

Relationship of biometric parameters on the concentration of tannins in two medicinal plants – a case study

[Relación de los parámetros biométricos sobre la concentración de taninos en dos plantas medicinales - un estudio de caso]

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Abstract

The Northeast region of Brazil has long been neglected because it mostly consists of semi-arid climate vegetation. However, this biome has an immense diversity, including various plants used for medicinal purposes. Two species widely used by local populations are *Myracrodruon urundeuva* Allemão and *Sideroxylon obtusifolium* (Humb. ex Roem. & Schult.) T.D. Penn. In order to identify parameters that could assist the pharmaceutical industry and local collectors in collecting samples with high yields of tannin, this study raised the following question: Do biometric parameters (diameter at breast height (DBH), bark thickness and height) relationship the concentrations of tannins in these species? The radial diffusion method was used to measure the tannin levels in all samples, and some were selected also measured using the method of Folin-Ciocalteu in order to compare results between methods. None of the biometric parameters evaluated showed any relationship on the concentration of tannins by either species. The radial diffusion method presented is safe and effective. Since it does not require sophisticated equipment and is inexpensive to implement, it is ideal for studies that use large numbers of samples.

Keywords: Caatinga; Semi-arid; seasonal dry forests; Bark extraction.

Resumen

La región nordeste de Brasil consiste fundamentalmente de una vegetación de clima semi-árido. Sin embargo, este bioma tiene una inmensa diversidad, incluyendo diversas plantas utilizadas para fines medicinales. Dos especies, ampliamente utilizadas por las poblaciones locales, son *Myracrodruon urundeuva* Allemão y *Sideroxylon obtusifolium* (Humb. ex Roem. & Schult.) TD Penn. Con el fin de analizar los parámetros que pueden ayudar a la industria farmacéutica y los coleccionistas locales en la recogida de muestras de corteza con una alta producción de tanino, este estudio plantea la cuestión siguiente: ¿los parámetros biométricos (diámetro a la altura del pecho (DAP), grosor de la corteza y la altura) presentan relación con las concentraciones de taninos en las especies? El método de difusión radial fue empleado para medir la concentración de taninos en todas las muestras, y algunas fueron seleccionadas también para análisis por el método de Folin-Ciocalteu a fin de comparar los resultados entre los métodos. Ninguno de los parámetros biométricos evaluados mostraron asociación con la concentración de taninos para cualquiera de las especies. El método de difusión radial se presenta seguro y efectivo.

Palabras Clave: Caatinga; semi-árido; bosques secos estacionales; extracción de corteza.

List of abbreviations: ASB – albumina sérica bovina; LPS – lipopolisacárido.

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INTRODUCTION

The Northeast region of Brazil has long been neglected because much of its area is covered by dry vegetation typically known as Caatinga. Currently, this important vegetation is a target of research aiming to evaluate the therapeutic use of plants used by local communities (Desmarchelier *et al.*, 1999; Desmarchelier & Barros, 2003; Viana *et al.*, 2003; Araújo *et al.*, 2008). Such research has changed the perception of this area, revealing great biodiversity with various plants used for medicinal and ritual purposes (Albuquerque & Andrade, 2002; Silva & Andrade, 2005; Almeida *et al.*, 2006; Albuquerque & Oliveira, 2007; Agra *et al.*, 2007; Albuquerque *et al.*, 2007a; Alviano *et al.*, 2008).

In the specific case of medicinal plants, the sampling by extraction and the increase in the marketing of products derived from these plants may result in the decline or near extinction of species of great therapeutic importance around the world (Melo *et al.*, 2009; Andel & Havinga, 2008). Accordingly, action is required to conserve the biodiversity of the Caatinga; it is under great pressure due to deforestation, irrational land use and extraction of medicinal species (Araújo *et al.*, 2007; Albuquerque *et al.*, 2009). Such practices have drastically reduced the population of plants such as *Schinopsis brasiliensis* Engl., *Myracrodruon urundeuva* Allemão and *Tabebuia aurea* (Silva Manso) S. Moore (Almeida *et al.*, 2005). *M. urundeuva* has been classified as vulnerable to extinction by the Brazilian Institute of the Environment and Renewable Natural Resources (IBAMA, 2008).

