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ORIGINAL ARTICLE

Determination of Flavan-3-ols by High-Performance Liquid Chromatography-Photodiode Array, Antioxidant Potential, Antimicrobial Activity, and Pharmacognostic Evaluation of *Maytenus obtusifolia* (Mart.) Leaves

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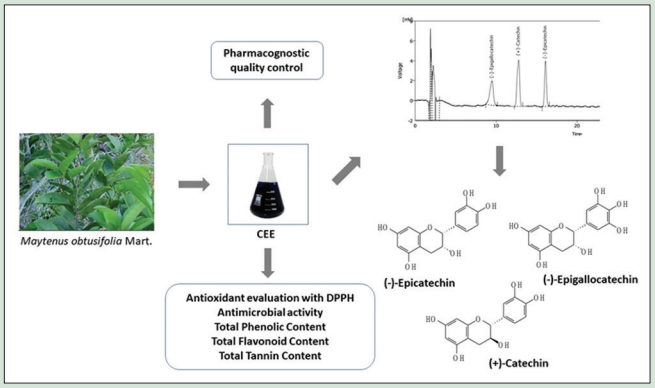
ABSTRACT

Background: *Maytenus obtusifolia* Mart. (Syn. *Monteverdia obtusifolia* Mart. Biral), *Celastraceae*, is a species located on the coast of Brazil, which is commonly used as anti-inflammatory and on the treatment of gastric ulcers. **Objectives:** The aim of this study consists of the determination of quality control parameters of the plant material, as well as to develop, validate high-performance liquid chromatography-photodiode array methodology for the quantification of its analytical markers and evaluate the antioxidant potential and antimicrobial activity of the extract. **Materials and Methods:** The plant material and ethanolic crude extract (ECE) were evaluated through pharmacopoeial and non-pharmacopoeial methods. Anti-oxidant assay was assessed using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method. Minimum inhibitory concentration was used to evaluate antimicrobial activity assay. **Results:** The assays have provided the following results: granulometry (d_{50} 0.82 mm), moisture content (6.41%–9.03%), pH of aqueous solution (5.41), apparent bulk density (0.41 mg/mL), heavy metals (<10 ppm), total ash (5.20%), and sulfated ash content (6.36%). Total flavonoid (2.14%; 6.24%), tannin (6.51%; 17.85%), and phenolic content (41.95; 134.76 mg AG/g) regarding leaves powder and its ECE, respectively. The validated method was specific, linear, precise, accurate, and robust. The content of (-)-Epigallocatechin, (+)-Catechin and (-)-Epicatechin on the ECE was 1.00%, 0.40%, and 0.33%, respectively. The anti-oxidant activity was evaluated by DPPH assay and have presented an IC_{50} at 18.01 µg/mL. In addition, the most important antimicrobial activity of the ECE was against *Aspergillus flavus* (MIC at 128 µg/mL). **Conclusion:** These data have demonstrated to be very useful toward the analysis and pharmacognostic quality control of *M. obtusifolia* Mart.

Keywords: Bom-nome, flavan-3-ols, *Maytenus obtusifolia*, pharmacognostic evaluation, quality control

SUMMARY

- The present study consists in the determination of quality control parameters of the plant material, as well as to develop, validate high-performance liquid chromatography-photodiode array methodology for the quantification of its analytical markers and evaluate the antioxidant potential and antimicrobial activity of the extract. The developed method was validated according to the requirements for the International Conference on Harmonisation and Brazilian National Agency of Health Surveillance guidelines. *M. obtusifolia* exhibited good DPPH-free radical scavenging property and antimicrobial potential against fungi.



Abbreviations Used: C: Catechin; DPPH: 2,2-difenil-1-picril-hidrazil; ECE: Ethanolic Crude extract; EP: Epicatechin; EPG: Epigallocatechin; GA: Galic acid; HPLC-PDA: High performance liquid chromatography-photodiode array; LOD: Limit of detection; LOQ: Limit of quantification; QM: Quadratic mean; QS: Quadratic sum; RSD: Relative standard deviation; SE: Standard error; TFC: Total flavonoid content; TPC: Total phenolic content; TTC: Total tannin content.

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INTRODUCTION

Maytenus obtusifolia Mart. (Syn. *Monteverdia obtusifolia* Mart. Biral),[1] *Celastraceae*, is an evergreen tree endemic to the Brazilian coastal regions occurring from the state of Pará to São Paulo. It is abundant in the restinga region, mainly from Rio de Janeiro, although it is also found in higher altitude regions.[2] This species is popularly known as "bom-nome" or "carne-de-anta" and is used in folk medicine as decoction from leaves for ulcer treatment,[3] as anti-inflammatory as well as anticancer. The stem-bark is also used as powder to treat skin ulcers.[4]

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Due to its traditional use, the ethanolic crude extract (ECE) of *M. obtusifolia* leaves was evaluated pharmacologically to verify its toxicity and confirm or not its popular indication. It was possible to observe that the ECE has presented antiulcerogenic activity and low toxicity, therefore, confirming its application on gastric ulcer treatments.[5] In addition, pharmacological activity of the chloroform extract from *M. obtusifolia* roots toward the central nervous system has been demonstrated, such as neuroleptic activity, possibly via central dopaminergic action, as well as depressant action, which was confirmed by the locomotor activity assay.[6] Antimicrobial activity of the essential oil from the species leaves inhibited the growth of several bacteria and fungi strains when applied *in natura*.[7] These findings have confirmed the pharmacological potential of *M. obtusifolia*. Previous Phytochemical studies performed with the roots of this species have reported several compounds, such as celastroids, i.e., pristimerine, tingenone, and 22-hydroxytingenone.[8] It was also reported the presence of friedelane, oleanane and ursane-type triterpenes, such as friedeline, 7-oxofriedeline, 3-oxo-29-hydroxyfridelane, 3-oxo-11α-hydroxy-20 (29)-lupene, 3 β-11α-dihydroxi-20 (29)-lupene, alkaloid N-methyl-flindersine, 3,4-seco-friedelan-oic acid, (-)-epicatechin (EP), (+)-catechin (C) and proanthocyanidin, obtained from the leaves of *M. obtusifolia*.[9-11]

