Abs_{control}}{Abs_{control}}) \quad (5)$$

Tyrosinase inhibition

The inhibition of tyrosinase by the extracts was evaluated following the method described by Hearing Jr. (1987). The extracts were prepared at a concentration of 10 mg·mL^{-1} and tested as follows: 20µL of the inhibitor was added to the microplate [extract, diluent standard (control) or (ac. Kojic)]. Afterwards, 80 µL of the enzyme tyrosinase solution was added and left to incubate for 5 min at 37 °C. Subsequently, 100 µL of DOPA color reagent was added, which was subjected to the immediate reading times: 5, 10, 15 and 20 min, with incubation of the plate at 37 °C. The plates were read in an ELISA plate reader and tyrosinase inhibition was calculated using the formula below:

$$\%Inhibition = 100 - [(\frac{Abs_{T20sample} - Abs_{T0sample}}{Abs_{T20control} - Abs_{T0control}}) \times 100] \quad (6)$$

Tyrosinase activity and melanin content assay

B16F10 cells were stimulated with IBMX (Isobutylmethylxantine) 25 µM for 24 h and then treated with 25 µg/mL of *P. nitida* for 48 h. Subsequently, the cells were placed in two microtubes (one for melanin content with 1 X 10^6 cells/microtube) and lysed with Triton-X 100 in PBS with 1mM PMSF (phenylmethanesulfonyl fluoride). Next, this solution was centrifuged at 10,000 g at 4 °C for 10 min to obtain the supernatant and the pellet for the measurement of tyrosinase activity and melanin content, respectively.

Tyrosinase activity was estimated by measuring the rate of L-DOPA oxidation, using the method reported by Tomita & Tagami (1992) with modifications. Protein concentrations were determined by the Bradford method, using bovine serum albumin (BSA) as standard. A total of 100 µL of supernatant containing 100 µg of total proteins were added to each well in a 96-well plate, and then mixed with 100 µL of L-DOPA at 3 mg/mL. After incubation at 37°C for 90 min, the dopachrome was monitored by measuring the absorbance at 475 nm.

Melanin content was measured as described by Hosoi *et al.* (1985) with modifications. After the lysis, the pellets of the B16F10 cells (1 X 10^6) were mixed with 250 µl of 1 N NaOH containing 10% DMSO for 1 h at 95 °C. The absorbance at 405 nm was measured using a microplate reader. The melanin content was determined from a standard curve prepared from a standard of synthetic melanin (Sigma, USA).

Phytochemical analysis

An LC-MS system (6,550 iFunnel, Agilent Technologies, Santa Clara, CA, USA) consisted of a liquid chromatography system coupled to a quadrupole time-of-flight mass spectrometer equipped with an electrospray ionization (ESI) source was used to explore the phenolic composition of extracts using an untargeted metabolomic approach (Koolen *et al.*, 2013). Chromatographic separation was performed in a Poroshell column (120 EC-

C18 2.7 μm, 4.6 mm i.d., 50 mm, Agilent Technologies, Santa Clara, CA, USA) using a binary mobile phase. Solvent A was water and solvent B was methanol. The gradient elution at 30 °C was as follows: 0–2 min, 5% B (v/v); 2–10 min, 5–70% B (v/v); 10–17 min, 70–100% B (v/v); 17–21 min, 100% B (v/v) at a flow rate of 0.5 mL/min. The autosampler temperature was maintained at 20 °C and the injection volume was 10 μL. The ESI source parameters were as follows: VCap, 3500 V; Nozzle voltage, 0 V; Fragmentor, 100 V; Skimmer, 65 V; gas temperature, 280 °C; gas flow, 14 L min^{-1}; nebulizer, 45 psi. The MS and MS/MS spectra were acquired at the *m/z* range of 50-700. Tentative identifications were performed using exact mass and comparison of the MS/MS spectral data with those previously published. The quantitative determination of phenolic compounds in the *P. nitida* leaves extract was performed using a previously described and validated method for UHPLC-MS/MS using the selected reaction monitoring (SRM) mode (Bataglion *et al.*, 2015). Results were expressed by means of μg/g of spray-dried extract (SDE).

RESULTS AND DISCUSSION

Granulometric analysis through sieving

From the analysis, it was noted that most of the particles have a particle size range of 0.25 to 0.71 mm. The powder is classified as a coarse powder in accordance with the classification of the Brazilian Pharmacopoeia 5th edition, which refers to particles that can pass in their entirety through a 1.70-mm sieve mesh and a maximum of 40% powder through 0.35-mm sieve mesh (Anvisa, 2010). The average particle size of the pulverized leaves dry was 0.506 (± 0.087) mm.

The granulometric evaluation is an important parameter because it has a direct influence on the efficiency of the extraction procedure (Migliato *et al.*, 2007). According to Sharapin, powders of a coarser nature are highly recommended for most botanical material because very fine powders can compromise the extraction (Fonseca, Silva, Leal, 2010; Sharapin, 2000). Therefore, the powder from *P. nitida* leaves showed good results regarding their particle size, since the range of 1.70 to 0.35 mm is considered coarse powder and, as such, favors the extraction of active compounds.

