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Article

Seasonal and pluviometric effects on the phenolic compound composition and antioxidant potential of *Licania macrophylla* Benth (Chrysobalanaceae), a medicinal plant from the Amazon rainforest

Ramon Diego Cunha Araujo[1]*, Anderson Luiz Pena da Costa[1], Jardel Barbosa Pinto[2], Luís Maurício Abdon da Silva[3], Gabriel Araujo da Silva[1,2]

[1]Postgraduate Program in Pharmaceutical Sciences, Federal University of Amapá (UNIFAP), Macapa, Brazil, [2]Collegiate of Chemistry, Amapá State University (UEAP), Macapa, Amapá, Brasil, [3]Institute of Scientific and Technological Research of Amapá (IEPA), Brazil

Licania macrophylla is a medicinal plant from the Amazon. It is mainly used in the form of a decoction and has been reported to contain several phenolic compounds. However, the effect of seasonality on the phenolic composition and antioxidant potential of this plant has not been well studied, especially in the Amazon region, an area affected by the rainy and less-rainy seasons. Therefore, we evaluated the seasonality of these aromatic compounds and the antioxidant potential of the extracts from *L. macrophylla* stem bark. We also determined the correlation between the extraction methods used and precipitation levels during each period for 1 year. The total flavonoid and phenolic content, DPPH-scavenging potential, percentage of phosphomolybdenum complex reduction, and iron-reducing power were quantified. The levels of phenolic compounds were the highest in June, whereas those of flavonoids were the highest in September and October; however, these differences were not significant. The extracts from April, November, and June showed the best results for DPPH scavenging, phosphomolybdenum reduction, and iron reduction power, respectively. Significant differences in the phenolic content and DPPH-scavenging activity were observed between the more- and less-rainy seasons. The total phenolic content was positively correlated with FRAP and DPPH, whereas flavonoid levels were negatively correlated.

Keywords: Anauera. Antioxidant activity. Extracts. Rain. Seasonality.

INTRODUCTION

Medicinal plants are widely used throughout the world, mainly by traditional communities and individuals without access to allopathic medicines. The resurgence in the use of medicinal plants is the result of the millennial transference of traditional knowledge, and this popular knowledge contributes to the choice of species to be studied scientifically, which may result in health benefits to humans (Pio *et al*., 2019).

From this perspective, there has been high interest in the pharmaceutical agents derived from natural extracts. This approach has contributed to massive searches for the products derived from these plants, especially those with already known medicinal properties that have used by the community (Lall, Kishore, 2014).

The antioxidant activity of plants can be affected (increased or decreased) by their chemical composition. Phenolic acids and flavonoids are particularly important antioxidants (Simões *et al*., 2010; Veiga *et al*., 2018). However, the search for new antioxidant compounds from natural resources continues, especially as these compounds are important for combating oxidative damage in various components in cells, such as DNA. Moreover, these compounds may protect against carcinogenesis,

***Correspondence:** R. D. C. Araújo. Programa de Ciências FarmaceuticasUniversidade Federal do Amapá. Rod. Juscelino Kubitschek, km 02 - Jardim Marco Zero. Cep: 68903-419, Macapá - AP, Brasil. Phone: +55(96) 9 8410-7446. E-mail: professorramonbiologia@hotmail.com. Orcid**:** 0000-0001-8540-9021. Anderson Luiz Pena da Costa ORCID: 0000-0002-7876-0187. Gabriel Araujo da Silva ORCID: 0000-0002-9073-1995

mutagenesis, cardiovascular diseases, and many forms of cancer (Esteban *et al*., 2019).

Licania macrophylla Benth (LmB), which belongs to the Chrysobalanaceae family, is an important species owing to its beneficial effects. This family is composed of approximately 18 genera and 531 species, including trees and shrubs that are widely distributed in the Amazon, and is also known as anauera in the Amapá state (Prance, 2007).

Ethnopharmacological data showed that extracts from the stem bark of anauerá are used as an anti-inflammatory agent for the reproductive system and a gastroprotective agent to treat worm infections. The seeds are used to treat diarrhea, in addition to having healing, amoebicidal, and antispasmodic actions. Moreover, anti-inflammatory and healing effects have been reported for the leaves (Gomes *et al*., 2006; Neto *et al*., 2013; Ramos *et al*., 2014). Recent studies have shown the antiulcer and potent gastroprotective properties of the stem bark extract (Sales *et al*., 2019).

In addition, the genus *Licania* has several species with biological and pharmaceutical potential. These biological activities include: anti-inflammatory, antioxidant, antibacterial and antifungal. Moreover, species from the *Licania* genus contain many secondary metabolites, mainly phenolic compounds, such as flavonoids (Medeiros, Medeiros, 2012; Silva *et al*., 2012; de Freitas *et al*., 2019; Moreira-Araujo *et al*., 2019; Santos *et al*., 2019; Shin *et al*., 2019; Lima de Medeiros *et al*., 2020). Data from the literature corroborate the chemotaxonomic knowledge of the genus and add value for the species, especially for *L. macrophylla*.

However, the pharmacological activities of LmB widely vary owing to biotic and abiotic factors, such as seasonality and precipitation, which must be considered during the collection of plant materials. As there is a correlation between the timing of species collection and the levels of secondary metabolites, isolating these plant materials at the correct time of year is crucial (Gobbo-Neto, Lopes, 2007; Gobbo-Neto *et al*., 2017).

In this study, we monitored the seasonal behavior of the phenolic compounds and antioxidant potential of crude hydroethanolic extracts isolated from LmB. Further, we correlated the level of these compounds with precipitation to determine the appropriate time to collect for LmB.