Although the easy access to the plant material, one of the main challenges related to natural products research, involves the analysis itself. Due to the complexity of the biological matrixes, it is hard to characterize and quantify all the plant compounds, which may have different polarities and vary its concentrations, due to several abiotic factors, such as climate, altitude, soil composition and light-dark cycles, as well as processing factors of the plant material, such as drying, grinding, solvent polarity, type of extraction, isolation technique and its degradation processes, reflecting on safety, pharmacodynamic and pharmacokinetic profiles. Therefore, for unambiguous identification, a combination of methodologies may be necessary, such as chemical analyses, anatomical and morphological characterizations.[12,13]

Despite phytochemical and pharmacological studies have been already performed with this species, there are no previous reports on scientific literature regarding specifications or analytical methodologies to assure its quality. Therefore, considering that there is no record of suitable quality control assays and that plant extracts represent a complex and dynamic mixture, comprising a great variety of chemical compounds, it is practically impossible to assure the reproducibility of the therapeutic effect among different batches or confirm the absence of interferents without the performance of such assays.[14,15] In addition, the standardization of plant extracts involves physicochemical and analytical methods capable of determining the characteristics of the plant material as well as providing its consistency among final products.[16]

Therefore, considering the pharmacological activities previously described, as well as the isolated compounds already reported from *M. obtusifolia*, the aim of this work is to stablish its analytical markers qualitatively and quantitatively through suitable analytical methods, enabling safety and efficiency of plant material analysis and its derivatives. Thus, this present study will perform the development and validation of a methodology involving high performance liquid chromatography-photodiode array (HPLC-PDA) technique for the quantification of the flavan-3-ols: (-)-epigalocatechin, (+)-C and (-)-EP from the leaves and its ECE obtained from the species, as well as to evaluate the anti-oxidant potential and antimicrobial activity of the extract.

MATERIALS AND METHODS

Chemicals and reagents

The standards of analytical markers used at HPLC analyses were purchased at Phytolab (Vestenbergsgreuth, Germany) presenting a high purity grade (>99%). The solvents of HPLC grade were purchased from Merck (Darmstadt, Germany), and the ultrapure water was obtained through the Milli-Q Gradient® equipment (Millipore, Bedford, MA, EUA), with a conductivity level lower than 0.50 μS/cm. Pyrogallol, gallic acid, and methenamine used for the analyses of total tannin (TTC), phenolic and flavonoid content respectively, were purchased from Sigma-Aldrich (Hamburg, Germany).

Plant material

The leaves of *M. obtusifolia* Mart., *Celastraceae*, were harvested in June 2014, in the city of Santa Rita, Paraíba, Brazil, (S 7°9'50, 2"/W 35°2'5, 22"). The species was identified by Professor Maria de Fátima Agra from the Center of Biotechnology of Universidade Federal da Paraíba (CBiotec/UFPB). A specimen was deposited at Lauro Pires Xavier Herbarium (JPB/UFPB), integrating the botanical collection of Agra *et al*. under the number 3230. The present study was also registered at the SISGEN (Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado) platform, with the reference number AF73B8C. The botanical material was dried at a heating oven with forced air circulation at 40°C for 72 h, then, it was submitted to a grinding process with a Harley mechanical mill, generating 1,512.1 g of leaves dried powder.

Extract preparation

An aliquot from the dried and pulverized botanical material was weighed (1000 g) and submitted to maceration with ethanol 96% in a suitable stainless steel reservoir for 72 h. The following procedure was repeated three times in order to increase yield extraction. The extracts were filtered and concentrated with a rotary evaporator (Büchi, R-3) under reduced pressure at 40°C. Thus, 321.3 g of ECE was then obtained after full evaporation of the solvent, with a yield of 32.13%.

Assays

Pharmacognostic quality control assays

The dried powder of *M. obtusifolia* leaves was submitted to several pharmacognostic quality control tests to assure the quality of the plant material. The assays were performed according to the general methods described in the 5th edition of the Brazilian Pharmacopoeia.[17] The granulometry was determined with the aid of a mechanical sieves shaker (Produtest®) comprising several grades (250, 425, 500, 1000, 1180 μm and 1,70 mm). The average diameter of the powder was determined by the curve of intersection between percent residues and percent passing. Three methods were employed to determine water content on plant material: gravimetric method (dessecation), Karl Fischer volumetric method (870 Titrino plus, Metrohm, Switzerland) using methodology previously described on Brazilian Pharmacopoeia (2010) and finally, quantification of water content of leaves powder (1 g) using the infrared scale (OHAUS-MB90).

The pH determination of the powder aqueous solution (1% w/v) was performed according to the 4th edition of the Brazilian Pharmacopoeia.[18] The solution was heated in a hot plate until the boiling point, then it was filtered and remained until reaching room temperature for pH measurement with a potentiometer previously calibrated (Gehaka, PG3000). The results were expressed as the mean of five determinations.

The apparent bulk density was evaluated by the addition of *M. obtusifolia* powder leaves to a graduated beaker, equivalent to

50 mL. The value was calculated according to the following equation: apparent bulk density (g/mL) = mass (g)/occupied volume (mL). The content of heavy metals on dried plant material was determined according to the limit test of heavy metals through the semi-quantitative I method.[17] The total ash and sulfated ash content were also determined by methodologies specified on Brazilian Pharmacopoeia.[17] The analyses were performed in quintuplicate. The ECE was analyzed by an HPLC methodology developed to verify its chromatographic profile and the quantification of the analytical markers: (-)-Epigallocatechin (EPG), (+)-C and (-)-EP.

