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Original Article

Qualitative and quantitative analysis of the phenolic content of *Connarus* var. *angustifolius*, *Cecropia obtusa*, *Cecropia palmata* and *Mansoa alliacea* based on HPLC-DAD and UHPLC-ESI-MS/MS

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ABSTRACT

The phenolic content of the medicinal species *Connarus perrottetti* var. *angustifolius* Radlk., Connaraceae, *Cecropia obtuse* Trécul, *Cecropia palmata* Willd., Urticaceae; and *Mansoa alliacea* (Lam.) A.H.Gentry, Bignoniaceae, collected in three different years was evaluated. Plant infusions and hydroalcoholic, butanol and ethyl acetate extracts were analyzed by high-performance liquid chromatography with diode array detection. In order to endorse these results, analysis by electrospray mass spectrometry was also performed. Were identified: gallic acid, catechin, caffeic acid, ferulic acid, rutin, quercitrin and resveratrol. *C. perrottetti* showed greater diversity of polyphenols. *M. alliacea* had the higher concentration of caffeic acid even though it was found in all species. Catechin was the major antioxidant, but was not detected in *M. alliacea*. However, we discuss the popular use of these species, as well as their phenolic constitution and the interannual distribution of phenolic compounds.

Introduction

The use of medicinal plants for prevention and recovery of infections as well as for health promotion is an ancient practice (Veiga et al., 2005; Schmitz et al., 2005; Alvim et al., 2006). This activity grew out of popular knowledge and for a long time was the only alternative treatment for health problems (Alvim et al., 2006).

Although modern medicine has been greatly advancing in the past decades, phytotherapy is still widely employed (Alvim et al., 2006). The World Health Organization estimates that about 80% of the world population uses this traditional medicine in its primary health care needs (MS, 2006). According to the Institute for Applied Economic Research (IPEA, 2010), about 30% of commercially available drugs derive from natural sources, while Lahlou (2013) asserts that approximately 40% of all drugs are either natural products or their semi-synthetic derivatives.

Brazil holds an expressive plant biodiversity, along with the largest rain forest on the planet: the Amazon (Paracampo, 2011). Although the employment of plants for medicinal purposes is very widespread in Brazil, knowledge about their chemical composition is rather limited, as most of them are applied with little or no scientific proof of action (Campelo, 2006). In order to change this situation, relevant studies concerning the chemical composition of these species and the arising pharmacological properties are of considerable importance.

Different parts of the plant may be used in herbal medicine, such as roots, bark, leaves, fruits and seeds (Rezende and Cocco, 2002). Tea, usually obtained by infusion, is a very popular way of getting the active compounds of different plant products (Schmitz et al., 2005).

Polyphenols have been increasingly investigated and consumed in recent years due to their nutritional potential and therapeutic value (Ajila et al., 2011). However, numerous species of medicinal interest still need to be studied regarding this and other classes of compounds. Amongst them are *Connarus perrottetti* var. *angustifolius* Radlk., *Cecropia obtusa* Trécul, *Cecropia palmata* Willd., Urticaceae, and *Mansoa alliacea* (Lam.) A.H.Gentry, Bignoniaceae, all

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native species of the Amazon rainforest, where they are extensively used by the locals with limited scientific information.

The species *C. perrottetti* var. *angustifolius*, popularly known as "barbatimão-do-pará", is a member of the Connaraceae family. This plant present antidiarrheal, anti-bleeding, anti-inflammatory, antibacterial, antifungal, antiviral and healing activities (Paracampo, 2011), being commonly used macerated, or as tea and syrup, for the treatment of genitourinary infections in women, uterine bleeding, vaginal discharge, headache, gastric diseases and cough (Coelho-Ferreira, 2009).

The species *C. obtusa* and *C. palmata*, popularly known in Brazil as "red-embaúba" and "white-embaúba" respectively, are both members of the Urticaceae family (Beringhs et al., 2015). Plants of this genus are usually employed as tea, and exhibit several recognized activities, such as antidiabetic (Freitas and Fernandes, 2006), expectorant, mucolytic, antiseptic, laxative, antimicrobial (Lameira et al., 2004), diuretic, antitussive and anti-inflamatory (Freitas and Fernandes, 2006; Lameira et al., 2004; Costa et al., 2011).

In Brazil, especially in the state of Pará, *M. alliacea* is popularly known as "cipó-d'alho" due to the characteristic smell of garlic released by its leaves when macerated (Zoghbi et al., 2009). This plant belongs to the Bignoniaceae family and is used by its anti-rheumatic (Ribeiro et al., 2009), antimalarial (Pérez, 2002), antifungal and antiviral (Zoghbi et al., 2009), activities and to the treatment of respiratory diseases (Ribeiro et al., 2009). Maceration, infusion, tea preparation and decoction are the main processes popularly used in this plant to obtain its active compounds (Zoghbi et al., 2009).

