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Physical and chemical characteristics of pulverized material from the stem bark of *Ximenia americana* L. and determination of its chromatographic profile by high performance liquid chromatography (HPLC-DAD)

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Abstract
Ximenia americana L., commonly known as white plum, features saponins, flavonoids and tannins. Due to its use by the general population, studies to specific potential pharmacological activities demonstrated antioxidant and analgesic action which directs the need for standardization of the contents of those metabolites. The present study aimed to physicochemical characterization of the stem of the species, starting from methodological protocol which included literature review, polyphenols content, pH and analysis by High-Performance Liquid Chromatography (HPLC) using factorial design tool for determining the best chromatographic profile. Results include an average polyphenols content of 6.60 ± 0.156, and a 5.73 value for pH. HPLC analysis allowed the identification of the compound gallic acid. As conclusions, the authors note the need to use statistical tools for the development of reliable analytical methodologies for the identification of direct markers for species and contribute significantly to the future development of more complex analytical methods.

Keywords: *Ximenia americana* L., phytochemical, physicochemical characterization, HPLC-DAD, factorial planning

Introduction
Brazil has the greatest biodiversity in the world, estimated at about 20% of the total number of species on the planet, but only 10% of them have been evaluated with respect to their biological characteristics and only 5% with phytochemicals goals. It also has a huge cultural diversity and also a large repertoire of plants with potential economic and medicinal value, but that is still little explored (Araújo *et al*., 2007) [3]

A region with a rich diversity of plants is the Northeast, possessing habitats ranging from tropical forest, occurring in northern Maranhão, Mata Atlântica, mangroves and coastal dune systems, to dry forests and savannas (Andrade-Lima, 1981) [2]. The study of the traditional uses of plants and its products in this area have gradually increased in recent years (Agra *et al*., 2007). However, there are few plant populations of this biome studied. Due to this ignorance, it is estimated that very little of their benefits are in fact exploited by man, such as the *Ximenia* (*X*.) *americana* L. (Silva *et al*., 2008) [11].

Belonging to the Olacaceae family, wild plum (*X. americana*) can also be found in the regions of New Zealand, Africa, India, South and Central America (Sacande & Vautier, 2006) and is easily located in tropical areas whose altitude ranges between zero to 2000 meters, with a variation in average temperature between 14° to 30 °C with mean values for precipitation between 300 and 1250 mm / year (Maia, 2004).

Also popularly known as wild plum and plum form the woods, it makes up the so-called shrub-tree extract Caatinga biome, representing one of its main species. During the driest periodes of the year - a time when most of the species loses its leaves - this species is notable for presenting fully green leaves, characterizing it as a species resistant to drought. Its fruiting period is rather short, concentrated in the months from December to January (Silva *et al*., 2008) [11].

Its bark, reddish, flat, demonstrates various therapeutic activities and is used for various purposes, such as the treatment for malaria, leprosy, headache, skin infection hemorrhoids and treatment of mucosal inflammation (Brasileiro *et al*., 2008) [5].

Considering its use by the population, the existing scientific literature to date does not show identification studies and quantification by high-performance liquid chromatography (HPLC) of its main secondary metabolites, with only studies involving other fractions of the plant such as root, fruit and seeds (Luna, 2006) [8].

Accordingly, it is necessary the presence of safe and reproducible method for qualitative and quantitative analysis via HPLC of the main secondary compounds present in the bark of *Ximenia americana* L. species, guiding principle of this research.

Materials and Methods

Library Survey

Bibliographical survey was conducted targeting methodologies to identify chemical markers present in *Ximenia americana* L. by High Performance Liquid Chromatography methodology coupled with diode array detector (HPLC-DAD) in the databases online Natural Products Alert (NAPRALERT^{sm}, Chemical Abstracts, Web of Science, Virtual Health Library (Bireme) and Scientific Electronic Library Online (SciELO).