Determination of loss through drying

The average loss through drying (9.08 ± 0.08) and the coefficient of variation (0.9 %) is in accordance with the specification limit for botanical material humidity, i.e., 8 to 14%, as established by the Brazilian Pharmacopoeia 5th edition (Anvisa, 2010). The moisture content is an important parameter to be evaluated, since excess moisture may allow the development of fungi and bacteria in the botanical material, besides favoring the action of enzymes whose activity can lead to degradation of the chemical constituents (Fonseca, Silva, Leal, 2010). The presence of excess moisture is directly related to the correct storage of the raw material (Migliato *et al.*, 2007). Thus, the results are within the established parameters for minimizing microbial contamination and chemical degradation and demonstrate the correct storage of the material.

Extractive content

The average of extractive content was 17.16 ± 0.05 and the coefficient of variation was 0.32%. This study was employed exclusively in order to assist in testing physicochemical characterization of the botanical material, but it is also related to water-soluble compounds present in the raw material, such as sugars, amino acids, glycosides and flavonoids, among others (Barni, Cechinel Filho, Couto, 2009).

Total ash

Determination of ash content allows the verification of non-volatile inorganic impurities that may be present as contaminants (Barni, Cechinel Filho, Couto,2009). Using the Brazilian Pharmacopoeia 5th edition as a reference (Anvisa, 2010), which sets the maximum limit of 10% for total ash in two other species, the *Passiflorae dulcis folium* and *Passiflorae acetum folium*, the result of the ash content in *P. nitida* was 5.43 ± 0.11 and the coefficient of variation was 1.99%, suggesting a low amount of non-volatile inorganic contaminants present in the sample.

Analysis of the particle size distribution

The distribution range of the particle size, the retention rate and the average particle size of the dried extract from the 5% (w/v), 7.5% (w/v) and 10% (w/v) extractive solutions are displayed in Table I. The average size of particles found in the DEPN classify it as a very fine powder, in accordance with the classification of the post by the Brazilian Pharmacopoeia 5th edition, which passes in its entirety through a sieve with a 125-µm nominal aperture (Anvisa, 2010). A comparison between the different kinds of DEPN demonstrated that 7.5% had the smallest average particle size (25.06 µm), and 95% of the particles have a size of ≤ 59.74 µm. The smaller particle size is desirable because it influences many factors such as rate of dissolution, content uniformity and stability. It also reduces the gritty sensation in formulations for topical use (Allen, Popovich, Ansel, 2007).

TABLE I - Characterization of particle size and surface area of DEPN

Sample	Particle Size Distribution	Retention Percentage	Average Particle Size	Surface Area	Pore Diameter
5%	95% ≤ 77.77µm	81%	27.35µm	2.5 $m^2 g^{-1}$	226.5 Å
7.5%	95% ≤ 59.74 µm	78%	25.06µm	2.74 $m^2 g^{-1}$	225.3 Å
10%	95% ≤ 229.6 µm	54%	37.68 µm	2.35 $m^2 g^{-1}$	221.0 Å

Specific surface area and pore diameter

Allen, Popovich, Ansel (2007) reported that a small particle size leads to an increase in the specific surface of the powder. Correlating the results of the specific surface area with a pore diameter shown in Table I and the results of the laser granulometry that indicated a very fine powder, the three kinds of DEPN presented a high specific surface area and the pores are classified as mesopores.

The porosity of a material determines important physicochemical properties such as adsorption, mechanical strength, dissolution characteristics and wettability (Porte, Leão, Porte, 2011; Schoonman *et al.*, 2001). The DEPN showed good results in relation to porosity. It is useful for a dry extract in pre-formulation or formulation that the porosity be in the range 20-500 Å, which ranks as mesopores, and confers increased specific surface area, greater cohesion between the particles and wettability. All these characteristics are important and should be considered in the formulation of the development phase.

Differential scanning calorimetry (DSC)

The DSC curve of the DEPN from the 5% (w/v) extractive solution showed four thermal events (Figure 1). The first event corresponds to its loss of water, the second and third events are related to thermal decomposition and the fourth event is associated with the removal of carbonaceous material. The DEPN from the 10% (w/v) extractive solution had well-defined endothermic events between 25 °C and 200 °C. These results were confirmed by the TG/DTG curves (Araújo *et al.*, 2006).

In the DSC curve of the DEPN from the 7.5% (w/v) extractive solution, two endothermic events were observed: the first between 25 °C and 100 °C, and the other above 400 °C. However, the TG/DTG curves of the DEPN from the 7.5 % (w/v) extractive solution had four weight loss events. Thus, the two intermediate events in the DSC curve of the DEPN from the 7.5 % (w/v) extractive solution are a junction of endothermic and exothermic events, making it impossible to clearly observe any changes in the baseline.