MATERIAL AND METHODS

Chemicals

The following reagents were used: potassium ferricyanide (Biotec®), ferric chloride (Biotec®), trichloroacetic acid (TCA) (Impex®), mono and dibasic sodium phosphate (Impex®), ethyl alcohol hydrate, 70% ethyl alcohol, and 30% water (Sol®), 2,2-diphenyl-1-picrylhydrazyl (Aldrich Chemistry®), sulfuric acid (Impex®), ammonium molybdate0 (Impex®), sodium carbonate (Isofar®), Folin-Ciocauteau (Merck KgaA®), aluminum chloride (Biotec®), ascorbic acid (Biotec®), quercetin (Biotec®), and distilled water.

Plant material

Samples of *L. macrophylla* stem bark were collected monthly over a period of 1 year (February 2018 to January 2019) from the same individuals (n = 3) in a forest area in the municipality of Porto Grande, Amapá, Brazil at the coordinates of 06°-80′84″N/51°50′68″O). An exsiccate of the specimen was prepared for identification a botanist, Dr. Tonny David Santiago Medeiros, Curator of the Herbarium of the Institute of Scientific and Technological Research of Amapá (IEPA) (LmB), Brazil, Amapá: Porto Grande, 02.II.2018, RDC Araujo, 02 (HAMAB).

The amount of sample collected from each plant was sufficient for the preparation of all extracts and did not compromise the integrity of the plants. Samples were always collected on the fifth working day of each month, and always in the morning (09:30–11:30 am BRT). In addition, the three individuals of LmB were healthy mature trees. It is worth mentioning that this species has been widely used by the IEPA for the production of tinctures (extracts) for the treatment of some diseases.

Preparation of crude hydroethanolic extract of *L. macrophylla*

After the collection and identification of the plant material, samples were sanitized and dried in an air circulating oven at 40 °C for 48 h, and then ground with a

knife mill at the Bioprospection Laboratory of the Federal University of Amapá (UNIFAP).

The powder (5 g) was soaked in a hydroalcoholic mixture of 70% ethanol in a ratio of 1:10 (m/v), and macerated for 5 consecutive days under gentle and periodic agitation. Next, the extract was filtered (with sterile filter paper with 45-µm porosity) and then dried in a rotary evaporator to obtain the crude extract (Brazilian Pharmacopoeia, 2010).

Determination of total flavonoid and polyphenol content

The Folin-Ciocalteu method was used to determine the total phenolic content of the hydroethanolic extracts (Singleton, Orthofer, Lamuela-Raventos, 1999). First, 0.250 mL aliquots of each solubilized extract at a concentration of 100 µg/mL were mixed with 1.25 mL Folin reagent (1:10 v/v) for 3 min, followed with 1.0 mL sodium carbonate (7.5%). Next, the mixture was incubated in the dark for 90 min, and its absorbance was measured using a spectrophotometer (BioSpectro SP-22) at 760 nm. Gallic acid was used as a standard, and the results were expressed as mg of gallic acid equivalent per g sample at concentrations ranging from 20–100 µg/mL. The calibration curve yielded the following equation: $y = 0.0109x - 0.0302$, with $r^2 = 0.9944$. All analyses were performed in triplicate. As a blank control,70% ethanol, Folin, and sodium carbonate were used as described above.

To determine the total flavonoid content, 2.5 mL aliquots of each solubilized extract at 500 µg/mL were mixed with 2.5 mL of 5% aluminum chloride. The samples were incubated in the dark for 30 min, and then the absorbance was measured at 420 nm using a spectrophotometer (BioSpectro SP-22). Interferences were also read (extract + ethanol) and subtracted by the first value obtained. Ethanol + aluminum chloride was used as a blank. The standard used was rutin, and the calibration curve yielded the following equation: $y = 0.0091x - 0.0033$, with $r^2 = 0.9983$. The results were expressed in mg equivalent rutin per g of sample (Woisky, Salantino, 1998).

DPPH radical-scavenging activity

The free radical-scavenging activity of the crude extracts was evaluated according to Sousa *et al*. (2007) and Lopes-Lutz *et al*. (2008), with small adaptations according to the structures and conditions of the research laboratory.

To initiate the tests, we prepared a hydroethanolic mixture of DPPH solution (40 µg/mL, i.e., the control) that was monitored at 517 nm. The mixture was then kept in the dark until analysis.

The extracts (samples) were diluted in 70% ethanol at different concentrations: 12, 25, 50, 75, and 100 **µg/mL. Subsequently, 2.7 mL** of the DPPH solution and 0.3 mL of each extract were pipetted into each concentration. As a blank control, we used 3 mL of solvent.

After 30 min, the preparations were read in a spectrophotometer (BioSpectro SP-22) at 517 nm. The assay was performed in triplicate, and the results were calculated to determine the percentage of free radical-scavenging activity (% FRS), which is matched using the DPPH radical consumption via the extracts, according to the following formula:

Abs control = (DPPH + ethanol 70);

Abs sample = (extract + DPPH solution)

The efficient concentration (EC_{50}) was also calculated using a linear regression after a chart was constructed, where the y-axis represented the % of the free radical consumption and the x-axis represented the different concentrations. This chart was used to obtain the line equation used for calculating the EC_{50}.

In this equation, the value of y was replaced with 50, (a and b) were replaced with the values of (intercept a) and (regression coefficient b), and x was the value of EC_{50}. This calculation determined the concentration value that decreased the amount of DPPH radical by 50%. The lower the EC_{50} value, the greater the consumption of free radicals and possibly the greater the antioxidant potential.

Phosphomolybdenum complex reduction

We prepared an 100 mL solution containing 0.1 mol/L sodium phosphate (28 mL), 3 mol/L sulfuric acid (20 mL), and 0.03 mol/L ammonium molybdate (12 mL). Next, 0.3 mL aliquots of each extract and 2.7 mL of the phosphomolybdenum complex solution were pipetted into falcon tubes. Then, the flasks were capped and incubated in a water bath (95°C) for 90 min. After cooling, the sample was subjected to spectrophotometry at 695 nm. For a blank control, we used 0.3 mL 70% ethanol and 2.7 mL of the phosphomolybdenum solution. All assays were performed in triplicate for each month analyzed (Prieto, Pineda, Aguilar, 1999).