Determination of total flavonoids and total tannins content

The content of total flavonoids (TFC) and TTC were determined using the colorimetric methods described by the Brazilian Pharmacopoeia for milled leaves and the ethanol crude extract.[19] All samples were performed in quintuplicate.

Determination of total phenolic compounds

The concentration of total phenolic compounds (TPCs) on the ECE of *M. obtusifolia* was determined by the spectrophotometric method.[20] 0.5 mL from ECE methanolic solution (1 mg/mL) were mixed with 2.5 mL of diluted Folin-Ciocalteau reagent (1:10) and 2.0 mL of sodium carbonate solution at 7.5% (m/v). After an incubation period of 2 h on the dark at room temperature, the absorbances of the following solutions were measured by spectrophotometer (Lambda 25, Perkin Elmer, USA) set at a wavelength of 765 nm. The results regarding TPCs were expressed as equivalent to galic acid (GA) (mg GA/g of extract), calculated through the construction of a calibration curve with standards in different concentrations varying from 5 to 200 μg/mL. The ECE of *M. obtusifolia* was solubilized with a solution of methanol:water (9:1) and was evaluated at a final concentration of 200 μg/mL. Concerning the evaluation of TPCs of the dry leaves powder from the species, 1 g of the powder was submitted to extraction with 10 mL of a methanol:water solution (9:1) for 1 h under constant agitation, then, an aliquot of 1 mL from the extract solution was collected, transferred to a volumetric flask and diluted to the concentration of 200 μg/mL.

High performance liquid chromatography method

The analyses were performed using a liquid chromatographer Young Lin® (South Korea), series YL9100, constituted of a quaternary pump system (YL9110), diode array detector (PDA) (YL9160), vacuum degasser (YL9101), column oven (YL9131) and automatic sampler (YL9150). As an analytical column, it was used a C-18 type from LiChrocart Purospher Star (Merck, Germany) (150 mm × 4,6 mm × 5 μm) with a precolumn (Merck, Germany) (4 mm × 4 mm) composed of the same stationary phase. The mobile phase A was composed of a phosphoric acid solution at 14 nM and the mobile phase B as acetonitrile. All the solutions were degassed and filtered through a membrane filter of 0.45 μm (Millipore, Bedford, EUA). The separation of the compounds was performed by gradient elution (0–38 min (5% to 25% B); 38–40 min (25% B) and 40–45 min (25% to 5%); with a mobile phase flow at 1.0 mL/min, oven temperature at 40°C and injection volume of 10 μL. The ultraviolet detection was performed at a wavelength set at 280 nm and a PDA detection range between 200 and 500 nm for peak purity determination. The solutions were prepared from *M. obtusifolia* ECE (1 mg/mL) and standard compounds with water:methanol (15:85) as the diluent and submitted to chromatographic analysis. Parameters such as retention time (Rt), peak area (Pa), peak resolution (Rs >1.0), and tailing factor (t < 1.5) were observed at all experiments. Data collection and statistical analysis were performed with the software YL-Clarity® (South Korea).

Validation of the high performance liquid chromatography method

The validation of the analytical method was performed according to previous guidelines stablished by the book of analytical and bioanalytical validation methods (RE 899/2003) from National Agency of Health Surveillance[21] and the validation Q2(R1) book from the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH).[22] The ECE obtained from the leaves of *M. obtusifolia* was analyzed during the validation process. The linearity was evaluated through the construction of three calibration curves obtained from the analyses of the following analytical markers: (-)-EPG, (+)-C and (-)-EP at eleven levels of concentration between 10 and 100 μg/mL, 4–40 μg/mL and 3.3–33 μg/mL, respectively. The specificity was evaluated through the comparison of Rts and purity profiles (≥0.99) of the three analytical markers. The chemical standards were analyzed at the following concentrations: (EPG) 20 μg/mL, C 8 μg/mL and (EC) 6.6 μg/mL. The ECE was analyzed at the concentration of 1 mg/mL. The mobile phase, diluent solution, and ethanol 96% used for extract preparation were evaluated with the aid of PDA, aiming to verify the existence of any coeluted peak at the same Rts of the analytical markers.

The limits of detection (LOD) and quantification (LOQ) were determined by mathematical extrapolation based on the equation obtained from the construction of the calibration curves of the chemical standards. The LOD and LOQ were defined by applying the formula 3 σ/S and 10 σ/S, respectively, where σ was correspondent to the standard deviation and S as the calibration curve inclination. Concerning the precision parameter, it was evaluated by two ways: repeatability, represented by the agreement among results obtained by the same analyst, using the same equipment, during a short period of time and the intermediate precision or inter-day precision, correspondent to the agreement among results obtained from the same laboratory, but obtained in different days by distinct analysts and/or equipment. The repeatability was performed using nine samples of ECE, prepared from 3 stock solutions at an initial concentration of 10 mg/mL, from which 1 mL was transferred to a volumetric flask of 5 mL; this final solution was termed as 100%. The intermediate precision was performed in a similar way, but this time, with the aid of two analysts on different days. The obtained results were expressed as mean ± standard deviation; besides, it was also determined the coefficient of variation (CV%). The results were considered valid when the CV was less than 5%.[21] Regarding accuracy, it was prepared three samples in triplicate at the concentration of 80%, 100%, and 120% related to the theoric concentration of the assay (total of 9 samples); where it was applied the method of recovery by standard addition, which adds known amounts of analytical markers to the ECE solution. Therefore, it was added increasing amounts of solutions containing (EPG) 100 μg/mL, (C) 40 μg/mL and (EP) 33 μg/mL. The sample solutions were prepared in the volumetric flask of 5 mL, with 1 mg of ECE, to which was added 30 μg of EPG, 13 μg of C and 9.9 μg de EP for the concentration of 80%; 50 μg of EPG, 20 μg of C and 16.5 μg of EP for the concentration of 100%; 70 μg of EPG, 28 μg of C and 23.1 μg of EP for the concentration of 120%. The known concentrations were determined according to the values obtained by the precision assay, and the experimental values were compared to the theoretical ones. The accuracy was evaluated by the recovered content, with a conformity parameter between 90% and 110%. Robustness was evaluated through the comparison of sample solutions and its analytical markers, submitted to slight variations on analytical parameters such as: pH 2.8 and 3.2, mobile phase flow