Several factors can coordinate or change the rate of production of phenolic compounds and other secondary metabolites in plants. The period of collection is one of them, since the concentration and even nature of such compounds may vary considerably over the year (Gobbo-Neto and Lopes, 2007).

Phenolic compounds range from simple to highly polymerized structures, which can withal be complexed to various other plant components. Therefore, different methods of extraction combined with solvents of different polarities are required to obtain them (Naczk and Shahidi, 2004).

This paper aims to identify and to quantify phenolic content, namely, gallic acid, catechin, caffeic acid, rutin, ferulic acid, quercitrin, myricetin, fisetin, resveratrol, quercetin, kaempferol, chrysin and flavone, present in the species *C. perrottetti* var. *angustifolius*, *C. obtusa*, *C. palmata* and *M. alliacea*. The extracts were obtained by ultrasound extraction using ethanol/water, n-butanol and ethyl acetate as solvents. Water infusions were also analyzed. The separation, identification and quantification of the aforementioned compounds were carried out using high-performance liquid chromatography with diode array detection (HPLC-DAD). An ultra-high-performance liquid chromatography-electrospray ionization-tandem mass spectrometry (UHPLC-ESI-MS/MS) analysis was performed in order to endorse the results previously obtained by HPLC-DAD.

Materials and methods

Chemicals

Analytical standards of gallic acid, (+)-catechin, caffeic acid, rutin, quercitrin, myricetin, fisetin, quercetin, kaempferol, chrysin and flavone were purchased from Sigma–Aldrich (St. Louis, MO, USA). Ferulic acid was purchased from Fluka (Buchs, Switzerland) and resveratrol from Tedia (Rio de Janeiro, RJ, Brazil). All standards used were of analytical grade (≥95% purity).

The mobile phase was prepared by diluting an 85% (v/v) phosphoric acid (PA) (F. Maya, Brazil) with ultrapure water (Milli-Q, Millipore Synergy UV, Bedford, MA, USA) to a concentration of 0.1% (w/w) in a volumetric flask. The solution was then filtered under vacuum system (Prismatec, 131, 2 V) through a 0.2 μm cellulose acetate membrane filter (Sartorius, Goettingen, Germany). Acetonitrile was also in the composition of the eluent and was obtained from Panreac ITW companis (Germany).

Stock solutions of 1000 mg/l were prepared by diluting each phenolic compound studied in HPLC grade methanol, supplied by Tedia (Rio de Janeiro, RJ, Brazil). They were stored in Falcon tubes at −30 °C. These solutions were then diluted in methanol to reach intermediate concentrations.

Plant samples were weighed on a digital analytical balance (Shimadzu/AUY 220) with a 0.0001 g precision.

Plant material

Barks of *Connarus perrottetti* var. *angustifolius* Radlk., *Connaraceae* (IAN 184393) and leaves of *Cecropia palmata* Willd. (IAN 185556), *C. obtusa* Trécul, Urticaceae (IAN 185555) and *M. alliacea* (Lam.) A.H.Gentry, Bignoniaceae (IAN 184394) were collected in April and May of 2012, 2013 and 2014, properly identified, dried and milled. All plants studied were provided by the herbarium IAN located at the Brazilian Agricultural Research Corporation (Embrapa) Eastern Amazon, Belém, PA, located at 1°27′21″ S and 48°30′14″ W, at an altitude of 10 m and an average annual temperature of 30 °C.

According to Ananias et al. (2010) the climate of the Amazon region is characterized by a dry period (July–October) and a rainy season (December–May), while June and November are considered transition periods. In addition, it is considered a hot and humid climate with very small temperature gradients. Therefore, samples studied were collected during the rainy season.

Extraction procedure

Plant extracts were prepared by ultrasound assisted extraction (Bandelin, Sonorex Super RK 510 H). Glass tubes containing approximately 0.2 g of the samples received 10 ml of the extraction solvent (70% hydroethanolic, butanol or ethyl acetate) and were placed in an ultrasonic bath for 4 h at room temperature. Then, the supernatant was withdrew and the remaining extract was filtered on a 0.22 μm membrane (Sorbline Tecnologie). Butanol and ethyl acetate were evaporated at 40 °C in a glass beaker. These extracts were later resuspended in HPLC grade methanol and filtered in a 0.22 μm membrane (Sorbline Tecnologie). The initial concentrations were maintained. The extracted samples remained stored at −30 °C until the analysis. Before injection into the chromatograph, all samples were diluted to 0.01 g/ml with HPLC grade methanol.

Infusions

A volume of 50 ml water at 90 °C were added to 1.5 g of dried plant. Thirty minutes later, the infusions were filtered, both by a paper filter and by a 0.22 μm membrane (Sorbline Tecnologie) and then stored at −40 °C until used. The samples injected were first diluted at 0.01 g/ml with HPLC grade methanol.