The extracts were evaluated at a fixed concentration of 120 µg/mL and compared with the same concentration and absorbance of the standard ascorbic acid, also at 120 µg/mL, which represented 100% antioxidant activity. The results were expressed as % reduction of the phosphomolybdenum complex (y-axis) vs analyzed month (x-axis) and plotted (Merino *et al*., 2015).

Iron-reducing power

In this test, 2.5 mL aliquots of the extracts at 2,000 µg/mL were mixed wtih 0.2 M sodium phosphate buffer (pH 6.6), and a mixture of 1% potassium ferricyanide was added. Next, the solution was incubated for 20 min at 50 **°C, and** then 2.5 mL of 10% TCA was added to stop the reaction. Afterward, the mixture was centrifuged at 1,000 rpm for 10 min. The supernatant (2.5 mL) was mixed with 2.5 mL of distilled water and 0.5 mL of 0.1% ferric chloride, and the absorbance was measured at 700 nm. We used 2,000 µg/mL ascorbic acid as a standard to compare and assess the iron-reducing power of each sample for each month evaluated (Oyaizu, 1986; Dorman *et al*., 2003).

Pluviometric data

To evaluate the seasonal effect on the pluviometric index, data on precipitation (mm) were collected from the National Weather Station (INMET) website, which use data from automatic stations, located in Macapa and Itaubal, that are close to the sample collection site.

According to the Köppen-Geiger climate classification, the climate in the Amazon—especially in Amapá—varies little in terms of temperature, light, and altitude, but has no variations in seasons (Vilhena, Silva, Freitas, 2018).

In this context, we evaluated the environmental factor that oscillates the most in the state, namely precipitation, with emphasis on two seasonal periods: the rainy and less-rainy seasons. For this research, seasons were divided into two periods: the rainy period (Rp), February 2018 to July 2018; and less-rainy period (Lrp), August 2018 to January 2019. The literature describes the period from May to August as a transitional period between the Rp and Lrp (Amanajas, Braga, 2012).

Statistical analysis

For statistical analysis, the GraphPad Prism 5 software and Bioestat 5.0 were used. A graph was constructed. The means, standard deviations (SD), and linear regressions were calculated. Analysis of variance (ANOVA) followed with Tukey's test was performed to compare the months and the means of the results beyond the Pearson correlation. $P < 0.05$ was considered to indicate statistical significance.

For Pearson correlation analysis, a comparison scale was used to determine the degree of correlation: weak correlation, $(r) < 0.30$; moderate correlation, $0.30 > (r) < 0.60$; and strong correlation, $(r) > 0.60$.

RESULTS AND DISCUSSION

Plants from the same genus and family as LmB have many uses in folk medicine, such as the treatment of malaria, epilepsy, diarrhea, and diabetes. The main isolated molecules from these plants are flavonoids, which have prompted research aimed at investigating these phenolic compounds and their biological/pharmacological activities, such as antioxidant properties (Neto *et al*., 2013).

Medeiros and Medeiros (2012) reported the isolation of the flavonoids 4′-O-methyl-epigallocatechin-3′-O-α-L-rhamnoside and 4′-O-methyl-epigallocatechin in crude extracts from LmB. This finding suggests the antioxidant

potential of this species due to the presence of aromatic compounds, which may include other simple phenolic compounds and flavonoids.

According to tests performed with larvae of *Artemia salina,* ethanolic extracts from LmB stem bark are not toxic as they display a lethal dose (LD) 50% of 1.250 µg/mL. However, phytochemical screenings performed with this same extract showed positive results for phenolics, tannins, organic acids, reducing sugars, saponins, and anthraquinones (Ramos, Rodrigues, Almeida, 2014).

Other studies performed with ethanolic extracts from LmB stem bark showed positive results against infection via *Staphylococcus aureus* using a disk-diffusion assay (Correa *et al*., 2008). In a previous study, LmB showed a lower minimum inhibitory concentration (MIC) of 20 µg/mL when compared to 32 µg/mL from *Licania tomentosa*, for the same test model (Silva *et al*., 2012).

Phenolic compounds are widely distributed in the plant kingdom and are present in all parts of the plant. These compounds exhibit redox properties against reactive oxygen species (i.e., free radicals). The production of these chemicals may be affected owing to several factors, such as the genetic variability of the plant or other extrinsic factors, e.g., environmental factors (Gobbo-Neto, Lopes, 2007).

Studies on the phenolic composition of plants of the Chrysobalanacea family are limited. However, some phytochemical studies have shown the presence of flavonoids and tannins in the stem bark of LmB (hydroethanolic extracts) (Gomes *et al*., 2006). In addition to chlorogenic acid, anthocyanins, kaempferol and many other flavonoid derivatives in other species of the genus *Licania* (de Freitas *et al*., 2019; Moreira-Araujo *et al*., 2019; Santos *et al*., 2019). Table I shows the total phenolic and flavonoid content of LmB.

As described in Table II, we measured the antioxidant potential, DPPH radical-scavenging activity, phosphomolybdenum complex reduction, and iron-reducing power over a period of 1 year.

Antioxidant substances can prevent or minimize the action of free radicals on cells by preventing diseases from developing and collaborating with the antioxidant defense system to prevent oxidative stress (Martelli, Nunes, 2014). Free radicals and other oxidizing agents are closely related to cardiovascular diseases, cataracts, diabetes mellitus type 1, and many forms of cancer (Sousa *et al*., 2007). In this sense, a diet containing anauerá extract could complement the antioxidant defense system exogenously by preventing and reducing oxidative stressors associated with the development of chronic diseases (Pereira, Cardoso, 2012). However, more research should be conducted to ensure the use of this natural product.