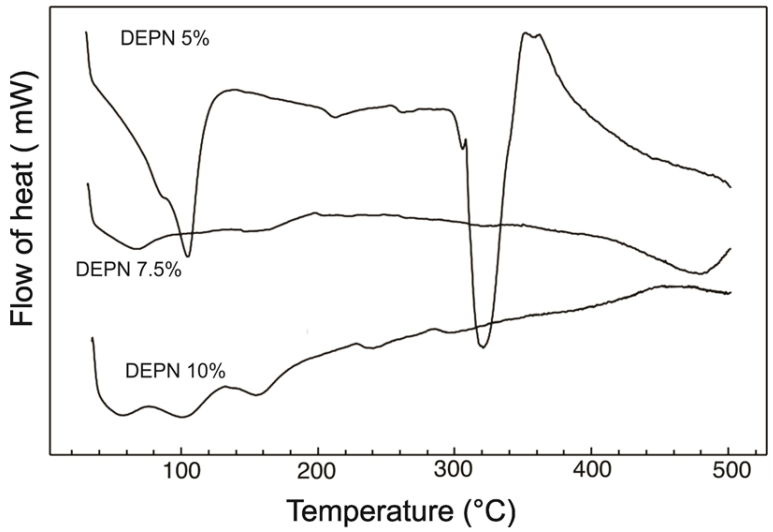

FIGURE 1 - Differential scanning calorimetry (DSC) of the DEPN 5%, 7.5% and 10% extractive solutions in a synthetic air atmosphere (50 mL·min^{-1}) at a heating rate of 10 °C min^{-1}.

Thermogravimetry (TG)

The TG/DTG curves of the three types of DEPN studied (Figure 2) showed four events of mass loss, of which the first event occurred between 25 and 150°C and is related to loss of surface water; this result is consistent with endothermic events that appear on the DSC curve. It is possible to observe three thermal events in the TG/DTG curve after dehydration. The first, between 150 and 400 °C, is associated with the thermal decomposition of organic compounds. The second stage of thermal decomposition, with the formation of carbonaceous material, occurs between 400 and 600 °C. The latter event happens above 600 °C, which is related to the loss of carbonaceous material formed in the previous step. Although there is a difference in the curve of the DEPN from the 10% (w/v) extractive solution, it has a derivative tending to zero because of the continuous loss of decomposition products (Araújo *et al.*, 2006; Melo *et al.*, 2013).

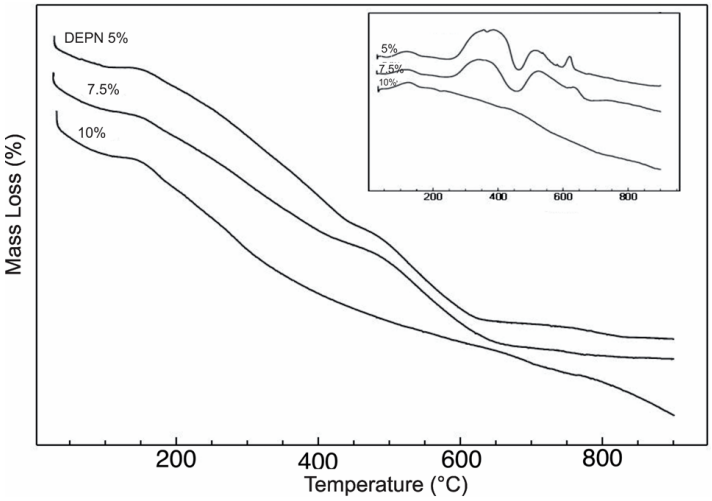

FIGURE 2 - Thermogravimetry (TG) and DTG of the DEPN 5%, 7.5% and 10% extractive solutions in nitrogen atmosphere (50 mL·min^{-1}) and a heating rate of 10 °C min^{-1}.

Wave dispersive X-ray fluorescence (WDXRF)

Many nutrients found in plants are absorbed from the soil and, among these, heavy metals or other elements may exist that cause disease or toxicity. Thus, to ensure the safe use of a plant raw material, the identification and quantification of the elements is of great importance. Various elements can be quickly found using the WDXRF technique (Rüdiger, Silva, Veiga-Júnior, 2009).

In all three types of the DEPN examined, the following chemical elements were found: Ca, K, Cl, S, P, Si, Mg, Na and Br, which probably originate from the soil from which the plant was removed. It was expected that the concentrations of the elements would increase with the increase in the concentration of the extracts, but it can be clearly seen that the 7.5% extract has a higher concentration of the elements observed, as shown the Table II. It is believed that this fact is related to the extraction process, where the concentration of 7.5% can be considered optimal for better extraction. Other data presented in this work, such as laser particle size, phenol and flavonoid content, among others, show the same standard of behavior, confirming the WDXRF data.

TABLE II - Concentration of chemical elements in *P. nitida* by WDXRF

Elements	% (m/m)		
	DEPN 5 %	**DEPN 7.5 %**	**DEPN 10 %**
Ca	0.00366	0.01207	0.005
K	0.03364	0.06786	0.04016
Cl	0.01355	0.0273	0.01933
S	0.01166	0.02064	0.01409
F	0.00491	0.00851	0.00609
Si	0.00045	0.00126	0.00062
Mg	0.02015	0.02762	0.01586
Na	0.0003	0.00045	0.00023
Br	0.00081	0.00162	0.00134

Determination of total phenolic content and total flavonoids

Table III shows the results of total phenolic content and total flavonoids for the DEPN. *P. nitida* presents a phenol content that is higher than that found in the species *P. edulis* and *P. alata* (Zhang *et al.*, 2013).