Obtaining and characterization Physical Chemistry

Obtaining and physicochemical characterization - consisting of the steps a) drying preparation and grinding of the material, b) determination of particle size distribution, c) determination of loss on drying and content of total ash, d) determination of moisture and ash content total thermal property, e) determination of extractable substances by alcohol, f) determination of pH, g) determining the content of total polyphenols, h) determining the foam index, i) determination of hemolytic activity and, j) survey methodology phytochemical - the kind of powder in question was performed using peels from the stem of *X. americana* L. collected in Serra Talhada-PE from December of 2013 and registered in the Herbário Dárdano de Andrade Lima do Instituto Pernambucano de Pesquisa Agropecuária (IPA 73349).

Research

The research was carried out on Laboratório de Tecnologia dos Medicamentos (LTM) from Universidade Federal de Pernambuco (UFPE), Campus Recife-PE and Central de Análise de Fármacos, Medicamentos e Alimentos (CAFMA) of Universidade Federal do Vale do São Francisco (UNIVASF), Campus Petrolina. Fresh material was then subjected to drying process in an oven with circulating air temperature of approximately

35 ºC for seven days followed by trituration process forage and standardization in a Wiley mill using 20 mesh knitting.

Particle size distribution

To determine the particle size distribution was held in triplicate analysis using sieves of

850, 425, 250 and 150□m, as recommended by the Brazilian Pharmacopoeia 5th Edition (2010). The loss on drying by gravimetric method and total ash, also according to the criteria of the Brazilian Pharmacopoeia (2010), was performed in triplicate. Calibration of the instrument was checked prior to testing and employing a standard calcium oxalate monohydrate, according to The American Society for Testing and Materials (Begley & Landes, 1972) [6].

Determination of loss by drying and total ash

The loss by drying by the gravimetric method, and total ash was performed in triplicate, as described in the Brazilian Pharmacopoeia 5th Edition (2010).

Determination of levels of total moisture and ash by thermogravimetry

The study of the thermal properties of the powder of *X. americana* was conducted by thermogravimetry (TG), derivative thermogravimetry (DTG) and differential thermal analysis (DTA). The TG / DTG curves / DTA were obtained in the temperature range between 25 and 800 °C using thermo balance Shimadzu® model DTG 60, under dynamic air atmosphere (50 mL min^{-1}), heating rate of 10 °C min^{-1} using platinum crucible containing sample mass around 20 mg. Calibration of the instrument was checked prior to testing and employing a standard calcium oxalate monohydrate, according to The American Society for Testing and Materials (1993), Silva (2010) and Araujo *et al*., 2006.

Determination of extractable substance by alcohol

Determination of substances extractable by alcohol followed the recommendation of the Brazilian Pharmacopoeia by the hot extraction method performed in duplicate. The method consists in weighing in a 250 mL Erlenmeyer flask (polished mouth) about 4, 0 g of dried plant drug, finely pulverized. It was added about 100 mL of water and weighed to obtain a total weight, including the bottle. Glassware was stirred vigorously and allowed to stand for 1 hour. Glassware was coupled to a reflux condenser and heated for 1 hour, followed by cooling and weighing. After the reflux, the corrected original weight solvent specified in the test for plant drug. After homogenization and filtration (dry filter), transferred 25 mL of the filtrate into a tared porcelain crucible in water bath at 105 °C, followed by evaporation to complete drying for 6 hours. After this process, the sample was placed in a desiccator for 30 minutes and then weighed immediately. Finally, we calculated the percentage materials extracted in mg / g dry material.

Determination of pH

The pH determination was performed in triplicate using a pHmeter MS Tecnopon® Instrumentation.

Determination of total polyphenols content

For determining the content of total polyphenol, an aliquot of 3 ml from the *X. americana* extract at a concentration of 240 ug / ml was transferred to 50 ml volumetric flask of glass and then was added 2 ml of the reactive Folin Ciocalteau. After 2 minutes, there was added 10 ml of sodium carbonate solution 15% (w / v) and the volume was completed with distilled water. The solution was allowed to stand for thirty minutes before being analyzed in a spectrophotometer at a wavelength of 760 nm. The clearing solution was prepared in the same manner, in the absence of the sample aliquot. The maximum reading was previously determined from scanning spectrum in the range 200 to 900 nm.