Among the several species of medicinal plants native to the Caatinga, two are widely used: *M. urundeuva* (Anacardiaceae), popularly known as "aroeira do sertão," and *Sideroxylon obtusifolium* (Humb. ex Roem. & Schult.) T.D. Penn. (Sapotaceae), also known by the common names "quixaba" and "rompe gibão". From the bark of the stems of these plants, extracts are prepared that are used primarily to treat

inflammation of the female genital tract (Desmarchelier *et al.*, 1999; Albuquerque *et al.*, 2007b). Furthermore, these species are widely distributed in the Northeast and have many medicinal uses, especially as anti-inflammatory. (Albuquerque *et al.*, 2007b; Santos *et al.*, 2008).

The biological activities of *M. urundeuva* are due in large part to the presence of tannins which are bactericidal, fungicidal, antiviral, anti-tumor and molluscicidal. Also help in the healing of wounds, burns and inflammation by forming a protective layer on the damaged skin or mucosa (Simões *et al.*, 2001). Souza *et al.* (2006) showed that the fraction rich in tannins from the crude extract of this species has anti-inflammatory and anti-ulcerogenic activities. Concerning *S. obtusifolium*, no works were found reporting which substances are responsible for its well-known therapeutic action (anti-inflammatory); however the antioxidant capacity of this species has already been proven (Desmarchelier *et al.*, 1999). Combining the popular use of *S. obtusifolium* and its antioxidant activity, tannins are believed to be the molecules responsible for part of its pharmacological actions.

The concentration of these secondary metabolites may be influenced by several factors such as seasonality, circadian rhythm and plant growth (Gobbo-Neto & Lopes, 2007). Several studies have examined the influence of seasonality (Monteiro *et al.*, 2006), hydric stress and plant part used (Chunlong *et al.*, 2008; Monteiro *et al.*, 2006) and biometric parameters (diameter and height of the trunk, for example) (Teixeira *et al.*, 1990; Caldeira *et al.*, 1998, Monteiro *et al.*, 2005) on the concentration of secondary metabolites in several species. In order to identify parameters that can direct the rational harvesting to collect samples with high yields of tannin, this study raised the following question: do biometric parameters (diameter at breast height (DBH), bark thickness and total height of the individual) relationship the concentrations of tannins in *M. urundeuva* and *S. obtusifolium*? The tannin levels were evaluated by the test of radial diffusion, a highly practical and appropriate

methodology for analysis of many samples (Hagerman, 1987). Additionally, results were verified by the widely-used Folin-Ciocalteu spectrophotometric method (Amorim *et al.*, 2008).

MATERIALS AND METHODS

Study site

Samples of the stem bark of *Myracrodruon urundeuva* Allemão and *Sideroxylon obtusifolium* (Humb. ex Roem. & Schult.) T.D. Penn. were collected in the municipality of Águas Belas, located in Agreste Pernambuco, 315 km from the city of Recife, over an area of approximately 885 Km². The semi-arid climate has an average annual temperature of 24.5°C and vegetation classified as Caatinga (seasonal dry forest) (CONDEP/FIDEM, 2008).

Botanical Material Sampling

Plant material was collected within 50 points-quadrant (Araujo & Ferraz, 2008), in an area of native vegetation, making a total area of 0.5 ha. All individuals of *M. urundeuva* (n=56) and *S. obtusifolium* (n=65) in the area were identified, georeferenced (by GPS), and their total height, bark thickness and diameter at breast height (DBH) were measured. Additionally, about 10 hours were spent walking in the area to locate and mark individuals of each species that were not sampled by the point-quadrant method. The bark thickness was obtained from four samples, at breast height (1.30 m), of each individual.