It is also interesting to note that increasing botanical material concentration does not necessarily mean that we will have a dry extract with a higher quantity of phenols and flavonoids. The results found in the DEPN from the 10% (w/v) extractive solution demonstrate this point since the levels were lower compared with the extracts from the 5% (w/v) and 7.5% (w/v) extractive solution. This occurs probably due to the saturation of the medium during the extraction process.

TABLE III - Antioxidant activity of the different DEPN concentrations

Tests	DEPN			Standard
	5%	**7.5%**	**10%**	**Ascorbic ac.**
Phenols (%)	16.7	18.9	17.9	
Flavonoids (%)	3.3	4.1	3.9	
IC_{50}(μg mL^{-1}) DPPH	23.5	31.5	25.9	2.7
IC_{50}(μg mL^{-1}) ABTS	14.9	21.8	20.4	4.8

IC_{50} = 50% inhibitory concentration

Determination of antioxidant activity

Table III shows the results of antioxidant activity of the DEPN. The analysis shows that there is antioxidant activity, though there are no significant differences in the results with the change in the types of extracts. These results, when compared to the values found for ascorbic acid, which was used as a standard, proved to be ± 10 times lower for DPPH and ± 5 times lower for ABTS. However, the antioxidant activity found in the DEPN was very good, especially compared to what has been reported

for other species of *Passiflora*. For example, Zhang *et al.* (2013) reported that *P. edulis* presents DPPH values between 875 and 1,100 mg·mL^{-1} and *P. incarnata* between 76 and 282 mg·mL^{-1}. In a previous study, Bendini *et al.* (2006) demonstrated excellent antioxidant activity of *P. nitida* through DPPH and ABTS assays.

Phenolic compounds are usually directly correlated with antioxidant activity, and their ability to donate hydrogens, which allows scanning of free radicals (Yilmaz *et al.*, 2013). Flavonoids are polyhydroxylated phenolic compounds that have antioxidant properties, which include free radical termination and chelating action of metals, besides inhibiting several enzymes. These multiple functions are related to their distinct chelating action, which occurs through their hydroxyl groups (Ziaullah, Warnakulasuriya, Rupasinghe, 2013). Previous studies have demonstrated the antioxidant activity of some flavonoids, such as orientin, isoorientin, vitexin and isovitexin, using HPLC in combination with the analytical results of DPPH (Demirkiran *et al.*, 2013; Yao *et al.*, 2012).

The antioxidant activity of *P. nitida* is related to phenols and flavonoids present in its composition. Teixeira *et al.* (2014) found four major peaks by using HPLC in the analysis of flavonoids from *P. nitida* leaves. Three flavonoids were identified, namely vitexin, swertisina, orientin, and one peak could not be identified when compared to the most common flavonoids.

Tyrosinase inhibition

In evaluating the inhibition of tyrosinase, the IC50 showed that the DEPN 5, 7.5 and 10% were respectively 654.4, 436.9 and 400.1 µg·mL^{-1}. The results of the standard, which is kojic acid (KA), was 1.0 ± 0.1 µg·mL^{-1} (Figure 3B). Despite the difference between the standard and the extract, the DEPN inhibits tyrosinase. This inhibiting activity is related to the flavonoids present and their metal chelating activity. The mode of inhibition of flavonoids is typically a competitive inhibition of the oxidation of L-DOPA by tyrosinase, and a key role is attributed to the chelation of copper. Flavonoids are the most investigated secondary metabolites of plants that show inhibition of tyrosinase; and quercetin, kaempferol, myricetin and morin act as competitive inhibitors of tyrosinase. Studies have shown that vitexin and isovitexin inhibit tyrosinase (Chang, 2009; Ziaullah, Warnakulasuriya, Rupasinghe, 2013; Teixeira *et al.*, 2014; Yao *et al.*, 2011). It has also been demonstrated that DEPN is a promising natural skin-whitening agent and could be used by the cosmetic industry for smoothing benign pigments (Lourith, Kanlayavattanakul, 2013).