0.8 and 1.2 mL/min, oven temperature 38°C and 42°C, mobile phase composition with trifluoracetic acid and an RP18 LiChrocart LiChospher chromatographic column (125 mm × 4 mm, 5 μ). In addition, it was verified the stability of the solutions after 24 and 48 h (maintained in the dark on refrigerator at 5°C). The solutions of the sample and biomarker standards were evaluated in triplicate.

Antioxidant evaluation assay with 2,2-diphenyl-1-picrylhydrazyl

Free radical scavenging activity was evaluated by 2,2-diphenyl-1-picrylhydrazyl (DPPH assay) methodology.[23] 0.1 mL of DPPH solution (0.03 mM) was added to 0.1 mL of *M. obtusifolia* ethanolic extract solution in different concentrations (0–200 μg/mL). The absorbance was measured at 518 nm after 30 min of incubation in the dark at room temperature. Ascorbic acid was used as reference and the antioxidante activity of the extract was expressed as IC_{50} in μg/mL.

Antimicrobial activity assay

Antimicrobial activity from *M. obtusifolia* ethanolic extract was evaluated towards several micro-organisms, such as bacteria: *Staphylococcus aureus* (ATCC-6538), *S. aureus* (M-117), *Pseudomonas aeruginosa* (ATCC-25853) and *P. aeruginosa* (LM-116); yeast: *Candida albicans* (ATCC-76645), *C. albicans* (LM-108), *Candida krusei* (LM-13), *C. krusei* (LM-08), *Candida tropicalis* (ATCC-13803) and *C. tropicalis* (LM-36); as well as filamentous fungi *Aspergillus flavus* (LM-247) and *Aspergillus fumigatus* (LM-210). The strains were acquired from Adolfo Lutz Institute, São Paulo and evaluated on the Laboratory of Mycology and Microbiology from the Department of Pharmacy of the Federal University of Paraíba. To maintain the strains, the micro-organisms were cultivated at appropriate culture medium, such as Nutrient Agar for bacteria and Sabouraud dextrose agar for fungi (DIFCO Laboratories/France/EUA), at 4°C and 35°C respectively.

The ECE obtained from the leaves of *M. obtusifolia* was diluted with dimethyl sulfoxide (Merck, Germany), at a proportion of up to 10%. The remained volume of the solution was completed with distilled and sterilized water until reaching 3 mL. The ECE solutions were evaluated in different concentrations, varying from 1024 to 32 μg/mL. The suspension of micro-organisms was prepared according to the McFarland Scale of 0.5, adjusted through the spectrophotometric measure (Leitz-Photometer 340–800), set at 530 nm and 90% of transmittance, corresponding, approximately to 10^6 CFU/mL.[24-26] Regarding antimicrobial activity control, it was used chloramphenicol (100 μg/mL), nystatin (100 UI/mL) and fluconazole (50 μg/mL) for bacteria, yeast and filamentous fungi respectively, which were purchased from Sigma-Aldrich® (Missouri, USA). The antimicrobial and antifungal activity evaluation were performed in liquid medium with nutrient broth for bacteria (DIFCO Laboratories/France/EUA) and RPMI 1640 (Acumedia/India) for yeast and filamentous fungi. The MIC determination of the ECE was evaluated by the microdilution technique in a microplate of 96 well. It was added to each well of the first row, 100 μL of Nutrient Broth and RPMI liquid medium, as well as 100 μL of the product solution, from which a serial dilution was performed. Finally, 10 μL of inoculum was added to each well, with each column representing a different micro-organisms strain. The microplates were sealed and incubated at 35°C for 24–72 h for bacteria and yeast assays. However, for filamentous fungi assay, the microplates were incubated for 7–10 days at 28°C–30°C.

Statistical analysis

The data were analyzed using Microsoft Excel® software, 2016 version, by a one-way analysis of variance (ANOVA) followed by Tukey's test, considering $P < 0.05$ as the significance level, as well as the *t*-test for two groups considering $P < 0.05$ as the significance level. Results were expressed as mean ± standard deviation (residual standard deviation (RSD%)). To determine the linearity of the method, line correlation tests and residual analysis were performed by simple line regression considering $r \geq 0.99$ and the residual sum of squares was evaluated.