Determination of the foam index

The determination of the foam index was performed using 1 g of powdered plant material, which was transferred to an Erlenmeyer flask containing 50 mL of boiling water. The sample was then cooled and filtered into a volumetric flask supplementing the volume to 100 mL. The decoct obtained was distributed in 10 vials (with cap) in order to form a series

increasing volumes (1 to 10 mL) of decoct supplementing up to a final volume of 10 mL (with deionized water). The tubes were then vigorously shaken in the vertical direction and up-down directions, up and down for 15 seconds followed by resting for 15 minutes to check the formed foam level.

Determination of hemolytic activity

For determination of hemolytic activity, sodium citrate was added (3,65% w / v) to a container with cover up to 10% of its capacity. Stir the flask and then added to fresh bovine blood, followed by further stirring and storage in a refrigerator at a temperature between 2 and 4 °C. Then it was diluted in a volumetric flask of 50 mL, 1 mL of blood with citrate in a quantity of phosphate buffer (pH 7,4) sufficient for a final volume of 50 mL. For the reference solution, 10mg of saponin transferred into a 100 mL volumetric flask and completed to volume with phosphate buffer. They were then carried out a) preliminary test (8 tubes) and b) main test (26 tubes).

Phytochemical screening

For the method for phytochemical screening, aliquots of 10 microliters were subjected to thin layer chromatography (TLC), using silica gel plates and cellulose. The presence of these secondary metabolites was verified: a) alkaloids (mobile phase: ethyl acetate - formic acid - acetic acid – water; Dragendorff as revealing and pilocarpine as standard); b) monoterpenes and sesquiterpenes (toluene-ethyl acetate as mobile phase, sulfuric vanillin as revealing and thymol as standard); c) triterpenes and steroids (mobile phase: toluene-ethyl acetate; Lieberman Buchard and beta-sitosterol as revealing and ursolic acid as standard); d) flavonoids, phenylpropanoid glycosides and cinnamic derivatives (mobile phase: ethyl acetate-acetic acid-water-formic acid; diethanolamine ester from diphenylboric acid as reveling and quercetin as standard); e) coumarins (mobile phase: toluene-ethyl etial; potassium hydroxide as reveling and umbelliferone as standard); f) leucoanthocyanidins and proanthocyanidins (mobile phase: ethyl acetate-acetic acidwater-formic acid; hydrochloric vanillin as reveling and epicatechin as standard); g) hydrolysable tannins (mobile phase: etial acetate-acetic acid-water-formic acid; diethanolamine ester from diphenylboric acid as reveling and dietanolamina acid and gallic acid as standard) and; h) saponosídeos through afrogenycit test (Petri dishes containing 12 mL of methanolic extract of *X. americana* L. were heated for removing the solvent followed by dilution with distilled water and transferred to test tubes and subsequent vigorous hand shaking and subsequent standing by approximately 2 hours).

Determination of Chromatographic profile by High Performance Liquid Chromatography (HPLC-DAD) and quantification of commercially available markers in bark of *Ximenia americana* L. species

The analyzes were performed using a Shimadzu liquid chromatograph (LC-20 AT) equipped with an autosampler (SIL-20 A) and a DAD diode array detector (SPDM20A) controlled by the LC-Solution 1.0 software, Shimadzu.

The initial approach begins with a gradient system of type A: B being in the ratio 25:75 B The methanol and water over 60 minutes at a flow 0,6□L / min in order to determine the overall chromatographic profile of the samples. Thereafter factorial design was carried out in 02 levels (n = 3) and the variables involved values for: a) sample injection flow (in □L / min) - 0.6 (-) 0.8 and (+); b) temperature (in °C) oven - 25 (-) 30 and (+) and; c) time analysis (in min) - 60 (-) 80 and (+) – as show in Table 1 for the development of a specific methodology. After conducting all experiments, it was determined the most value for Hierarchical Response Function Chromatographic (HCRF), based on the equation shown in Figure 1 below:

Table 1: Planning matrix for determination of Best chromatographic method for *Ximenia americana* L. specie.