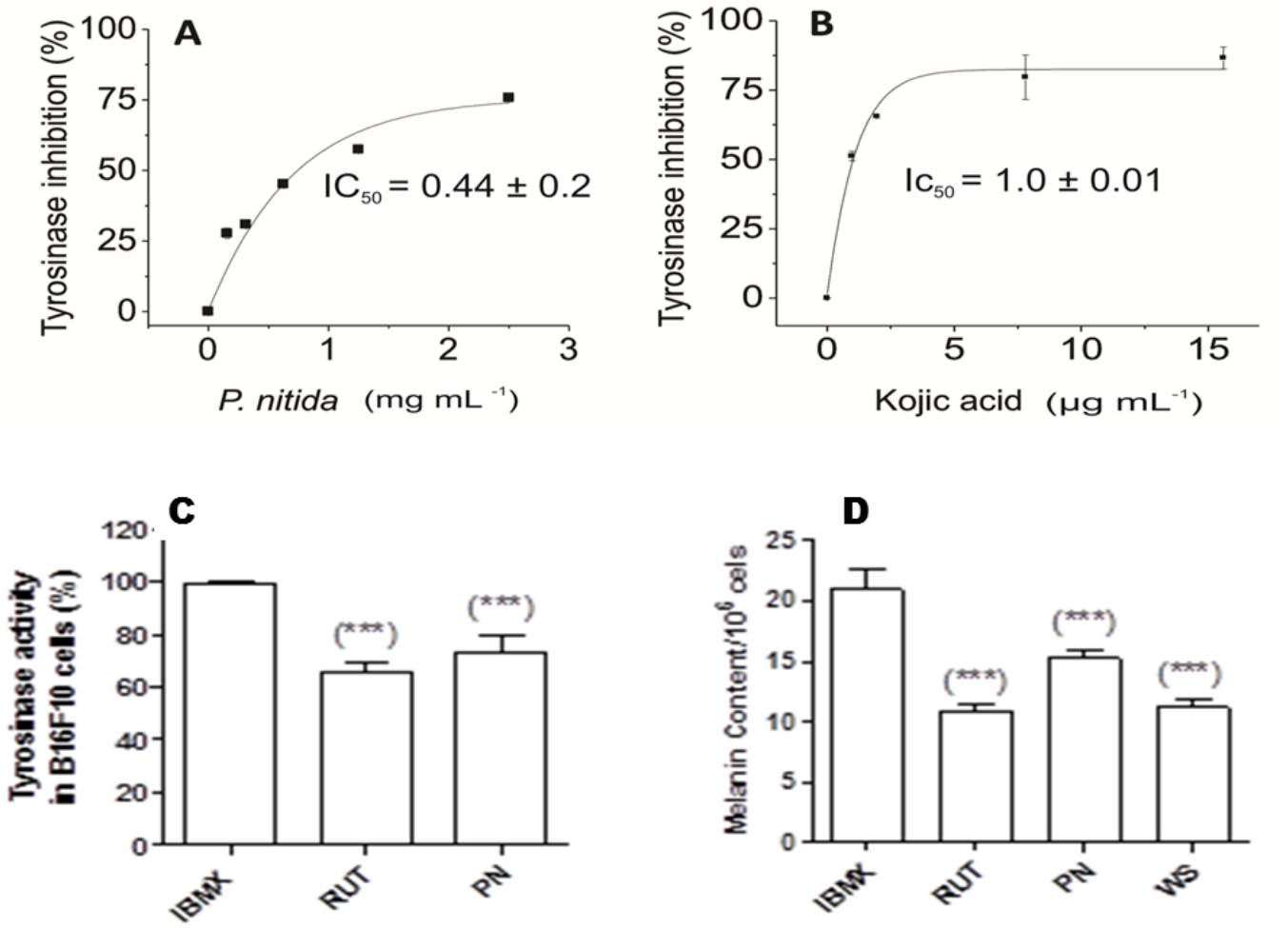

FIGURE 3 - (A) Inhibitory activity of tyrosinase in *P. nitida* (IC50: 0.44 ± 0.2) and (B) standard kojic acid (IC50: 1.0 ± 0.01). (C) Inhibitory effect *P. nitida* Kunth on tyrosinase in B16F10 cells. Cells were stimulated for 24 h in the presence of 25 µM IBMX and, subsequently, B16F10 cells were treated with *P. nitida* Kunth and the standard, rutin (RUT), at 25 µg.mL^{-1} for 48h. The difference in cellular tyrosinase activity between *P. nitida* and RUT was not statistically significant. (D) Effects of *P. nitida* on cellular melanin content. Melanin contents were determined in B16F10 cells treated with *P. nitida* and the standard, RUT, at 25 µg.mL^{-1} for 48h after stimulation for 24 h with 25 µM of IBMX. Unstimulated cells with IBMX (WS - without stimulation) were also quantified for comparison. Melanin levels were measured at 405 nm. (C), (D) Values represent mean ± SD (n=3). ***p < 0.001 compared to cells treated with 25 µM IBMX.

Tyrosinase activity and melanin content

The inhibitory effect of *P. nitida* Kunth on the tyrosinase activity of B16F10 cells treated with 25 µM IBMX (Figure 3C) inhibited tyrosinase activity by 26.6 % when compared with cells treated with IBMX alone. When compared with the standard, rutin, the cellular tyrosinase activity was not statistically significant (Figure 3A). Melanin synthesis was effectively inhibited (Figure 3D). The *P. nitida* Kunth dried extracts showed a statistically significant decrease in melanin content (27.1%), thus indicating depigmentation activity.

These results demonstrate that the depigmentation activity in *P. nitida* is due to inhibition of tyrosinase. Kojic acid is reported as an excellent inhibitor of mushroom tyrosinase and studies reported low activity in B16F10 cells and the need for high concentrations and combination with other depigmenting substance in order to have a strong

effect *in vivo* (Ahn *et al.*, 2011; Cho *et al.*, 2012). Because of this, rutin was used as standard on cells in tests. In addition, rutin has been reported as a metabolite that is present in fruits and plants with great depigmentation potential (Drewa *et al.*, 1998; Si *et al.*, 2012).