All individuals were processed by noting the date, biometric parameters, the collectors and the morphological characteristics in the field record for the specific location. Reference material was incorporated into the Herbarium of Prof. Vasconcelos Sobrinho (PEUFRP), Department of Biology, Universidade Federal Rural de Pernambuco.

Samples preparation

The total of 56 individuals of *M. urundeuva* and 65 *S. obtusifolium* were analysed (biometric parameters and tannins concentration). For the determination of tannins, the Radial Diffusion method was used (Hagerman, 1987), because it is fast, inexpensive and ideal when using a large number of samples. The Folin-Ciocalteu method was used with some samples of each species, totaling 12 samples of

M. urundeuva and 18 of *S. obtusifolium*. This method was used for comparison purposes.

All samples were pulverized and standardized by passing through a domestic sieve. For the radial diffusion assay, plant material was extracted for one hour at room temperature using methanol:water 50% (v/v) in a plant:solvent ratio of 50:1 and 100:1 (mg:ml) for *M. urundeuva* and *S. obtusifolium*, respectively. In the Folin-Ciocalteu test the solvent used was methanol:water 80% (v/v). The extract of *M. urundeuva* was prepared with a plant:solvent ratio of 100:50 (mg:mL) and that of *S. obtusifolium* was prepared with a ratio of 250:50 (mg:mL) in decoction for 30 minutes.

Determination of tannins by the method of radial diffusion

The radial diffusion method, as described by Hagerman (1987), was used. A solution of 50 mM acetic acid and 60 µM ascorbic acid was prepared, and the pH was adjusted to 5.0, the optimum pH for the tannin-protein interaction (Lopez *et al.*, 2004). The gel was prepared using 1% agarose (type I) (Sigma-Aldrich) in the solution previously described. The mixture was heated and stirred until boiling to complete homogenization of the agarose, and bovine serum albumin (BSA) fraction V free fatty acids (Sigma-Aldrich) were subsequently added after cooling to a temperature of 45°C. Aliquots of 10.0 mL of solution were distributed in Petri dishes 9.0 cm in diameter and left capped to solidify. Wells with capacity of approximately 8 µL were made using a punch 4 mm in diameter, 2.0 cm distant from each other and from the edges of the plates. Three successive aliquots of 8 µL of each extract were micropipetted into the wells. All samples were processed in triplicates to obtain the standard curve; an aqueous solution of tannic acid with a concentration of 25 mg/mL was prepared. Aliquots of 2, 4, 8, 12, 16 and 20 µl were placed in wells in triplicate, and the aliquots higher than the capacity of the well were added after fractionation.

The assay described is based on the observation that tannins form a stable complex with proteins, forming a clearly visible ring whose area is linearly proportional to the amount of tannin placed in the well. According to Hagerman (1987), to facilitate the calculations the squares of the diameters obtained are used instead of the areas. The plates were scanned, and the program Corel Draw© X3 Version 13 was

used to measure the diameter of the rings. Around each ring, a circular line was drawn, and two perpendicular diameters were recorded and used to calculate the average diameter of each ring. The data for the standard curve were plotted on a dispersion graph in order to obtain the line equation by linear regression using Microsoft Excel, version 2003.

Determination of tannins by the Folin-Ciocalteu method

The standard protocol of Amorim *et al.* (2008) was used. In this protocol, the levels of total and residual phenols are obtained, and the difference between them is the level of tannins contained in each plant species. Samples were processed in triplicate. To determine the total phenols, 1 mL of crude extract was transferred to a volumetric flask of 100 mL, where 5 mL of 10% Folin-Ciocalteu (v/v) and 10 mL of aqueous solution of sodium carbonate 7.5% (w/v) were added, and the volume completed with distilled water. After 30 minutes, the absorbance at 760 nm was measured in a spectrophotometer. In a flask of 25 mL 6 mL of crude extract, 10 mL of distilled water and 1 g of casein were mixed. The sample was stirred for 3 hours to promote the precipitation of tannins by binding proteins. After this period, the concentration was filtered through Whatman paper (9 mm) into a volumetric flask of 25 mL, adding distilled water to the filtrate to complete the volume. A 5 mL volume of this solution was transferred to the 100 mL volumetric flask and was treated as described for the determination of total phenols, thus resulting in the concentration of residual phenols. The readings of absorbance were replaced in the standard curve equation, calculated as follows: a 0.1 mg/mL solution of tannic acid was prepared, and five to seven increasing aliquots (0.5, 1.0, 1.5, 2.0, 2.5) were distributed in volumetric balloons of 100 mL that were processed in the same manner described for determination of total phenols. The readings were plotted on a dispersion graph and the equation obtained through linear regression.