HPLC-DAD apparatus

The chromatographic analysis was performed in a Knauer chromatograph (Berlin, Germany) equipped with a manual Knauer injector (20 μl loop). The system consisted of a Smartline Pump 1000, a Smartline Manager 5000 and a Smartline UV detector 2600 with photodiode array technology. Instrument control and data acquisition and processing were managed by the ChromGate® V 3.3.1 Knauer software.

Table 1
Data from the chromatographic separation via HPLC-DAD.

Phenolic compound/equation of analytical curve	t_R (min)[a]	λ (nm)[b]	LOD (mg/kg)[c]	LOQ (mg/kg)[d]
1. Gallic acid/$y = 9387.5x + 5270.2$	4.40	220	0.7	1.0
2. (+)-Catechin/$y = 13992x + 23821$	8.65	220	1.7	2.8
3. Caffeic acid/$y = 14013x + 7961.9$	10.10	320	0.3	1.0
4. Rutin/$y = 16178x + 1196.8$	12.45	254	0.6	2.4
5. Ferulic acid/$y = 15927x + 3286.3$	14.75	320	0.5	1.0
6. Quercitrin/$y = 18225x + 5576.9$	18.00	254	0.4	0.8
7. Resveratrol/$y = 24317x + 1592.9$	28.45	320	0.4	0.8

[a] t_R: retention time.
[b] λ: wavelength.
[c] LOD: detection limit.
[d] LOQ: quantification limit.

Identification and quantification of phenolic compounds were performed using the methodology developed which, besides the low limits of detection and quantification presented by this chromatographic method (Table 1), the analytical validation confirmed its selective, linear, precise and accurate character (Table 2).

Compounds were separated with a gradient reverse-phase system. A 250 mm × 4.6 mm chromatographic column packed with C18 (5 μm particle diameter, Phenomenex) and a guard column with similar composition were employed. The mobile phase consisted of orthophosphoric acid solution (0.1%, w/w) as solvent A and acetonitrile as solvent B. The elution conditions were: 90–80% A and 10–20% B (0–5 min); 80–75% A and 20–25% B (5–35 min); 75–0% A and 25–100% B (35–55 min). The flow rate of the mobile phase was 0.8 ml/min (0–35 min) and 0.8–1.0 ml/min (35–55 min). Room temperature (21 ± 2 °C) was monitored during all chromatographic runs. The wavelengths scanned were 220 nm, 254 nm, 320 nm and 360 nm.

Identification of compounds was based on the correspondence of retention times of chromatographic signals of plant extracts and reference materials, the ultraviolet (UV) spectra and by the addition of three different concentrations of the standard solutions to the samples.

Following, compounds were quantified by the standard addition method, which correlates the concentration of the standard solution (added in μmol/l) to the peak area. According to Ribani et al. (2004) the concentration of the samples analyzed may be determined through straight line extrapolation, corresponding to the point where it cuts the axis of the abscissae (x).

Table 1 shows the equation for the analytical curve, retention times of the antioxidant compounds found in plant species, as well as the detection wavelength (nm), limit of detection and limit of quantification of the chromatographic separation via HPLC-DAD.

The limits of detection (LOD) and of quantification (LOQ) were determined by the signal-to-noise ratio, in acordance to National Institute of Metrology (2016). The area and standard deviation of the retention times were computed through the injection of 7 replicates of the blank (matrix free of the compound of interest), in this case methanol (HPLC grade). $LOD = X + t_{(n-1)} \cdot s$ and $LOQ = X + 10 \cdot s$, where: X = mean of blanks, s = standard deviation of blanks, t = number of injections (7-1) = 6 degrees of freedom. Considering the degrees of freedom, the unilateral t, at a 99% confidence level, is 3.143.

Table 2 shows the linear range, the correlation coefficient, the intraday precision, expressed by the relative standard deviation (RSD%), the inter-day precision, expressed by the coefficient of variation (CV%), and the accuracy (%), determined by recovery assays.

UHPLC-ESI-MS/MS method

The method developed and applied by Faccin et al. (2016) to detect the molecular ions through mass spectrometry was employed for confirmation purposes.

Results and discussion

The methodology employed to determine the phenolic constituents by HPLC-DAD enables the separation and identification of thirteen antioxidants as shown in Fig. 1(A). Among these, seven were detected in the studied plants, namely, gallic acid (1), catechin (2), caffeic acid (3), rutin (4), ferulic acid (5), quercitrin (6) and resveratrol (9).

Determination by HPLC-DAD of phenolic compounds in plant extracts from different years of collection

Fig. 2 shows the concentration of antioxidants found in the medicinal species studied relating the extracts to the respective collection period. The mean coefficient of variation over the triplicate extractions was 28–30% for all extracts obtained.