The data show that the DEPN has greater cell penetration activities and that these are higher than in KA, which suggests a greater ability of the extract to penetrate the cells and inhibit tyrosinase. This activity is possibly related to the flavonoid compounds found in *P. nitida* leaves.

Phytochemical analysis

The qualitative chemical analysis by LC-MS/MS of the spray-dried extracts from *P. nitida* (Figure 4) showed that the phenolic composition is dominated by two main flavonoid glucosides, tentatively identified as vitexin (1, *m/z* 431.0962) (Zucolotto *et al.*, 2012) and kaempferol-3-*O*-galactosyl-rhamnosyl-glucoside (2, *m/z* 755.2071). Compound 1 was previously identified in the leaves of *P. edulis*, *P. tripartita*, and *P. manicata* (Zucolotto *et al.*, 2012), while compound 2 has not been previously identified in any *Passiflora* species, however, it has been described in oolong tea extracts (Dou *et al.*, 2007) and *Camelia sinensis* (Fraser *et al.*, 2013). The crude hydroethanolic extract from the leaves of *P. nitida* displayed a similar composition of a previous study (Teixeira *et al.*, 2014), being identified as *C*-glucoside flavonoids isoorientin, vitexin, and swertinin. Since the metabolite composition of the spray-dried extract displayed just two phenolic compounds, a targeted quantitative analysis was performed to investigate the minor compound of the obtained sample.

Operating in SRM mode, the LC-MS/MS indicated that the extract possesses trace amounts of phenolic acids and flavonoid aglycones. Gallic acid (182.9 µg/g SDE), ferulic acid (63.1 µg/g SDE), and chlorogenic acid (56.5 µg/g SDE) were the main phenolic compounds besides compounds 1 and 2. Other phenolic acids, such as *p*-coumaric (40.7 µg/g SDE), caffeic (29.6 µg/g SDE) and protocatechuic acids (31.3 µg/g SDE) were also observed. Additionally, two flavonoid aglycones were identified and quantified as being quercetin (25.3 µg/g SDE) and kaempferol (27.8 µg/g SDE), both sharing similar amounts in the spray-dried extract.

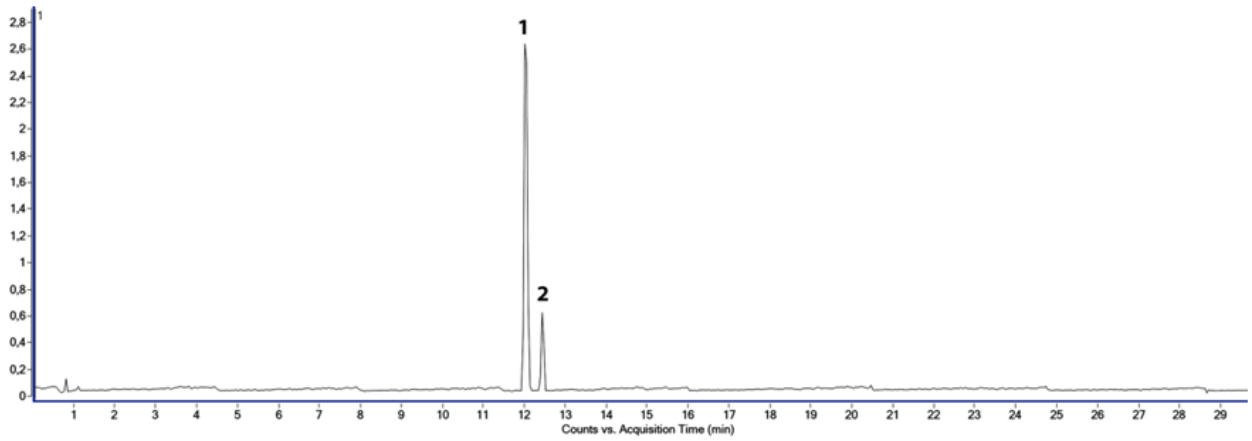

FIGURE 4 - LC-MS/MS total ion chromatogram of the spray-dried extract from *P. nitida*. Tentatively identified compounds: vitexin (1) and kaempferol-3-*O*-galactosyl-rhamnosyl-glucoside (2).

CONCLUSIONS

Dried leaf extracts of *P. nitida* can be used as plant raw material for developing new products with antioxidant or depigmenting properties. This characterization allowed for the determination of parameters for quality control within pharmacopoeia specifications and for the use of new techniques in characterizing the material with improved understanding of the new raw material. Moreover, the dry leaf extract of *P. nitida* shows moderate antioxidant and tyrosinase inhibitory activities, although further studies of efficacy and safety are required. Among the three DEPN studied, this was the best choice of extract for future use in developing formulations from the dry extract of *P. nitida* 7.5% (w/v). Therefore, the results of this study can be used as the basis for developing potential dermocosmetics containing *P. nitida* Kunth leaves.