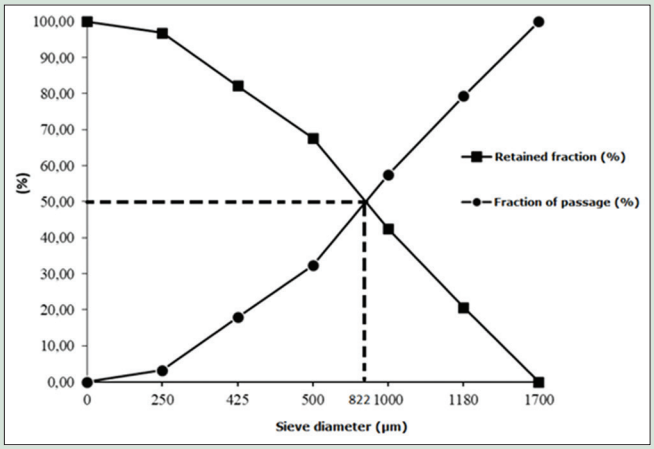

Figure 1: Curve of retained and passing material from *Maytenus obtusifolia* leaves dried powder

RESULTS AND DISCUSSION

Pharmacognostic quality control assays

Granulometric analysis from *M. obtusifolia* leaves powder led to the verification of a heterogeneous size distribution [Figure 1]. Predominantly represented by particles with a diameter >500 μm, comprising 49% of the whole material. Knowing the granulometric distribution of the plant powder might be useful on drug development, as particle size distribution might interfere on the final product, suggesting the previous calibration of powder for better efficiency.[27] The average size of the particles (d_{50}) 822 μm was correspondent to the curve of intersection between percent residues and percent passing. The powder must be classified as thick, once <40% of the particles passed through the sieve with a grid diameter of 355 μm.[17] This characteristic enhances extractive processes, unlike thin powder, which may bond to bigger particles, increasing viscosity, therefore creating a barrier that hinders solvent penetration, besides enabling filter clogging.[28] Once the granulometric distribution determines the contact surface of the particles on solvent-drug interaction, leading to the correspondent drug extract, the results represent an important parameter to maintain reproducibility and homogeneity of extractive processes with *M. obtusifolia*.[29]

This work is the first attempt to standardize *M. obtusifolia* and quantify analytical markers for this species. The results regarding quality control assays of the leaves powder (granulometry, moisture content, pH of the aqueous solution, apparent bulk density, heavy metals, total ash and sulfated ash content, as well as TFC, tannin, and phenolic content) and its ECE (TFC, tannin, and phenolic content) are presented on Table 1.

The results regarding the moisture content of plant material have presented inferior values when compared to previously stablished limits from official monographs (8%–14%).[19] The excess of water in plant material favors the enzymatic action, besides enabling fungus and bacteria growth.[30] Among the different assessment techniques applied, it was observed substantial differences regarding moisture content on plant material, which may be related to the presence of essential oil and/or other thermosensitive constituents, dependent on the heat time exposure. This behavior was also observed where water content evaluation using oven

and infrared radiation have determined not only moisture content loss but the other volatile constituents, dragged along by water steam.[31,32] Therefore, the moisture content results of *M. obtusifolia* plant material related to oven drying and infrared radiation techniques were consistent with moisture losses and volatiles compounds. On the other hand, the analysis performed by the volumetric method has preserved the volatile and thermosensitive compounds.

The pH of the plant material aqueous extract provides important information about the nature of the molecules, as well as the information related to the stability of the chemical compounds. The aqueous extract obtained with water at pH 6.22 has provided a solution with pH 5.41, indicating the presence of acid compounds. These results are corroborated by the phytochemical profile of the species, once its therapeutic properties are mainly related to flavonoids, which preset slight acid characteristics.[33]

The apparent bulk density is an important parameter for extraction process control of plant material, which may predict filling and compaction properties, as well as providing important information about the drying process of plant extracts.[34] Due to the absence of quality control studies, there are no reference parameters for this species whatsoever.

The heavy metals limit assay provides the generation of a suspension, consisted of solid sulfide particles of heavy metals, followed by a visual comparison of color intensities between samples and standard. This assay is semi-quantitative, represented by the sum of contaminant concentrations within the sample. The obtained results from *M. obtusifolia* plant material have demonstrated acceptable levels of heavy metals (<10 ppm) when compared to the reference.[17] These metals may come from soil, irrigation water or atmosphere, which increases according to environment pollution. In addition, the proximity to highways may increase the soil contamination, with pollutants dragged by rain. Agricultural excipients, such as fertilizers containing cadmium as well as fungicides and insecticides containing organophosphate, are also considered sources of contamination.[35]

Concerning the total ash and sulfated ash assays for plant materials, it was observed the values of 5.20% and 6.36% respectively, which were in agreement with the preestablished values between 4% and 15% for plant materials.[19] The total ash assay refers to the total amount of residual organic material after plant material incineration, including natural ashes generated from the vegetal tissue itself and the nonphysiological ashes, such as sand and dust that may be on the plant material surface. These ashes are constituted of carbonates, phosphates, silicates and silica. Regarding sulphated ash content, the plant material is treated with sulphuric acid, promoting the conversion of oxides, chlorides and carbonates into sulphates which presents higher thermal stability.[30,36] Therefore, the difference observed between total ash and sulfated ash content may be related to the transformation of oxalates, halides, and carbonates into sulfates.

Table 1: Results regarding quality control assays of *Maytenus obtusifolia*

Assay	Results
Granulometry (d_{50})	0.82 mm
Moisture content[a]	6.93% ± 0.09 (1.3%)
	6.41% ± 0.14 (2.2%)
	9.03% ± 0.06 (0.6%)
pH of aqueous solution[a]	5.41±0.05 (0.9%)
Apparent bulk density	0.41 g/mL±0.01 (2.0%)
Heavy metals content	<10 ppm
Total ash content[a]	5.20% ± 0.14 (2.6%)
Sulphated ash content[a]	6.36% ± 0.12 (1.9%)
TFC[a]	2.14% ± 0.08 (3.8%)
TTC[a]	6.51% ± 0.14 (2.19%)
TPC[a]	41.95 mg AG/g±0.38 (0.9%)
TFC - ECE[a]	6.24% ± 0.06 (1.0%)
TTC - ECE[a]	17.85% ± 0.23 (1.3%)
TPC - ECE[a]	134.76 mg AG/g±0.86 (0.6%)
EPG - ECE[a]	1.00% ± 0.01 (1.1%)
C - ECE[a]	0.4% ± 0.01 (1.4%)
EP - ECE[a]	0.33% ± 0.01 (1.7%)