The DPPH free radical-scavenging assay evaluates whether an extract donates hydrogens or electrons to the radical, thereby stabilizing it via reduction. This method allows different interpretations owing to the various analytical procedures (Oliveira, 2015).

TABLE I - Precipitation values and phenolic content of extracts from LmB stem bark that were collected in a rural area of Porto Grande, Amapá, Brazil. Values are expressed as average and SD

Months	Rainfall	Total phenolic content	Total flavonoid content
Rainy period	**mm**	**mg/g**	**mg/g**
February	220	49.4 ± 0.56^{a}	19.84 ± 0.56^{a}
March	280	52.27 ± 0.43^{b}	19.26 ± 0.44^{a}
April	615	60.40 ± 0.30^{c}	22.33 ± 0.73^{b}
May	500	47.57 ± 0.50^{a}	19.33 ± 0.48^{a}
June	220	70.23 ± 0.21^{d}	12.52 ± 0.47^{c}

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Months	Rainfall	Total phenolic content	Total flavonoid content
Rainy period	**mm**	**mg/g**	**mg/g**
July	110	49.50 ± 0.05^{a}	24.06 ± 0.39^{b}
Less-rainy period	**mm**	**mg/g**	**mg/g**
August	75	49.57 ± 0.17^{a}	18.41 ± 0.56^{a}
September	195	55.77 ± 0.88^{b}	26.62 ± 0.24^{d}
October	50	53.93 ± 0.51^{b}	26.68 ± 1.87^{e}
November	180	47.90 ± 1.80^{a}	11.86 ± 1.06^{c}
December	200	47.70 ± 0.20^{a}	11.64 ± 0.57^{c}
January	210	$43.23 \pm 0.3^{l}e$	14.64 ± 0.54^{c}

Values are expressed as the mean ± SD from three experiments. Different lowercase letters in the same column correspond to significant differences, as assessed using ANOVA followed with Tukey's test ($p < 0.05$). Phenol and flavonoid content is expressed as mg of gallic acid, rutin equivalent to g of extract.

TABLE II - Antioxidant activity of extracts from LmB stem bark that were collected in a rural area of Porto Grande, Amap**á**, Brazil. Values are expressed as the mean ± SD

Months	DPPH (EC_{50})	Phosphomolybdenum	FRAP
Rainy period	**µg/mL**	**%**	**OD**
February	71.30 ± 0.71^{a}	23.32 ± 0.63^{b}	0.041 ± 0.0005^{a}
March	82.05 ± 1.28^{b}	54.15 ± 2.37^{d}	0.031 ± 0.0005^{b}
April	65.63 ± 0.36^{d}	40.57 ± 2.91^{a}	0.042 ± 0.001^{a}
May	69.46 ± 0.59^{a}	43.28 ± 0.23^{a}	0.039 ± 0.001^{a}
June	76.68 ± 0.32^{c}	30.82 ± 0.17^{c}	0.054 ± 0.001^{c}
July	75.20 ± 1.33^{c}	47.13 ± 0.21^{a}	0.032 ± 0.0005^{b}
Less-rainy period	**µg/mL**	**%**	**OD**
August	68.74 ± 1.27^{a}	33.02 ± 0.77^{c}	0.049 ± 0.001^{d}
September	78.83 ± 0.27^{c}	43.12 ± 2.62^{a}	0.031 ± 0.003^{b}
October	77.31 ± 0.34^{c}	43.15 ± 0.85^{a}	0.033 ± 0.0005^{b}
November	77.72 ± 0.33^{c}	64.44 ± 3.36^{e}	0.045 ± 0.0005^{a}
December	84.56 ± 1.04^{b}	39.65 ± 1.39^{a}	0.035 ± 0.0005^{b}
January	96.02 ± 1.77^{e}	42.93 ± 2.34^{a}	0.033 ± 0.001^{b}
Quercetin	5.57 ± 0.41	-	-
Ascorbic acid	-	100	0.521 ± 10.25

Values are expressed as the mean ± SD from three experiments. Different lowercase letters in the same column correspond to significant differences, as assessed using ANOVA followed with Tukey's test ($p < 0.05$). µg/mL, concentration; %, reduction percentage of phosphomolybdenum; OD, optical density or absorbance.

Total flavonoid and phenolic content

Figure 1 shows the results of the total phenolic compounds in the crude extracts from LmB collected over throughout the year. Specifically, we observed that the extract from LmB collected in June had the highest rate of phenols (70.23 ± 0.21 mg/g), whereas the extract collected in January had the lowest rate (43.23 ± 0.31 mg/g). Analysis of the extract collected during the Rp and Lrp revealed a significant difference in total phenolic content ($p = 0.024$). Extracts collected during the Rp presented higher averages of phenolic compounds, possibly owing to the accumulation of rainfall during this period (1,945 mm).

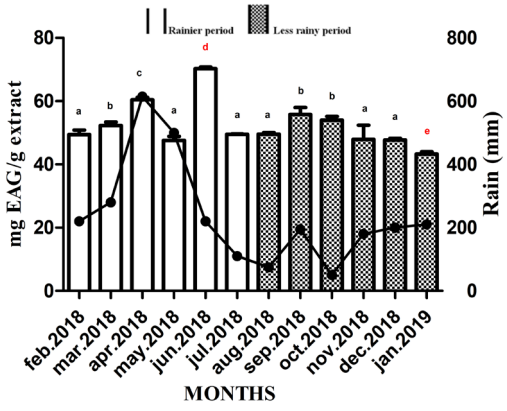

FIGURE 1 - Seasonal variation of the total phenolic content of LmB. Each value in the column chart is the average of three replicates ± SD. Different lowercase letters correspond to significant differences in concentration, as assessed using Tukey's test ($p < 0.05$). The line graph represents precipitation, expressed in mm.