Statistical analysis

The relationship between the Radial Diffusion Method (Hagerman, 1987) and the Folin-Ciocalteu method (Amorim *et al.*, 2008) was assessed by Pearson's correlation and by simple linear regression analysis using BioEstat 5.0 software (Ayres *et al.*,

2007). Data normality was tested using the Kolmogorov-Smirnov test. To assess the relationship of the parameters diameter at breast height (DBH), total height and bark thickness to tannin levels the Pearson correlation coefficient was used. A simple linear regression analysis ($p < 0.05$) was performed on the relationship obtained between the parameters DBH and bark thickness (two important variables to determine possible impact of the extractivist pressure). The samples were grouped into classes of diameter and bark thickness. For *M. urundeuva* the distribution of diameters was as follows: 3.00 - 4.99, 5.00 - 6.99, 7.00 - 8.99, 9.00 - 10.99 and 11.00 - 12.99 cm. In relation to bark thickness, the samples were grouped into three classes: 2.00 - 3.99, 4.00 - 5.99 and 6.00 - 7.99 mm (Fig. 1). Individuals of *S. obtusifolium* were included in the following classes of diameters: 2.00 - 3.99, 4.00 - 5.99, 6.00 - 7.99 and 8.00 - 9.99 cm. For the bark thickness classes, the distribution was: 0.00 - 1.99, 2.00 - 3.99 and 4.00 - 5.99 mm (Fig.1). The levels of tannins grouped in these classes were normally distributed for both species. Thus, the analysis of variance (ANOVA) one way test was used to highlight differences between the classes. All tests were performed by the software BioEstat 5.0 (Ayres *et al.*, 2007).

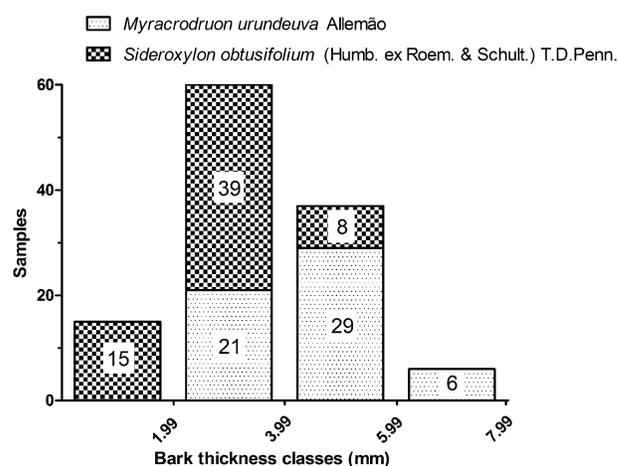


Figure 1. T Size classes of bark thickness of *Myracrodruon urundeuva* Allemão and *Sideroxylon obtusifolium* (Humb. ex Roem. & Schult.) T.D. Penn..

RESULTS

Relationship between methods

The results of the Radial Diffusion and Folin-Ciocalteu methods were linearly related, and the absolute levels of the assay Radial Diffusion are, in most cases, lower than the levels found by the Folin-Ciocalteu method (Fig. 2). That was not a surprise, since the methodologies are based on different chemical properties of tannins. In the radial diffusion assay, the tannins contained in the sample react with the protein contained in the gel, forming insoluble, stable complexes clearly visible to the naked eye, without the need for staining. This type of reaction can

be compared to that which occurs in the radial immune-diffusion tests, where the antibody contained in the sample interacts with the antigen previously incorporated into the gel, forming rings of insoluble precipitates (Hagerman, 1987). In the Folin-Ciocalteu assay, the phenol present in the sample, in an alkaline medium, react with the phosphotungstic and phosphomolybdic acids contained in the reagent Folin Ciocalteu, forming blue compounds that can be quantified by spectrophotometric reading (Amorim *et al.*, 2008).