Connarus perrottetti *var.* angustifolius

Among the analyzed species, *C. perrottetti* var. *angustifolius* presented the highest diversity of phenolic compounds. Although catechin was the predominant antioxidant, corresponding to

Table 2
Validation parameters of the HPLC–DAD method.

Phenolic compound	Linear range (μmol/l)	(r)[a]	Intraday (RSD%)[b]	Interday (CV%)[c]	Accuracy (%)
1. Gallic acid	2.94–588	0.9990	0.6	1.9	104.7
2. (+)-Catechin	8.61–344.4	0.9997	1.2	3.5	83.8
3. Caffeic acid	5.5–550	0.9997	0.8	2.3	103.4
4. Rutin	0.82–164	0.9996	0.9	2.8	88.2
5. Ferulic acid	5.15–515	0.9997	0.5	1.6	97.6
6. Quercitrin	2.23–223	0.9984	1.2	3.7	94.6
7. Resveratrol	4.38–438	0.9996	0.6	1.9	100

[a] r: correlation coefficient.
[b] RSD: relative standard deviation.
[c] CV: coefficient of variation.

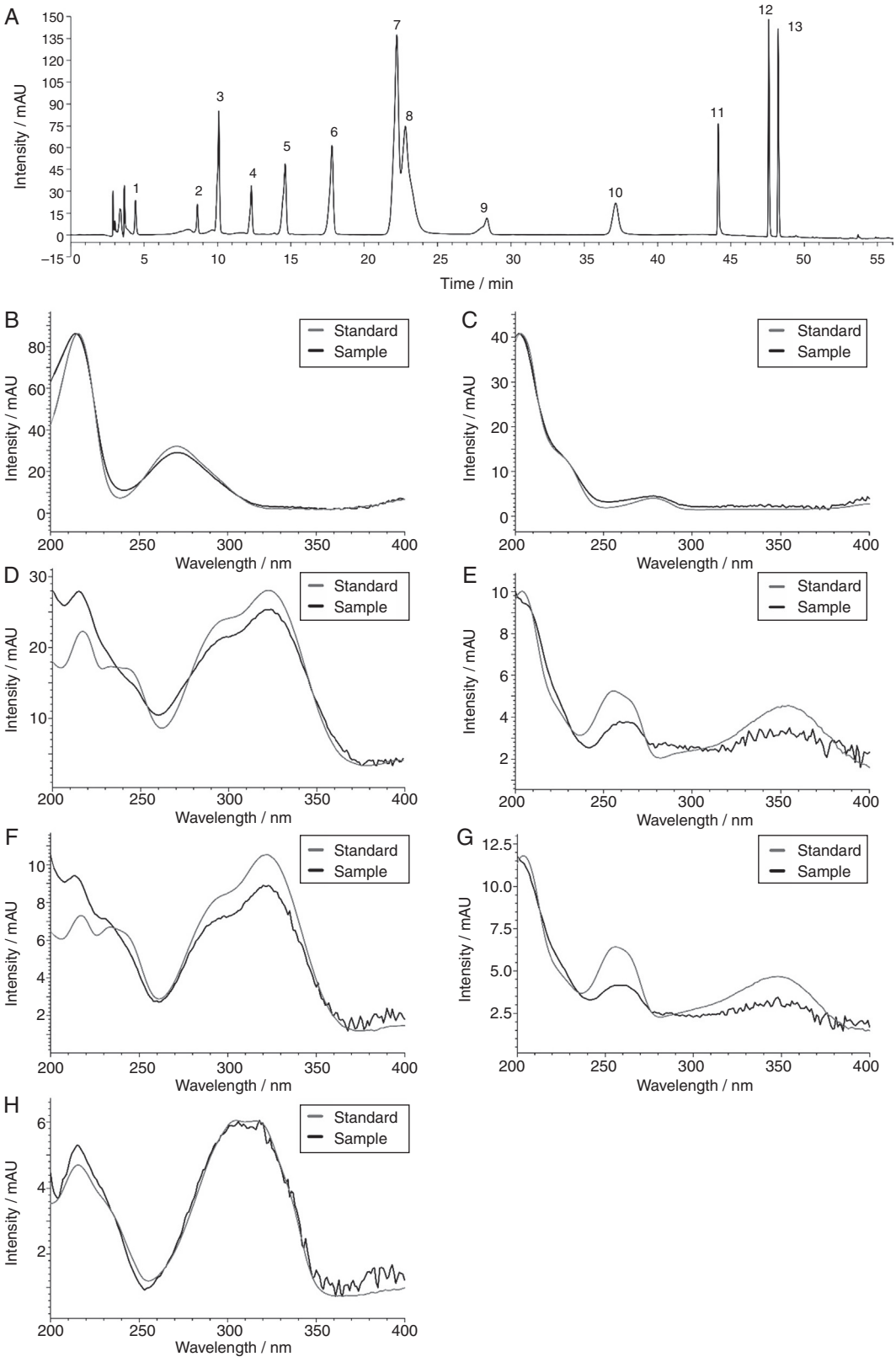

Fig. 1. (A) Chromatographic separation of the thirteen antioxidants standards by DAD (20.5–218.25 μmol/l). 1. gallic acid, 2. catechin, 3. caffeic acid, 4. rutin, 5. ferulic acid, 6. quercitrin, 7. myricetin, 8. fisetin, 9. resveratrol, 10. quercetin, 11. kaempferol, 12. chrysin and 13. flavone; (B) Standard spectrum of gallic acid/sample: *Cecropia obtusa* hydroalcoholic extract 2013; (C) Standard spectrum of catechin/sample: *Cecropia palmata* butanolic extract 2013; (D) Standard spectrum of caffeic acid/sample: *M. alliacea* butanolic extract 2013; (E) Standard spectrum of rutin/sample: *C. perrottetti* var. *angustifolius* ethyl acetate extract 2014; (F) Standard spectrum of ferulic acid/Sample: *Cecropia palmata* butanolic extract 2013; (G) Standard spectrum of quercitrin/sample: *C. perrottetti* var. *angustifolius* butanolic extract 2014; (H) Standard spectrum of resveratrol/sample: *C. perrottetti* var. *angustifolius* ethyl acetate extract 2013.