The total polyphenols can provide numerous beneficial effects against chronic diseases in humans; thus, the discovery of the month or period with the highest rates of these compounds becomes important. However, high concentrations of phenolic substances may also be linked to some infectious processes in plants (Ko *et al*., 2018). The ecological aspects of the stresses caused by phytopathogens in plants represent another valuable point concerning the chemical set present in plants.

Extracts from LmB collected during September and October showed the highest levels of total flavonoids (26.62 ± 0.24 mg/g and 26.68 ± 1.87 mg/g, respectively), but the levels subsequently decreased considerably (Figure 2). Collectively, extracts from LmB collected during February, March, and April showed an increase in the levels of these compounds compared with the lowest levels observed at the end of the Lrp and in June. There was no difference in the levels between extracts from LmB collected at the two periods ($p = 0.487$).

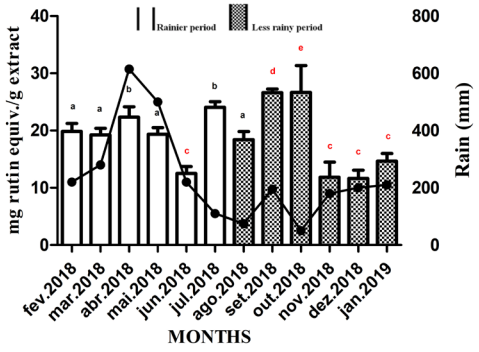

FIGURE 2 - Seasonal variation of total flavonoid content of LmB. Each value in the column chart is the average of three replicates ± SD. Different lowercase letters correspond to significant differences in concentration, as assessed using Tukey's test ($p < 0.05$). The line graph represents precipitation, expressed in mm.

The pharmacological (gastroprotective) actions of extracts from LmB stem bark may be associated with phenolic compounds, mainly flavonoids (Sales *et al*., 2019). In this sense, a collection guideline based on seasonal data of the phenolic and antioxidant profile of this plant may improve the effectiveness of this extract as a herbal medicine.

From an ecological point of view, flavonoids are responsible for the protection of plants against solar radiation (Simões *et al*., 2010). This can corroborate our results as the maximum production of flavonoids occurred in September and October (months with the least amount of rain), when air humidity levels were low and the temperature was mildly increased.

The production of compounds of different subclasses but similar to flavonoids, such as flavones and anthocyanins, may be altered (concomitantly) by the effect of associated or isolated factors (Yao *et al*., 2005). Environmental factors can affect certain substances but not others simultaneously, thereby culminating in fluctuations of the chemical compositions over months or seasons (Lavola *et al*., 2003; Araujo *et al*., 2015).

In this study, as the total flavonoid content was investigated, it may be possible that a given class was affected by the low amounts of precipitation, resulting in different concentrations throughout the year (Figure 2).

As an example to support this statement, grapes cultivated under direct sunlight had high concentrations of flavonoids and low concentration of anthocyanins (Spayd, Tarara, Mee, 2002), which indicates that both temperature and solar radiation affect phenolic and flavonoid compositions (Mori *et al*., 2007). Studies have shown that low amounts of precipitation and increased irradiation may contribute to increased flavonoid production (Dalmagro *et al*., 2018). This phenomenon occurred in October, thereby corroborating our results on flavonoid content.

DPPH radical-scavenging activity

All samples showed DPPH radical-scavenging activity and were effective in scavenging 50% of the radical. Extracts from LmB collected in April showed the highest activity (65.63 ± 0.36 µg/mL), whereas those collected in January were the least effective (96.02 ± 1.77 µg/mL; Figure 3). There was a significant difference ($p = 0.0056$) in activity between the periods, with Rp-collected samples showing better EC_{50} values. Moreover, there was a subtle increase in the EC_{50} value at the end of the Rp until the end of the Lrp.

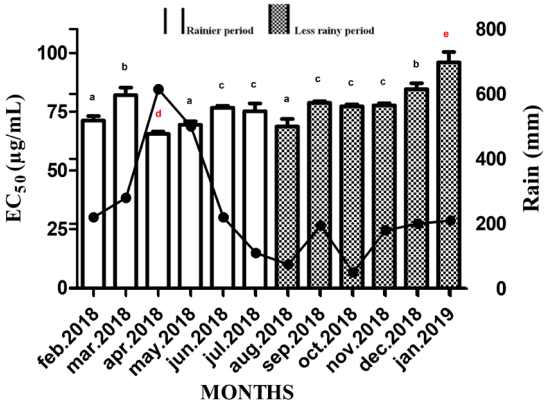

FIGURE 3 - Seasonal variation of the EC_{50} value (µg/mL) of LmB. Each value in the column chart is the average of three replicates ± SD. Different lowercase letters correspond to significant differences in EC_{50} value, as assessed using Tukey's test ($p < 0.05$). Quercetin ($EC_{50} = 5.57 \pm 0.41$ µg/mL) was used as a control. The line graph represents precipitation, expressed in mm.

When compared to the hydroethanolic extracts isolated from the leaves of *L. tomentosa*, LmB presented with better EC_{50} values during April, May and August, i.e., mainly during the Rp (Silva *et al*. 2012). These data corroborate our hypothesis that the seasonal profile correlates with the maximum antioxidant potential of these extracts, thereby helping to find appropriate times to harvest these plants and contribute to species conservation (Botha *et al*. 2018).

Table III shows the antioxidant activity of LmB hydroethanolic extracts at different concentrations over a period of 1 year. All samples showed moderate to strong capacity to scavenge DPPH radicals, mainly at a concentration of 100 µg/mL. In addition, when we evaluated the concentrations of these radicals from each month, we observed that there was a statistical difference between all concentrations (12–100 µg/mL). In the individual analysis of the concentrations tested for DPPH sequestration, LmB extracts showed better radical consumption percentages in most samples, especially when compared with leaf extracts from *L. tomentosa* (Silva *et al*. 2012).