[a]$\bar{x}$ ± SD (RSD %): $\bar{x}$: Mean; SD: Standard deviation; RSD: Relative standard deviation; ECE: Ethanolic crude extract; TFC: Total flavonoid content; TTC: Total tannin content; TPC: Total phenolic content; EPG: Epigallocatechin; C: Catechin; EP: Epicatechin

The TFC, TTC, and TPC of the leaves powder and ECE have evidenciated the richness of the polyphenolic content of the species, which were reinforced by previous studies involving *M. aquifolium*, highlighting the presence of flavonoids[37] and corroborating with the phytochemical profile of the genus.[37-40] Besides, it was possible to verify that the flavonoid content of *M. aquifolium* vary according to the seasons, reinforcing the necessity of plant material quality control analysis.[37] Moreover, the tannin content regarding *M. obtusifolia* was superior than the values already reported by literature regarding *M. ilicifolia* and *M. aquifolium*.[38,40] These values are higher than the recommended parameters of 2%, for TTC on *Maytenus ilicifolia* plant material.[19]

The quantification of the analytical markers EPG, C, and EP on the ECE was performed by HPLC and have demonstrated the concentrations of 1.00 % ± 0.01 (1.1%), 0.40% ± 0.01 (1.4%) and 0.33% ± 0.01 (1.7%). These results support the performance of quality control assays of this species, upon which the analytical markers may be established as quality control parameters for herbal medicine or Phyto cosmetics development. Posterior analyses related to antiulcerogenic activity from different samples will establish the minimal limits of analytical markers, thus, contributing to the production of a monograph for *M. obtusifolia*.

High performance liquid chromatography method development

The method was developed to separate the phenolic compounds present on the ECE of *M. obtusifolia* [Figure 2]. Methanol and acetonitrile were chosen as eluents, once they have worldwide acceptance and application on international protocols, as well as for being often used for flavanols separation and detection.[41,42] To avoid ionization of the constituents and minimize peak tailing, aqueous mobile phase was evaluated with

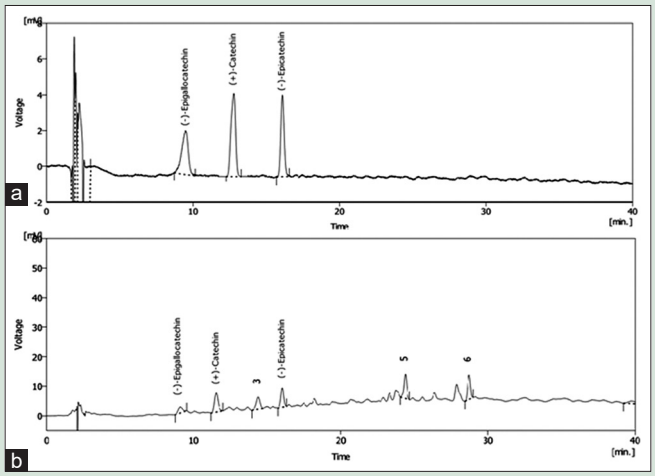

Figure 2: Chromatographic profile of the analytical markers: EPG; C; EP (a). ECE of *Maytenus obtusifolia* at 1 mg/mL (b)

several types of acids, in different concentrations, such as phosphoric, acetic and formic acids.[43] Distinct models of chromatographic columns were tested (Shim-pack® C_{18} 150 mm × 4.6 mm × 5 μm, Shimadzu; Gemini® C_{18} 150 mm × 4.6 mm × 5 μm, Phenomenex; Luna® C_{18} 150 mm × 4.6 mm × 5 μm, Phenomenex). The LiChrocart Purospher Star C_{18} (Merck) has presented the most suitable chromatographic profile, generating the best separation of the three analytical markers, as well as the other compounds. The chosen mobile phase flow rate was set at 1.0 mL/min, once the flow rate at 0.8 mL/min has promoted a slight enlargement of the peaks and increased the chromatographic run time. Furthermore, the flow rate at 1.2 mL/min has generated the coelution of a few important compounds and augmented the column pressure, which could reduce the useful life of the equipment for high throughput analyses. The wavelength usually chosen for flavanols detection is set at 280 nm or between 210 and 230 nm.[41-43] At a wavelength between 210 and 230 nm, the analytical markers have presented an intense absorption; however, as there is also a great number of substances that strongly absorb in this region, it has promoted a disturbance at the baseline, which could affect the precision of the results. Therefore, the wavelength of 280 nm was chosen as the most appropriate, once the maximum absorbance of flavan-3-ols may be detected.[44]

In relation to the diluent solution, it was observed no significant separation of the compounds when applying methanol solutions at concentrations higher than 60%. The decrease of the methanol proportion and consequently, the increase of water percentage have promoted a better separation of the analytical markers. Methanol solutions between 0% and 15% have generated the best separations; however, the use of water at 100% has reduced the detection of catechins by 20%.[42] Therefore, the chosen proportion concerning the diluent solution was water:methanol (85:15).

High performance liquid chromatography method validation

The chromatogram analyses have evidenciated no existence of peak coelution [Figure 2]. Additionally, it was also verified the purity of the analytical markers (>0.99) and the absence of solvent, mobile phase, and diluent solution effects. The system suitability parameters for analytical methods such as HPLC and its recommended limits were: peak asymmetry ≤1.5, number of effective theoretical plates >2000, peak resolution >2.0, and capacity factor >1.5.[45] The calibration curve was prepared with the analysis of samples prepared in triplicate at several different concentrations. The ANOVA of linear regression leaded to the determination of the existence of a significant angular coefficient and the validation of the linear regression [Table 2]. The calibration curves and its correlation coefficients were determined as y = 4148,6x– 16,071 (r = 0,9995) (*n* = 3) for EPG, y = 15387x + 0,9041 (r = 0,9998) (*n* = 3) for C and y = 11612x + 0,9107 (r = 0,9998 (*n* = 3) for EP. These results were in accordance with the parameters recommended r > 0.99.[21] The residues calculation of each biomarker has confirmed the validity of the adopted calibration model by observing the random distribution of residues close to zero, its homoscedasticity and the absence of atypical samples.