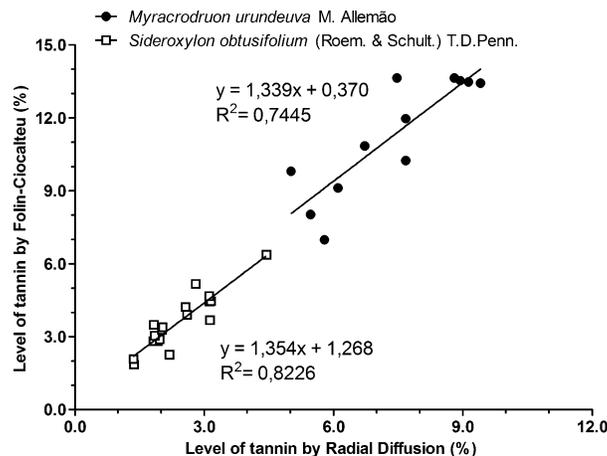


Figure 2. Relationship between tannin levels of *Myracrodruon urundeuva* Allemão and *Sideroxylon obtusifolium* (Humb. ex Roem. & Schult.) T.D. Penn. obtained by the Folin-Ciocalteu and Radial Diffusion methods.

Relationship between tannin levels and biometric parameters

For both species no relationship was found between the tannin levels and the biometric parameters evaluated, as can be seen in figures 3a-d and 4a-d. Chunlong *et al.* (2008) investigated the variation in the phenolic compound concentrations in *Populus euphratica* Oliv., a tree native to the desert areas of Inner Mongolia, in relation to the part of the plant and the amount of soil moisture. They observed that the concentration of phenolic compounds from the stem bark of different individuals with diameter at breast height ranging from 16.2 to 17.5 showed no statistically significant difference. However, a much smaller range of diameters was examined in that study than in our work.

M. urundeuva showed a statistically significant relationship between diameter at breast height and

bark thickness, although the correlation explained by this model was low ($R^2=0.1528$; $p<0.005$), presenting a Pearson correlation coefficient of 0.3966 (Figure 3d). *S. obtusifolium* presented a stronger relationship between DBH and bark thickness than *M. urundeuva* with $R^2=0.4938$ and a Pearson correlation coefficient of 0.7027 ($p<0.0001$) (Figure 4d). Williams *et al.* (2007) report that the bark thickness is always related to the diameter of the stem for two species used medicinally in South Africa, *Albizia adianthifolia* W. Wight and *Warburgia salutaris* (G.Bertol.) Chiov. This ratio generally increases with the size of the tree and decreases with the height of the stem.

There were no differences in tannin levels for different classes of diameter or of bark thickness for either species ($p > 0.05$). Teixeira *et al.* (1990) evaluated the change in the tannin concentration of the stem bark of *Stryphnodendron adstringens* (Mart.) Coville in relation to diameter. Individuals were