Comparing all the evaluated periods revealed that the extract did not present a better EC_{50} (Figure 03) than the control in any month; however, it is important to note that the 100 µg/mL extract-treated group showed higher DPPH-scavenging activity than the control in April. In another study, it was verified that at low concentrations, the extracts showed higher radical-scavenging activity than in the standard, but it did not present better EC_{50} values than the control (Falcão *et al*., 2006).

In comparison, 120 µg/mL extracts of the leaves of *Licania rigida* and *L. tomentosa* showed lower DPPH radical-scavenging activity than the LmB extract (Macêdo, 2011). These results showed the high antioxidant power of LmB, thereby supporting the use of anauerá as a nutraceutical candidate that can be used to treat oxidative disorders. According to the EC_{50} values, the LmB extract was more effective than seed extracts from *L. tomentosa* and *L. rigida* (Farias *et al*., 2013), which further corroborate previous findings.

In addition, between April and January, there were no differences in radical-scavenging activity among the different extract concentrations. Owing to the increased

concentrations, the LmB extracts that were collected in April captured more radicals, which surpassed the 100 µg/mL control (Figure 4). It is possible that the extracts from LmB exert their effect in a dose-dependent manner, especially as the radical-scavenging activity increased with increasing concentrations in all of the months.

TABLE III - Seasonal effect on the antioxidant activity of different concentrations of LmB extracts, as assessed by DPPH assay and expressed as % of free radical sequestration (% FRS)

µg/mL	Control	Feb 2018	Mar 2018	Apr 2018	May 2018	Jun 2018	Jul 2018
12	39.21 ± 0.79[a]	23 ± 1.26[a]	18.73 ± 0.59[a]	17.77 ± 0.31[a]	18.08 ± 0.70[a]	15.58 ± 0.72[a]	20.06 ± 0.78[a]
25	64.78 ± 0.68[b]	28.47 ± 0.48[b]	22.86 ± 0.36[b]	26.14 ± 1.35[b]	26.86 ± 0.35[b]	23.44 ± 0.47[b]	29.80 ± 2.60[b]
50	65.46 ± 0.21[b]	45.15 ± 0.56[c]	35.96 ± 0.92[c]	43.27 ± 1.54[c]	42.59 ± 0.92[c]	36.40 ± 1.26[c]	37.32 ± 1.26[c]
75	66.04 ± 0.10[c]	50.73 ± 0.92[d]	47.89 ± 0.31[d]	55.58 ± 1.34[d]	53.05 ± 0.05[d]	46.9 ± 0.82[d]	52.50 ± 0.61[d]
100	66.82 ± 0.21[d]	62.76 ± 0.54[e]	58.45 ± 0.89[e]	70.52 ± 0.21[e]	65.63 ± 0.89[e]	64.71 ± 0.27[e]	60.74 ± 0.3[e]
µg/mL	**Control**	**Aug 2018**	**Sep 2018**	**Oct 2018**	**Nov 2018**	**Dec 2018**	**Jan 2019**
12	39.21 ± 0.79[a]	23.78 ± 1.14[a]	21.80 ± 1.05[a]	22.49 ± 1.32[a]	16.30 ± 0.57[a]	20.06 ± 0.50[a]	18.83 ± 0.35[a]
25	64.78 ± 0.68[b]	29.22 ± 0.17[b]	24.98 ± 0.97[b]	28.64 ± 0.59[b]	24.13 ± 0.42[b]	25.05 ± 0.30[b]	25.84 ± 0.54[b]
50	65.46 ± 0.21[b]	44.30 ± 1.13[c]	27.38 ± 0.99[c]	40.68 ± 0.42[c]	40.16 ± 1.09[c]	37.05 ± 0.82[c]	35.28 ± 0.41[c]
75	66.04 ± 0.10[c]	50.97 ± 1.03[d]	51.54 ± 0.78[d]	50.52 ± 0.42[d]	51.72 ± 0.36[d]	44.57 ± 2.63[d]	43.10 ± 0.36[d]
100	66.82 ± 0.21[d]	65.63 ± 1.57[e]	62.62 ± 0.31[e]	58.62 ± 0.33[e]	58.45 ± 0.47[e]	57.74 ± 1.20[e]	51.21 ± 0.51[e]

Values are expressed as the mean ± SD from three experiments. Different letters in each column, for each month, correspond to significant differences based on calculations using ANOVA followed with Tukey's test ($p<0.05$). Quercetin was used as a control, and its concentrations were expressed as µg/mL.

LmB presented notable free radical-scavenging activity, especially during February, April, and May (Rp), suggesting that this time of year may be the best period to collect LmB. Results of the same method in another *Licania* species corroborated their antioxidant potential during the same time period (Pessoa *et al*., 2016), thereby adding value to their pharmacological attributes.