The method has presented high sensitivity, as demonstrated by the low values of LOD and LOQ. The LOD values for EPG, C, and EP were 2.08 μg/mL, 0.60 μg/mL, and 0.49 μg/mL, respectively, whereas the LOQ values for the same analytical markers were 6.94 μg/mL, 2.00 μg/mL and 1.63 μg/mL.

Concerning repeatability and intermediate precision, it was used analytical markers at a concentration of 100%, generating an RSD lower than 5.0%, which complies with the recommended parameters [Table 3].[21,22]

The variances of RSD were not significant, which demonstrates that the method is precise and admits variance from different analysts. The accuracy, which represents the proximity between the analysis of a sample with a known concentration and its calculated value obtained by the calibration curve, have presented average results for the three levels of concentration evaluated, which were 100.14% ± 0.21 (0.21%), 100.26% ± 0.14 (0.13%) and 100.12% ± 0.09 (0.09%) for EPG, C and EP, respectively, complying with the confidence interval between 95% and 105%,[21,22] therefore, confirming that the method is accurate for the quantification of *M. obtusifolia* analytical markers.

The analysis concerning the *t*-test, with two presumably equivalent samples, in 2 different days, has demonstrated no significant statistical differences considering the confidence interval of 95%, once the calculated *t* values for EPG, C, and EP were (*t* calc = 0.12 < *t* crit = 2.23), (*t* calc = 0.11 < *t* crit 2.23) and (*t* calc = 0.66 < *t* crit 2.23) respectively. Furthermore, regarding the ANOVA of accuracy, it was also observed no significant differences among the concentration levels, once the *P* values were higher than 0.05 for the analytical markers EPG (*p* calc = 0.540 > 0.05), C (*p* calc = 0.668 > 0.05) and EP (*p* calc = 0.932 > 0.05).

The robustness was evaluated by the alteration of chromatographic analysis parameters, and its results were analyzed according to the mean and relative standard deviation for each biomarker analysis. Although slight variations may exist on the results regarding area, Rt and resolution imposed by these parameter alterations, the method has demonstrated to be robust, considering that the evaluation of the three analytical markers on sample analysis has generated an RSD lower than 2.4%.[21,22]

In relation to the modification of the original mobile phase flow rate, it was observed that the reduction of the flow rate has increased the Rts

Table 2: Statistical data used at regression equations of calibration curves from epigallocatechin, catechin, and epicatechin, developed by high-performance liquid chromatography method

Regression	Calibration curve for EPG		Calibration curve for C		Calibration curve for EP	
Slope of a line (se)	4148.6 (17.92)		15,387 (47.72)		11,612 (35.64)	
Intercept (se)	−16.071 (0.85)		0.9041 (0.90)		0.9107 (0.56)	
Correlation coefficient (*r*)	0.9995		0.9998		0.9998	
F value signification	9.16×10^{-52}		3.19×10^{-56}		2.33×10^{-56}	
ANOVA	$F_{1,31}$=53,609.20, *P*<0.05		$F_{1,31}$=103,975.93, *P*<0.05		$F_{1,31}$=106,121.68, *P*<0.05	
Limits of confidence (95%) inclination	4112.0; 4185.1		15,289; 15,484		11,539; 11,684	
Limits of confidence (95%) intercept	−17.800; −14.341		−0.9384; 2.7466		−0.2247; 2.0461	
	QS	QM	QS	QM	QS	QM
Regression	444,224.38	444,224.38	977,785.03	977,785.03	378,993.04	378,993.04
Residue	256.88	8.29	291.52	9.40	110.71	3.57
Total	444,481.25		978,076.55		379,103.75	

se: Standard error; QS: Quadratic sum; QM: Quadratic mean; ANOVA: Analysis of variance; EPG: Epigallocatechin; C: Catechin; EP: Epicatechin

Table 3: Data of precision and accuracy regarding the analytical markers epigallocatechin, catechin, and epicatechin

Precision											
	EPG (µg/mL)			C (µg/mL)				EP (µg/mL)			
Repeatability - day 1	20.0±0.0003 (1.45%)			8.01±0.0001 (0.65%)				6.5±0.0001 (1.07%)			
Repeatability - day 2	20.1±0.0003 (1.60%)			8.0±0.0001 (0.73%)				6.5±0.0001 (0.89%)			
Intermediate precision	20.0±0.0003 (1.45%)			8.01±0.0001 (0.66%)				6.5±0.0001 (0.96%)			
Accuracy											
EPG (µg/mL)				**C (µg/mL)**				**EP (µg/mL)**			
TC (µg/mL)	**MC (µg/mL)**	**Recovery (%)**	**Average Recovery[a]**	**TC (µg/mL)**	**MC (µg/mL)**	**Recovery (%)**	**Average Recovery[a]**	**TC (µg/mL)**	**MC (µg/mL)**	**Recovery (%)**	**Average Recovery[a]**
16	15.966	99.76	100.35±0.52	6.4	6.384	99.75	100.40±0.58	5.28	5.249	99.42	100.14±0.67
	16.130	100.81	(0.52%)		6.455	100.86	(0.58%)		5.319	100.75	(0.67%)
	16.072	100.46			6.438	100.60			5.291	100.23	
20	20.074	100.37	100.14±0.20	8	8.013	100.16	100.13±0.23	6.6	6.618	100.28	100.20±0.37
	20.012	100.06	(0.20%)		7.990	99.88	(0.23%)		6.635	100.53	(0.37%)
	19.998	99.99			8.026	100.33			6.585	99.77	
24	24.026	100.11	99.93±0.52	9.6	9.637	100.38	100.27±0.11	7.92	7.861	99.26	100.02±0.68
	24.082	100.34	(0.52%)		9.624	100.25	(0.11%)		7.965	100.57	(0.68%)
	23.844	99.35			9.616	100.17			7.937	100.22	
Total	100.14±0.21 (0.21%)			100.26±0.14 (0.13%)				100.12±0.09 (0.09%)			