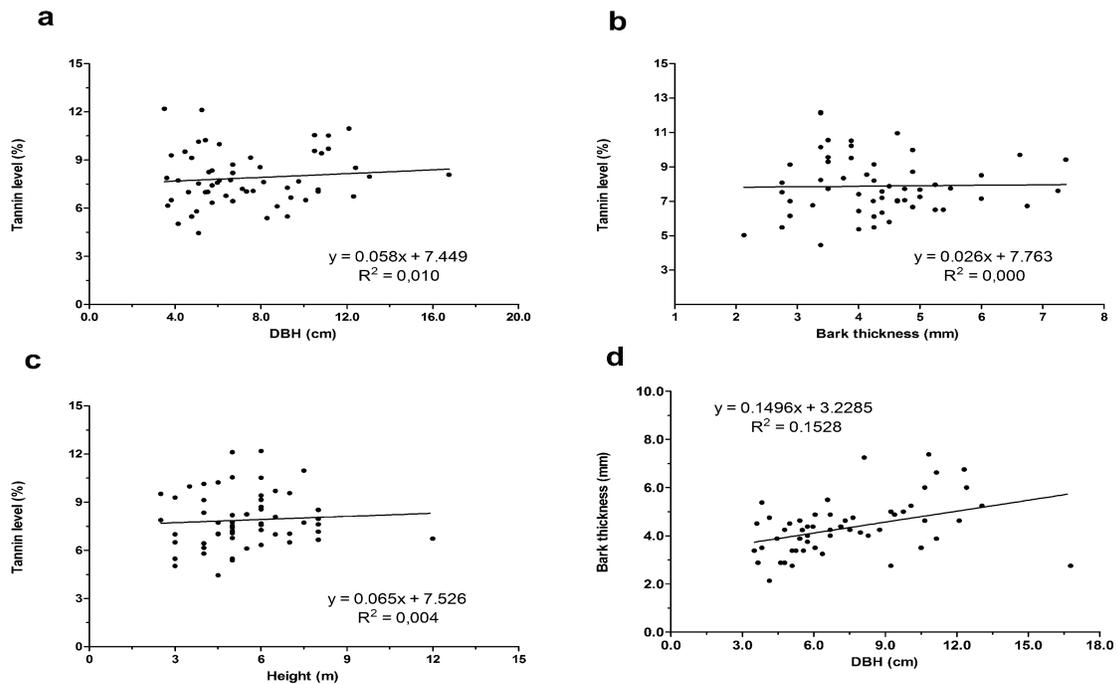


Figure 3. Graphical representation of the results for *Myracrodruon urundeuva* Allemão. (a) Relationship between tannin levels and diameter at breast high (DBH) (b) Relationship between tannin levels and bark thickness (c) Relationship between tannin levels and height (d) Relationship between the diameter at breast height (DBH) and bark thickness.