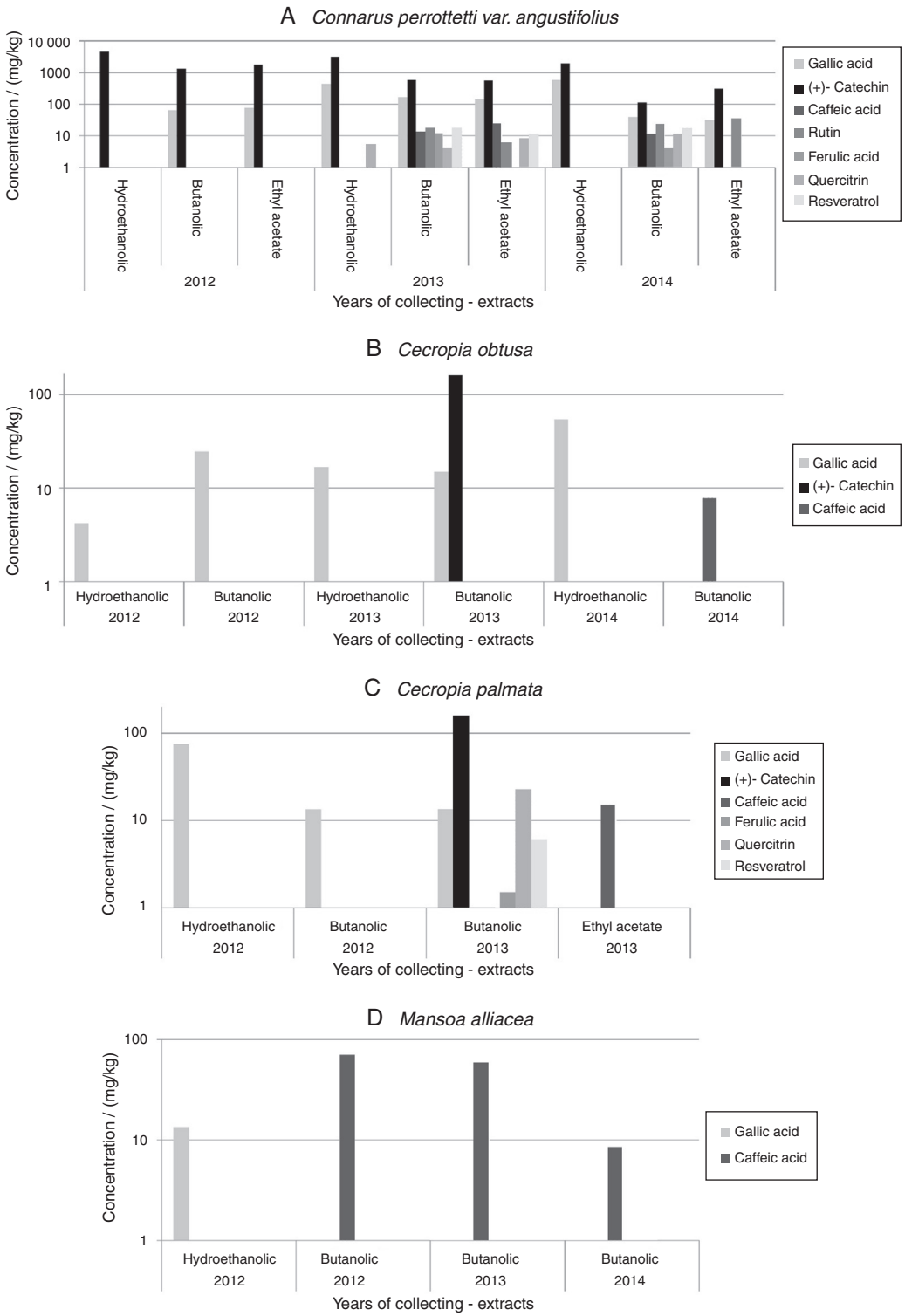

Fig. 2. Phenolic compounds found in the studied species via HPLC-DAD: (A) *C. perrottetti* var. *angustifolius*, (B) *Cecropia obtusa*, (C) *Cecropia palmata* and (D) *M. alliacea*; coefficient of variation: 28–30% over extracts triplicate.

approximately 80%, all seven compounds determined in the studied plants were encountered in this species.

Fig. 2 shows a decrease on the concentration of catechin between collections of 2012 and 2014. This decrease was observed for all extracts analyzed, but there was no clear correlation to the concentration.

The highly polar phenolic compounds are not completely extracted with pure organic solvent. The addition of water to ethanol increases the polarity, facilitating the extraction of the polar constituents (Stalikas, 2007), i.e. gallic acid and catechin. Accordingly, the greatest amounts of these compounds were detected in the hydroethanolic extracts. Silveira et al. (2015) extracted the sample collected in 2012 with the same solvents reported in this study and analyzed the extracts by liquid chromatography with amperometric detection, also finding the presence of gallic acid and catechin.

The use of solvents with lower polarities, such as butanol and ethyl acetate, enabled the identification of less polar constituents. This observation is supported by Naczk and Shahidi (2006), who reports that the solubility of phenolic compounds is affected by the chemical nature of the plant constituents and by the polarity of the solvent extractor. Therefore, various organic solvents and aqueous mixtures with alcohols are reported in the extraction of these metabolites (Ignat et al., 2013).

Caffeic and ferulic acid were present in higher concentrations in the sample collected in 2013. Rutin and quercitrin, on the other hand, reached higher concentrations in plants collected in 2014, what is supported by Fig. 1(E) and (G), that shows the spectra used to identify rutin and quercitrin in the species.

There was no significant variation on resveratrol concentration when comparing butanolic extracts of *C. perrottetti* var. *angustifolius* collected in different years (2013 and 2014). Nonetheless, extracts using ethyl acetate as a solvent exhibited a decrease of approximately 34% on this concentration from 2013 to 2014. This is a strong evidence of the lower affinity between this solvent and resveratrol, whose presence in the extract is supported by Fig. 1(H). Fig. 2(A) shows that resveratrol was not detected in plants collected in 2012.