ACKNOWLEDGMENTS

For this study, the authors acknowledge CNPQ and FAPEAM (PRONEM EDITAL) for their financial support and Centro de Tecnologias Estratégicas do Nordeste – CETENE, for light scattering (laser granulometry) and specific surface area analysis. The authors also are grateful to Central Analítica (UFAM) for the chemical analysis.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

REFERENCES

Ahn SM, Rho HS, Baek HS, Joo YH, Hong YD, Shin SS, et al. Inhibitory activity of novel kojic acid derivative containing trolox moiety on melanogenesis. Bioorg Med Chem Lett. 2011;21(24):7466-9.

Allen LV, Popovich NG, Ansel HC. In: Formas Farmacêuticas e Sistemas de Liberação de Fármacos. Artmed, Porto Alegre. 2007.

Agência Nacional de Vigilância Sanitária. ANVISA. Farmacopeia Brasileira volume 1. 5th ed. Brasília: ANVISA. 2010.

Araújo AAS, Mercuri LP, Seixas SRS, Storpirtis S, Matos JR. Determinação dos teores de umidade e cinzas de amostras comerciais de guaraná utilizando métodos convencionais e análise térmica. Braz J Pharm Sci. 2006;42(2):269-77.

Barni ST, Cechinel-Filho V, Couto AG. Caracterização química e tecnológica das folhas, caules e planta inteira da Ipomoea pes-caprae (L.) R. Br., Convolvulaceae, como matéria-prima farmacêutica. Braz J Pharmacogn. 2009;19(4):865-70.

Bataglion GA, Silva FMA, Eberlin MN, Koolen HHF. Determination of the phenolic composition from Brazilian tropical fruits by UHPLC – MS / MS. Food Chem. 2015;180:280-7.

Bendini A, Cerretani L, Pizzolante L, Toschi TG, Guzzo F, Ceoldo S. et al. Phenol content related to antioxidant and antimicrobial activities of Passiflora spp. extracts. Eur Food Res Technol. 2006;223(1):102-9.

Bundesvereinigung. Deutscher Apothekerverbände, Arzneimittel – Codex. 1986. Frankfurt: Govi; Stuttgart: Deutscher Apotheker. 1986. v.1:Codex – Probe 4,9.

Burlando B, Cornara L. Revisiting amazonian plants for skin care and disease. Cosmetics. 2017;4(25):1-12.

Carvalho MJ, Pedrosa TN, Simplicio FG, Nunez CV, Ohana DT, Pereira MM, et al. Estudo farmacognóstico e atividade in vitro sobre a coagulação sanguínea e agregação plaquetária das folhas de Passiflora nitida Kunth (Passifloraceae). Acta Amaz. 2010;40(1):199-206.

Chang C, Yang M, Wen H, Chern J. Estimation of Total Flavonoid Content in Propolis by Two Complementary Colorimetric Methods. J Food Drug Anal. 2002;10(3):178-82.

Chang T. An Updated Review of Tyrosinase Inhibitors. Int J Mol Sci. 2009;10(6):2440-75.

Cho JC, Rho HS, Baek HS, Ahn SM, Woo BY, Hong YD, et al. Depigmenting activity of new kojic acid derivative obtained as a side product in the synthesis of cinnamate of kojic acid. Bioorg Med Chem Lett. 2012;22(5):2004-7.

Demirkiran O, Sabudak T, Ozturk M, Topcu G. Antioxidant and Tyrosinase Inhibitory Activities of Flavonoids from Trifolium nigrescens Subsp. petrisavi. J Agric Food Chem. 2013;61(51):12598-603.

Drewa G, Schachtschabel DO, Palgan K, Grzanka A, Sujkowska R. The influence of rutin on the weight, metastasis and melanin content of B16 melanotic melanoma in C57BL/6 mice. Neoplasma. 1998;45(4):266-71.

Figueiredo CBM, Alves LDS, Silva CCAR, Ferreira PA, Marques GS, Santana ASCO, et al. Physical-chemical characterization, anatomical and seasonal evaluation of

Thuja occidentalis L. (Cupressaceae). Int J Pharm Sci Res. 2014;5(5):1721-31.

Fonseca FN, Silva AH, Leal LKAM. Justicia pectoralis Jacq., Acanthaceae: Preparation and characterisation of the plant drug including chromatographic analysis by HPLC-PDA. Braz J Pharmacogn. 2010;20(6):871-7.

Fraser K, Lane GA, Otter DE, Hemar Y, Quek SY, Harrison SJ, et al. Analysis of metabolic markers of tea origin by UHPLC and high resolution mass spectrometry. Food Res Int J. 2013;53(2):827-35.

Hearing VJ. Mammalian monophenol monooxygenase (tyrosinase): Purification, properties, and reactions catalyzed. Method Enzymol. 1987;142:154-65.

Hosoi J, Abe E, Suda T, Kuroki T. Regulation of Melanin Synthesis of B16 Mouse Melanoma Cells by 1a,25-Dihydroxyvitamin D3 and Retinoic Acid. Cancer Res. 1985;45(4):1474-78.

Janssens, K. X-Ray Fluorescence Analysis. In: Gauglitz G (ed), Vo-Dinh T (ed). Handbook of Spectroscopy. Weinheim: Wiley-VCH. 2003.

Koolen HHF, Silva FMA, Gozzo FC, Souza AQL, Souza ADL. Antioxidant, antimicrobial activities and characterization of phenolic compounds from buriti (Mauritia flexuosa L. f.) by UPLC–ESI-MS/MS. Food Res Int J. 2013;51(2):467-73.