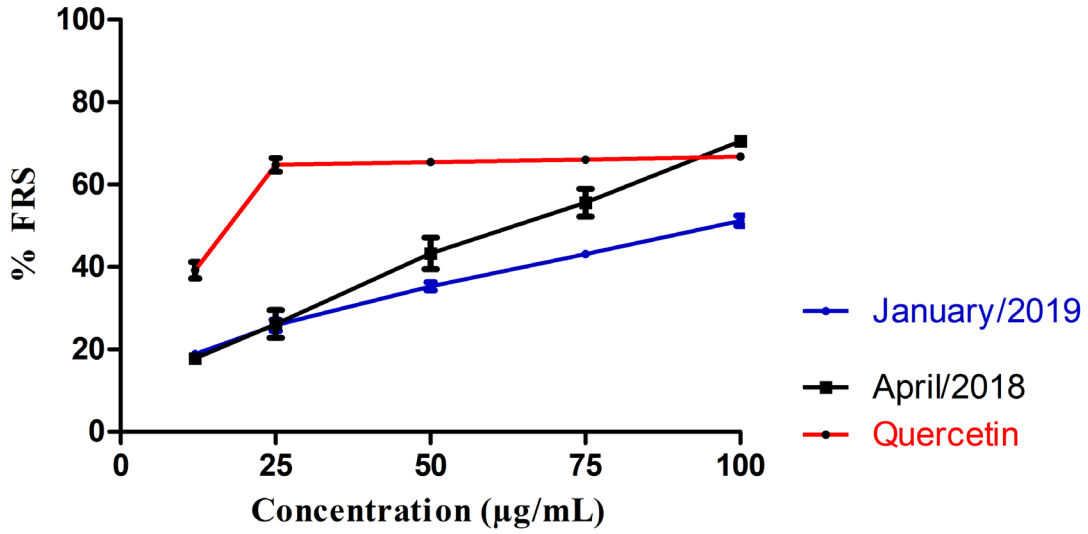

FIGURE 4 - Percentage of free radical sequestration for the months that presented higher and lower consumption of DPPH compared with the control. Each point on the graph represents the average of three replicates ± SD.

Phosphomolybdenum complex reduction

When investigating the percentage reduction of the phosphomolybdenum complex, the LmB extract collected in November (64.44 ± 3.36%) had the greatest phosphomolybdenum complex reduction, whereas samples collected in February (23.32 ± 0.63%) showed the lowest values (Figure 5). A clear oscillation was observed throughout the year, but there was no significant difference between the time periods ($p = 0.2242$).

The phosphomolybdenum method, a low-cost and uncomplicated procedure, evaluates the complex reduction capability of the extracts (complex mixtures) or isolated substances, which in turn represent their lipophilic and hydrophilic characteristics (Prieto, Pineda, Aguilar, 1999). However, studies showed that apolar fractions present better results than polar extractives (Merino *et al*., 2015). The plant used in this study showed relevant results in some months of the year (Figure 5), even when extracted with the polar hydroalcoholic method.

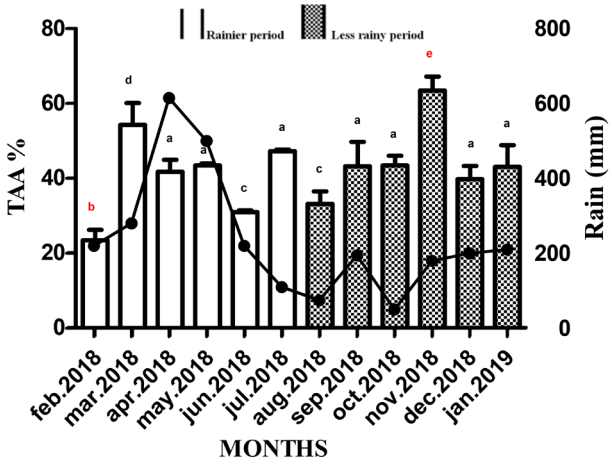

FIGURE 5 - Seasonal variation of % reduction of the phosphomolybdenum complex by LmB extract. Each value in the column chart is the average of three replicates ± SD. Different lowercase letters correspond to significant differences in percentage, as assessed using Tukey's test ($p < 0.05$). Ascorbic acid was used as a standard and represents 100% antioxidant activity. The line graph represents precipitation, expressed in mm.

Ferric-reducing power

Transition metals, such as iron, are atoms of relevant importance to the human body; however, depending on their oxidative state, they can damage cells and lead to pathological conditions. Oxidative stress is induced

via reactions between hydrogen peroxide and other substances, which produce radicals such as hydroxyl. This radical is considered one of the most reactive and dangerous (Mahomoodally *et al*., 2019).

By reacting with proteins, lipids, and other molecules, hydroxyls can trigger lipid peroxidation. In this context, the chelation and reduction of these metal ions can reduce their activity to decrease the production of radicals and to balance oxidative stress (Lobo *et al*., 2010).

Extracts from LmB collected in June presented the highest iron-reducing power (0.054 ± 0.001 OD), although fluctuations were predominant throughout the year. However, there were no differences between the evaluated periods (p = 0.396; Figure 6).

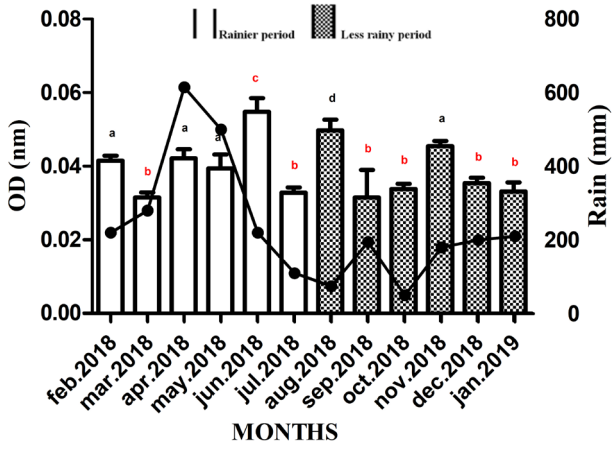

FIGURE 6 - Seasonal variation of the iron-reducing power of LmB. Each value in the column chart is the average of three replicates ± SD. Different lowercase letters correspond to significant differences in optical density, as assessed using Tukey's test ($p < 0.05$). The extracts were standardized in a single concentration (2,000 µg/mL). Ascorbic acid was used as a standard (0.521 nm). The line graph represents precipitation, expressed in mm.