Cecropia obtusa

From 2012 to 2014, a significant increase (92.2%) was observed on the concentration of gallic acid in the hydroethanolic extract of this species, as shown in Fig. 2(B). This may be a result of some stress suffered by the plant, since, as reported in literature, many environmental factors such as seasonality, temperature, water availability, ultraviolet radiation, nutrients and altitude may interfere with the biosynthesis of secondary metabolites (Gobbo-Neto and Lopes, 2007; Morais, 2009). In order to endorse the results concerning the identification of gallic acid, the spectrum measured for this species was compared to its reference spectrum. This comparison is reported in Fig. 1(B).

Catechin was the major compound in *C. obtusa*, but it was detected only in the butanolic extract of plants collected in 2013. These extraction conditions also detected caffeic acid in the sample collected in 2014.

Besides presenting little variety of antioxidants in its constitution, *C. obtusa* has shown very irregular behavior during the analyzed period. This may be related to the defense strategy of the plant against the influence of biotic and/or abiotic factors, as mentioned by Morais (2009) and Coutinho et al. (2010).

Cecropia palmata

Regarding the studied compounds, *C. palmata* exhibited a greater diversity when compared to the other species of the same genus analyzed, as shown in Fig. 2(C).

The hydroethanolic solvent extracted the highest concentration of gallic acid on plants collected in 2012. The similarity between polarities (both high) of solvent and extracted compound justifies this result. However, this condition did not extracted any other compound of interest.

Butanolic extraction of *C. palmata* collected in 2013, on the other hand, enabled the separation of less polar compounds which have not been identified in this plant so far, i.e., in the hydroethanolic extract. Fig. 1(F) shows that ferulic acid is also present in this species. In addition, the following compounds were extracted: catechin, quercitrin and resveratrol. This solvent was also able to extract caffeic acid, despite its higher affinity with ethyl acetate.