Lourith N, Kanlayavattanakul M. Antioxidant activities and phenolics of Passiflora edulis seed recovered from juice production residue. J Oleo Sci. 2013;62(4):235-40.

Maruki-Uchida H, Kurita I, Sugiyama K, Sai M, Maeda K, Ito T. The protective effects of piceatannol from passion fruit (Passiflora edulis) seeds in UVB-irradiated keratinocytes. Biol Pharm Bull. 2013;36(5):845-9.

Maximiano FP, Costa GHY, Souza J. Caracterização físico-química do fármaco antichagásico benznidazol. Quim Nova. 2010;33(8):1714-9.

Melo MS, Santana RF, Batista CM, Pinheiro MS, Albuquerque Júnior RLC, Padilha FF, et al. Thermal characterization of mangaba-based films. J Therm Anal Calorim. 2013;111:2097-102.

Migliato KF, Moreira RRD, Mello JCP, Sacramento LVS, Corrêa MA, Salgado HRN. Controle da qualidade do fruto de Syzygium cumini (L.) Skeels. Braz J Pharmacogn. 2007;17(1):94–101.

Molyneux P. The use of the stable free radical diphenylpicryl-hydrazyl (DPPH) for estimating antioxidant activity. J Sci Technol. 2003;26(2):211-19.

Porte LHM, Leão MHMR, Porte A. Avaliação da porosidade de microcápsulas contendo proteína bioativa por porosimetria de mercúrio e adsorção de nitrogênio. Quim Nova. 2011;34(9):1582-87.

Re R, Pellegrinia N, Proteggentea A, Pannalaa A, Yanga M, Rice-Evansa C. Antioxidant activity applying an improved abts radical. Free Rad Biol Med. 1999;26(9-10):1231-37.

Rüdiger AL, Silva CC, Veiga Júnior VF. EDXRF Analysis of Amazonian Burseraceae Oleoresins. J Braz Chem Soc. 2009;20(6):1077-81.

Schoonman A, Mayor G, Dillmann M, Bisperink C, Ubbink J. The microstructure of foamed maltodextrin / sodium caseinate powders: a comparative study by microscopy and physical techniques. Food Res Int. 2001;34(10):913-29.

Sharapin, N. Fundamentals of technology Herbal Products. Cyted. Santa fé de Bogotá. 2000.

Si YX, Yin SJ, Oh S, Wang ZJ, Ye S, Yan L, et al. An Integrated Study of Tyrosinase Inhibition by Rutin: Progress using a Computational Simulation. J Biomol Struct Dyn. 2012;29(5):999-1012.

Singleton VL, Rossi JA. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. Am J Enol Vitic. 1965;16:144-58.

Teixeira LS, Lima AS, Boleti AP, Lima AA, Libório ST, Paula L. et al. Effects of Passiflora nitida Kunth leaf extract on digestive enzymes and high caloric diet in rats. J Nat Med. 2014;68(2):316-25.

Tomita YMK, Tagami H. Melanocyte-Stimulating Properties of arachidonic acid metabolites: possible role in postinflammatory pigmentation. Pigm Cell Res. 1992;5(5 Pt 2):357-61.

United States Pharmacopeia Convention – USP. United States Pharmacopeia 30th edition. Rockville. 2008.

Yao H, Chen Y, Shi P, Hu J, Li S, Huang L, et al. Screening and quantitative analysis of antioxidants in the fruits of Livistona chinensis R. Br using HPLC-DAD– ESI/MS coupled with pre-column DPPH assay. Food Chem. 2012;135(4):2802-07.

Yao Y, Cheng X, Wang L, Wang S, Ren G. Mushroom tyrosinase inhibitors from mung bean (Vigna radiatae L.) extracts. Int J Food Sci Nutr. 2011;63(3):358-61.

Yilmaz BS, Altun ML, Orhan IE, Ergene B, Citoglu GS. Enzyme inhibitory and antioxidant activities of Viburnum tinus L. relevant to its neuroprotective potential. Food Chem. 2013;141(1):582-8.

Zhang DY, Luo M, Wang W, Zhao CJ, Gu CB, Zu YG, et al. Variation of active constituents and antioxidant activity in pyrola [P. incarnata Fisch.] from different sites in Northeast China. Food Chem. 2013;141(3):2213-19.

This is an open-access article distributed under the terms of the Creative Commons Attribution License.

Ziaullah KS, Warnakulasuriya SN, Rupasinghe HPV. Biocatalytic synthesis, structural elucidation, antioxidant capacity and tyrosinase inhibition activity of long chain fatty acid acylated derivatives of phloridzin and isoquercitrin. Bioorg Med Chem. 2013;21(3):684-92.

Zucolotto SM, Fagundes C, Reginatto FH, Ramos FA, Castellanos L, Duque C, et al. Analysis of C-glycosyl Flavonoids from South American Passiflora Species by HPLC-DAD. Phytochem Anal. 2012;23(3):232-9.

Received for publication on 20th May 2019
Accepted for publication on 12th May 2021