Due to their high antioxidant potential, the hydroethanolic extracts from *L. macrophylla* can serve as candidates for pharmaceutical applications, as well as in the food and cosmetics industry for samples with a higher content of phenolic compounds. Moreover, this trend is observed in other species, such as the ethanol extracts from *L. tomentosa*, that contain high phenolic content and high antioxidant activity, as well as 12 compounds by UPLC, namely flavonoids (Lima de Medeiros *et al*., 2020). These results suggest a similar application for LmB extracts upon establishing its chemical profile, a process that benefits from our corroborating data of seasonal variations.

Table IV presents Pearson coefficients between amounts of precipitation and the antioxidant parameters tested in this study. Our data demonstrate a significant positive correlation between the amount of precipitation and DPPH radical-scavenging activity. Levels of phenols were also positively correlated with the level of rainfall; however, both presented a weak correlation.

TABLE IV - Pearson coefficients of the whole experimental period for LmB

L. macrophylla annual correlation	Total phenols	Total flavonoids	DPPH	Iron-reducing	Phosphom.	Rainfall
Total phenols	1					
Flavonoids	0.09	1				
DPPH	0.34*	-0.35*	1			
Iron-reducing	0.50*	-0.48*	0.46*	1		
Phosphom.	-0.28	-0.05	-0.26	-0.35*	1	
Rainfall	0.20	0.01	0.36*	0.07	0.03	1

*p values <0.05.

The total levels of phenols showed a significant positive correlation with DPPH-scavenging activity and iron-reducing power, unlike flavonoids, which displayed an inverse relationship. These observations corroborate the weak correlation between levels of phenols and flavonoids throughout the year. The DPPH-scavenging activity inversely correlated with iron-reducing power and molybdenum complex reduction; these results corroborated the differences and intrinsic similarities between the parameters.

The accumulation of secondary metabolites can be affected by several environmental factors, such as growth cycle, rain, temperature, herbivory, and parasitism. However, the effect of an isolated factor may not reveal or hinder its intervention in the chemical composition and antioxidant activity in the extracts, even with the environmental factor analyzed (Yao *et al*., 2016). Nevertheless, we found strengthening values according to the Pearson correlation (Table IV). The parameters that did not present a strong positive correlation with the antioxidant potential and the analyzed compounds were phenolic compounds and total flavonoids, suggesting the importance of investigating other metabolites that may be strongly linked to the antioxidant activities on a seasonal basis. Leitão *et al.* (2017) evaluated extracts and substances isolated from Mexican orange leaves, and found that alkaloids exhibited sufficient antioxidant capacity, despite not being hydroxylated aromatic compounds, such as flavonoids.

The antioxidant activity of all the extracts varied during the analyzed seasons, which corroborated previous findings on seasonal variation, interspersing months, or periods of time with lower and higher biological activity (Siatka, Kasparová, 2010; Araujo *et al*., 2015; Yao *et al*., 2016; Tálos-Nebehaj, Hofmann, Albert, 2017; Bhota *et al*., 2018; Dalmagro *et al*., 2018; Ribeiro *et al*., 2020).

These oscillations we observed may be linked to the antioxidant evaluation techniques used or to the detriment of plant metabolism caused by distinct mechanisms during the oxidation process. Thus, the amount of substances involved in this biological event may change throughout the year, but this may not necessarily apply for other compounds (Bulbovas, Rinaldi, Delitti, 2005; Araujo *et al*., 2015). From this perspective, antioxidant activity can be increased or decreased depending on the time of collection as well as environmental factors.

To investigate, extract, or isolate phytocompounds, a seasonal study is important to potentiate the specific synthesis of the metabolites, mainly on an industrial scale, to produce a new phytotherapeutic drug (Dalmagro *et al*., 2018). This assertive selection of the most appropriate period for sample collection will contribute to biodiversity preservation.

This study provided seasonal data for understanding changes in the total polyphenols and flavonoids in LmB extracts, favoring new research and experimental

designs regarding these compounds and their antioxidant properties (Ribeiro *et al*., 2020).

In this context, this research is relevant, as the identification of a time period when LmB presents its greatest antioxidant potential or optimal chemical composition (phenolic compounds). Determining the optimal time for harvesting LmB can help preserve this medicinal species and direct its phytopharmaceutical production. Moreover, this approach corroborates findings from other studies on the relevance of guided exploration of parts of this plant on a seasonal basis (Botha, Prinsloo, Deutschlander, 2018). The best collection period may be related to the method used, especially as one methodology may be more appropriate than another for measuring a pharmacological property of interest (Bujor *et al*., 2018). All procedures in this research were performed to measure antioxidant capacity. However, different characteristics were observed throughout the study (Table II, Figures 3–6), which contributed to the different "convenient" times of harvest according to the desired outcome.

In this study, we have demonstrated the novel antioxidant potential of hydroethanolic extracts isolated from LmB stem bark, a medicinal plant widely used in Amapá and within the Amazon.

In addition, as LmB is marketed as decoction tincture by the pharmacy of the IEPA. Due to its growing use, these results can subsidize and direct new research, such as further characterizing and identifying compounds found within LmB extracts using chromatographic tests, thereby contributing to the quality control and development of herbal medicines from this plant.

CONCLUSION

There was significant seasonal variation in the total level of phenolics and DPPH free radical-scavenging activity between the Rp and Lrp over the course of 1 year. This research showed a comprehensive *in vitro* spectrophotometric analysis of the antioxidant potential of extracts from LmB stem bark. These results showed that the hydroalcoholic extract from LmB exerted moderate to strong antioxidant potential, as well as weak ferric-reducing power, throughout the year. However, further research is needed to examine the antioxidant effect of anauerá extracts *in vivo*. In addition, the effect of more variables —such as soil composition, herbivore consumption, and pathogen attack — on the seasonal variation of phenolic compounds and antioxidant activity should be evaluated. Moreover, these extracts should be further characterized using chromatographic fingerprinting or other quantitative techniques. Collectively, this study provided previously unavailable data on the seasonal variation of an important medicinal plant in the Amazon rainforest, which will certainly be used as a basis for future studies.

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