[a]$P<0.05$. $\bar{x}$ ± SD (RSD %). TC: Theoretical concentration; MC: Measured concentration; $\bar{x}$: Mean; SD: Standard deviation; RSD: Relative standard deviation; EPG: Epigallocatechin; C: Catechin; EP: Epicatechin

of the analytical markers (EPG-11.29 min; C-14.20 min; EP–18.0 min) and the chromatographic run time, whereas the increase of flow rate has reduced its Rts (EPG-7.23 min; C-9.80 min; EP–14.2 min). This behavior is correlated to the interaction between the compounds and the stationary phase, as well as the mass transfer effect promoted by the mobile phase. Moreover, the temperature parameters of the oven have generated slight alterations on analytical markers' Rts, once higher temperatures diminish superficial tension and viscosity of the solvents; therefore, favoring substance separation. The other proposed modifications on chromatographic analysis parameters did not promote any alteration, either on chromatographic profiles or analytical markers' quantification. The samples and standard solutions, storaged at 5°C, have demonstrated to be stable for 48 h, by maintaining its peaks areas and contents (RSD% ≤1.5%).

Antioxidant evaluation assay with 2,2-diphenyl-1-picrylhydrazyl

The ECE of *M. obtusifolia* has presented an IC_{50} of 18.01 ± 1.02 µg/mL, whereas the ascorbic acid has demonstrated an IC_{50} of 16.17 ± 2.32 µg/mL. This strong antioxidant activity of the crude extract may be related to the presence of phenolic compounds, especially flavonoids and flavanols. Previous *in vitro* assays have demonstrated that the antioxidant activity of flavonoids is superior to vitamins E and C,[46] and that (-)-EPG, (+)-(C) and (-)-EP have substantial antioxidant activity.[47] The antioxidant effect of phenolic compounds has been attributed to its hydroxyl groups, enabling radical scavenging.[48] The compounds with major free radical scavenging are the flavanols with hydroxyl groups at the positions 3', 4' and 5' of the B ring and/or C3 position.[49] This type of hydroxylation provides great stability to the phenoxy-catechin radical, by the electron relocation, which is considered an important antiradical mechanism, thus, justifying the great antioxidant activity of gallocatechin.[48,50,51]

There is no previous research regarding antioxidant activity of *M. obtusifolia*, however previous studies performed with *M. ilicifolia*,[52-55] *M. krukovii*,[56] *M. aquifolium*[57] and *M. imbricata*[39] have confirmed the richness of phenolic compounds of the *Maytenus* genus, corroborating with the antioxidant potential of the *M. obtusifolia* species.

Evaluation of antimicrobial activity

The ECE was evaluated in several concentrations, between 1024 and 32 µg/mL, by the microdilution technique. It could be observed that the extract at 1024 µg/mL inhibited the growth of four bacterial strains: *S. aureus* ATCC-6538, *S. aureus* M-117, *P. aeruginosa* ATCC-25853 and *P. aeruginosa* LM-116, among which, *S. aureus* ATCC-6538 was also sensitive to the extract at 512 µg/mL. On the other hand, the fungal species have indicated to be the most sensitive to the extract. At a concentration of 1024 µg/mL the extract has inhibited the growth of seven strains and at 512 µg/mL, the extract has inhibited the growth of six strains, including *C. albicans* LM-108, *C. tropicalis* ATCC-13813, *C. tropicalis* LM-36, *C. krusei* LM-13, *A. flavus* LM-247 and *A. fumigatus* IPP-210. In which *A. flavus* LM-247 was the most sensitive of all micro-organisms tested (128 µg/mL).

According to preestablished guidelines described,[58] the results of the antimicrobial assays of *M. obtusoflia* Mart. crude extract at concentrations of 1024, 512, 256 and 128 µg/mL against bacteria and fungi may be considered as moderate (1024 µg/mL) and great (512–128 µg/mL) activity. Previous related studies, using root bark methanolic extract of *M. senegalensis* towards strains of *B. subtilis* ATCC 6051; *S. aureus* ATCC 12600; *Escherichia coli* ATCC 11775 and *Klebisiella pneumoniae* ATCC 13883, have reported antibacterial activity against *B. subtilis* and *S. aureus*.[59] Furthermore, no significant inhibitory growth using bark ethanolic extract of *M. krukovii* against bacterial strains was observed;[56] however, this extract has demonstrated promising results concerning fungal inhibitory growth, similar to *M. obtusifolia* activity, suggesting a fungal inhibitory profile of the genus *Maytenus*.[60,61]

CONCLUSION

The lack of a current official monograph describing *M. obtusifolia* species, raises the necessity to develop assays and determine control parameters to contribute to the pharmacognostic control of the plant material and its products. The analytical methodology developed by HPLC-PDA and its validation has offered suitable chromatographic conditions for the analysis of the analytical markers EPG, C, and EP in this species. The method was considered specific, sensitive, precise, linear, accurate,

and robust, thereby providing parameters and specifications of the plant material, that supports its application for herbal drug development. The ECE of *M. obtusifolia* leaves has presented antioxidant activity due to the high concentration levels of TFC, TTC and TPC, besides the antimicrobial activity aimed at fungal strains.

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Conflicts of interest

There are no conflicts of interest.

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