Similarly to what was observed to *C. obtusa*, catechin was the major compound, but was only detected on butanolic extracts of the sample collected in 2013. Comparing both species in relation to the catechin concentration, a minor difference of 1.3% – higher for *C. obtusa* – is observed. Fig. 1(C) shows the spectrum employed on the identification of catechin in *C. palmata.*

There are several studies regarding species of *Cecropia* genus reported in literature. Franck (1998) analyzed phenolic acids and flavonoids in extracts of the leaves of *C. hololeuca*, *C. pachystachaya* and *C. glaziovii* using thin-layer chromatography. *C. glaziovii* was also studied using liquid chromatography by Luengas-Caicedo et al. (2007) who identified catechin, and by Arend et al. (2011) and Beringhs et al. (2015) who detected caffeic acid. These results support the ones presented here for the two species of *Cecropia* investigated.

Mansoa alliacea

This species showed the lower diversity of phenolic compounds, as shown in Fig. 2(D). In contrast, it presented the highest concentration of caffeic acid, detected in the butanolic extract, Fig. 1(D). Considering the entire period of collection, i.e. 2012–2014, there was a decrease of 87.9% in the concentration of such compound. Similarly to the other plants evaluated in this study, gallic acid was detected in *M. alliacea*, but only in samples collected in 2012.

Phytochemical studies involving the leaves of *M. alliacea* revealed the presence of polyphenols and flavonoids (Ribeiro et al., 2009; Patel et al., 2013) and the absence of catechins (Ribeiro et al., 2009). Also, flavonoids like luteolin and apigenin were detected in the flowers (Zoghbi et al., 2009), and phenols in the root (Patel et al., 2013). The chemical composition of this species may be better understood due to the results presented in this study, since they agree with the aforementioned works and provide additional information on the phenolic acids composition, such as caffeic and gallic.

According to the National Institute of Meteorology (INM, 2015), the average rainfall during the years of collections (2012, 2013 and 2014) was 416.7 ± 80.4 mm and the coefficient of variation stayed around 20%. The temperatures varied between a minimum of 23.3 ± 0.3 °C and a maximum of 32.8 ± 0.3 °C. Becho et al. (2009) showed that compounds with higher polarity can be eliminated from plants by leaching. Whereas the samples analyzed were collected during the rainy season, these data support the behavior observed concerning the period of collection.

Although the chemical composition of plants is largely determined by the genetic characteristics of the species, various environmental factors (Diniz et al., 2007) and the employment of different extraction methods can change it (Naczk and Shahidi, 2004). For this reason, studies concerning these variables are paramount in order to assure the desired concentrations of active compounds.

Analysis of infusions by HPLC-DAD

Investigated compounds were detected only on infusions of *C. perrottetti* var. *angustifolius* and *C. obtusa*. Considering the use of these species in the form of tea, a concentration of 0.81 mg/100 ml of gallic acid was quantified in samples of *C. perrottetti* var. *angustifolius* collected in 2013 and 0.58 mg/100 ml in samples collected in 2014. Concentrations of catechin were considerably higher on this species: 7.9 mg/100 ml in samples collected in 2013 and 3.5 mg/100 ml in samples from 2014. Samples of *C. obtusa* collected in 2012 and 2013 showed no variation on the concentration of gallic acid, which was of 0.14 mg/100 ml.

Regarding medicinal purposes, the consumption of tea obtained by infusion of barks of *C. perrottetti* var. *angustifolius* can provide reasonable amounts of active compounds to the organism, such as gallic acid and catechin, which are associated to different

Table 3
Tandem mass spectrometry (MS/MS) parameters of investigated phenolic compounds.

Phenolic compound	Fragmentador voltage	Quantification transition[a]	Confirmation transition[a]
1. Gallic acid	106	169.0 > 125.1 (10)	–
2. (+)-Catechin	134	289.1 > 245.1 (10)	289.1 > 203.2 (15)
3. Caffeic acid	106	179.0 > 135.1 (10)	–
4. Rutin	201	609.1 > 300.1 (31)	–
5. Ferulic acid	88	193.1 > 134.1 (9)	193.1 > 178.1 (7)
6. Quercitrin	164	447.1 > 301.1 (17)	–

[a] The energy collision (*V*) is given in brackets.

pharmacological effects and especially recognized for their antioxidant activity.

Confirmation of the identity of antioxidant compounds by UHPLC-ESI-MS/MS

In order to endorse the results concerning the phenolic constitution of the medicinal plants studied, data obtained by HPLC-DAD and by UHPLC-MS/MS were compared.

Negative chemical ionization was employed for all phenolic compounds analyzed and the retention times ranged from 0.39 to 3.66 min. The mass spectral data obtained are summarized in Table 3.

More than 60% of the results reported in Fig. 2 are in good agreement with the ones from analysis by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). For samples containing catechin and ferulic acid, these compounds were detected by HPLC-DAD and LC-MS/MS.