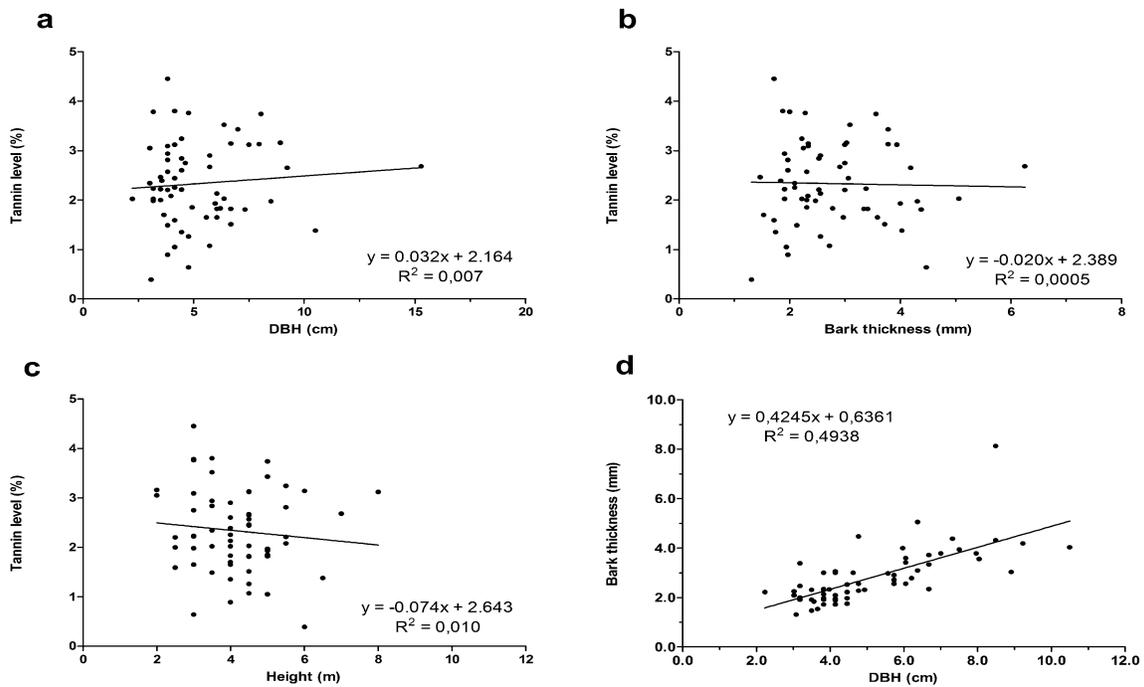


Figure 4. Graphical representation of the results for *Sideroxylon obtusifolium* (Humb. ex Roem. & Schult.) T.D. Penn (a) Relationship between tannin levels and diameter at breast high (DBH). (b) Relationship between tannin levels and bark thickness. (c) Relationship between tannin levels and height. (d) Relationship between the diameter at breast height (DBH) and bark thickness.

grouped into classes with stem diameters of 5-10 cm, 10-15 cm and 15-20 cm. In this study no evidence was found for the Relationship of diameter at breast height on tannin levels. However their sample presented several sources of variation as each class of sample consisted of ten individuals belonging to ten different places in Minas Gerais State. Silva & Frizzo (1985) determined the concentration of tannin in the bark of *Acacia mearnsii* de WILD. at different heights of the trunk from the roots up to the apex. Significant variations in tannin levels were observed along the stem of the species studied. In contrast, Caldeira et al. (1998), when studying the same species, found no difference in tannin levels along the stem. Monteiro et al. (2005) evaluated the tannin levels in samples of the stem bark and leaves of *M. urundeuva*, *Anadenanthera colubrina* (Vell.) Brenan and *Caesalpinia pyramidalis* Tul. The authors did not observed statistically significant relationships between the tannin levels of these species with the diameter at breast height and the total height of the individual, consistent with the results of this work. The tannin concentration in *M. urundeuva* found by Monteiro et al. (2005) ranged from 7.04% to 10.38%, which corroborates our finding of an average of 7.87% tannin concentration.

Implications for sustainable collection

The collection of the stem bark of trees is very damaging, because part of the vascular system of the plant is removed, causing a deficiency in crude sap conduction and reducing the intake of nutrients and water. When this type of extraction is light, the plant can recover the damaged tissue. But massive collection, which girdles the trunk, can kill the tree and may even cause it to fall because the sap can no longer reach the leaves. For this reason, since the biometric parameters did not Relationship the production of tannins in *M. urundeuva* and *S. obtusifolium*, it is proposed that the collection of the stem bark be performed only in individuals of greater size, or larger diameter at breast height, since they can better withstand the extractivist pressure. Moreover, as bark thickness did not explain higher tannin levels and plants with larger diameters tend to have thicker bark, they can offer higher amount of biomass with less damage to the plants.

CONCLUSIONS

The biometric parameters were not related to the production of tannins in the species evaluated. However, the DBH and the bark thickness are positively correlated, and this relationship is more pronounced for *S. obtusifolium*. For these reasons, the collection of barks of *M. urundeuva* and *S. obtusifolium* should be directed to individuals of greater size, which are better able to withstand extraction and offer greater amount of biomass and tannic compounds.

The methods of radial diffusion and Folin-Ciocalteu showed high correlation; however, the levels measured by radial diffusion are generally lower, because the tests are based on different chemical properties. Despite this, the radial diffusion method was very accessible and inexpensive and can be used for analysis of a large number of samples.

Studies such as this one are important to mitigate the impact of the extraction of plant resources, enabling the collection to be targeted in a sustainable manner, while still providing raw materials with high levels of tannins. However, we believe that is necessary to take studies in various environments, because multiple environments (locations, years, biomes, seasons, climates, soils, etc.) are needed to draw even robust conclusions that might provide broad application.

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