The results not supported by LC-MS/MS were: caffeic acid, rutin and quercitrin on samples of *C. perrottetti* var. *angustifolius* collected in 2013, as well as resveratrol for all periods assessed for this species; gallic acid on infusion and samples of *C. obtusa* collected in 2012 and 2014; gallic acid on *M. alliacea* samples; gallic acid, quercitrin and resveratrol on *C. palmata* samples. This disagreement is believed to be due to the difference in the extraction methods used, as all these were obtained from cold maceration.

According to Naczk and Shahidi (2006), the extraction of phenolic compounds in plants is influenced by several factors, such as chemical nature of the species, extraction method, sample particle size, processing, amongst others. Each extraction method affects the selectivity of the compounds, especially when it involves the study of antioxidant components, since they are sensitive to the action of light, oxygen and heat (Andreo and Jorge, 2006; Pereira and Meireles, 2010).

There is no standard or fully satisfactory procedure for the extraction of all phenolic compounds or a specific class of vegetable material (Naczk and Shahidi, 2004). Given the vast number of studies on the subject, however, the extraction assisted by ultrasound proves to be quite effective in isolating polyphenols (Luque and Priego-Capote, 2007; Routray and Orsat, 2013).

According to Dal Prá et al. (2015), extraction assisted by ultrasound involves the formation of cavitation bubbles, which assist the release of the vegetable content, increasing the mass transfer. In studies with *Brassica oleracea*, the extraction assisted by ultrasound had a positive effect, increasing the efficiency in approximately $36.1 \pm 15.5\%$ when compared to conventional extraction (maceration).

Some phenolic compounds are very photosensitive. Therefore, time-consuming extraction may result in degradation. Thereby techniques involving ultrasound have been widely used mainly in order to prevent the degradation of resveratrol (Guamán-Balcázar et al., 2016). Resveratrol belongs to the class of stilbenes, which present high sensitivity to external agents such as air, light and oxidative enzymes. Accordingly, there are many food additives being developed aiming the increase on solubility and photostability of these compounds (Silva et al., 2014).

Studies regarding the solid phase extraction of polyphenols in wine demonstrated a low recovery rate for the gallic acid. Even tough, quantification was still possible, given the large amounts of this compound present in this matrix (Villiers et al., 2004). Hence, when occurring in lower concentrations, as in some plant species, the detection may not be possible, depending on the extraction procedure.

According to Jaques et al. (2010), who studied the change on phenolic compound composition of blackberry pulps during storage, gallic acid suffered the major degradation. This result is related to the redox reactions resulting from its highly hydroxylated structure.

Furthermore, according to Waterhouse (2002), gallic acid is widely used as a standard in the Folin-Ciocalteu method for the determination of total polyphenols, because it is relatively inexpensive and pure and its dry form is very stable. However, the standard solution loses about 5% of its effectiveness after one week at room temperature and the decomposition is even faster when the solution is exposed to the air.

Taking into account all parameters involving the determination of the polyphenols, which were here demonstrated, along with the numerous studies on this subject, we can affirm that both analysis performed in this study, by diode array detection and by mass spectrometry, yielded satisfactory results.

Conclusions

One or more phenolic compounds, e.g. phenolic acids and flavonoids, are present in the chemical constitution of the extracts of medicinal plants analyzed. Among the investigated compounds, gallic and caffeic acid were the only antioxidants found in all species when analyzing the extracts with diode array detection. In *C. perrottetti* var. *angustifolius*, catechin, rutin, ferulic acid, quercitrin and resveratrol were also present. Among these, only rutin was not detected in *C. palmata*. Catechin was not present only in *M. alliacea*, being the major compound in all other species. The infusion with *C. perrottetti* var. *angustifolius* may provide important amounts of gallic acid and catechin, supporting its employment with medicinal purposes.

When comparing the results obtained via LC-MS/MS to the ones obtained by HPLC-DAD, over 60% agreement was reached. Of all compounds, only resveratrol could not be detected by mass spectrometry. However, this result, as well as the fact that other components were also not detected in some periods of collection or even in some plant species, may be a consequence of the extraction method used.

Therefore, the results obtained in this work show the importance of studies concerning the investigation of chemical constituents of plants collected in different periods in order to ensure the adequate concentration of the active ingredients in therapeutic applications of such species.

Authors' contributions

FBP carried out the research. These results form a part of her Master degree work. CBD, DM, and VDP supported the ultrasound extraction. HF performed the LC-MS analysis. LMC, CV and MBR created the Project and were responsible for arranging the scholarship and financial support. MBR was the advisor of FBP. OL collected the plants and performed the voucher. FOL performed the chromatographic validation. All the authors have read the final manuscript and approved its submission.

Conflicts of interest

The authors declare no conflicts of interest.

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