Experiment	Flow (□L/min)	Temperature (ºC)	Analysis time (min)
1	0,6	25	60
2	0,8	25	60
3	0,6	30	60
4	0,8	30	60
5	0,6	25	80
6	0,8	25	80
7	0,6	30	80
8	0,8	30	80

$$HCRF = 1.000.000n + 100.000R_{min} + (t_m - t_l)$$

Fig 1: Hierarchical Chromatographic Response (HCRF) equation.

Where n corresponds to number of peaks in the chromatogram, R_{min} to the resolution of the latter to separate peaks and t_m, and t_l correspond to the maximum acceptable value for analysis time and holding time for the last peak chromatogram respectively (Ji *et al*. 2005) [7].

After determining the best method, it was used in new chromatographic analysis which analyzed commercially available markers and isolated in the laboratory aiming the determination and quantification of these secondary metabolites in the sample of assessed species. The values for the sample retention times were compared to the values obtained for the standards considering an absorbance band equal to 270nm, as previously performed to study the species *Ximenia caffra* (Zhen *et al*., 2015) [12,].

Results

Particle size distribution

As show in Figure 2 below, 55, 44% of the particles were found in the sieve with holes 425□m. Most of the plant was found in 300 to 500□m tracks (there were no particles with sizes above 1000□m).

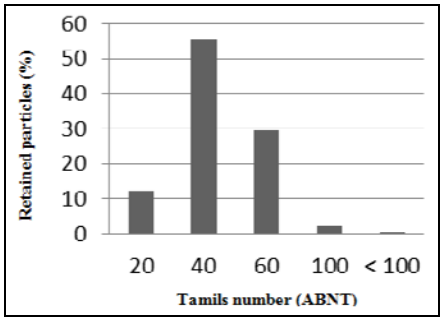

Fig 2: Particle size distribution from *Ximenia americana* L. powder.

Moisture content

The value found was equal to 9,17 ± 0,02. For the ash contentthe value was equal to 3,66 ± 0,09.

Thermal Characterization

TG DTG curves showed a loss of surface water temperatures of between 50 and 150 °C (Δm value of 9,8%). The DTG

curve also revealed the occurrence of four different thermal events related to weight loss (Δm). The occurrence of the first two between 247 and 303 ºC (Δm value of 22%) and between 332 and 354 ºC (Δm value of 14%). The 3th event occurred in the temperature range between 490 and 505 ºC, involving weight loss of 2.8%, resulting in formation of carbonaceous material. The last event (Δm equals 1,8%) corresponded to the burning material formed in the previous step. At a temperature exceeding 600 °C, the ash content was representative of the minerals and impurities present in the sample. The characteristic curve can be seen in Figure 3 below.

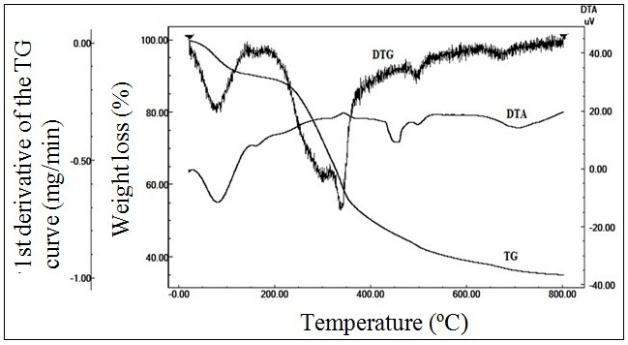

Fig 3: TG/DTG/DTA curves from *X. americana* L. powder obtained with 10 ºC.min^{-1} heating rate and dynamic N_2 atmosphere (50mL.min^{-1}) respectively.

Determination of extractable substance by alcohol

The determination of extractables alcohol, after following the methodology described above resulted in a value of 0,034mg of extractable substances per gram of vegetable sample.

Average value of pH

The average value after carrying out reading of triplicate digital pHmeter showed a value of 5,73.

Content of total polyphenols

The content for total polyphenols found (mean value) was 6.60 ± 0.156 (coefficient of variation equal to 2,31%) in terms of catechin. The foam index value found (based on the highest obtained (equal to 1,7748cm) was 572,08.

Determination of hemolytic activity

The determination of hemolytic activity was confirmed in both tests.

Phytochemical screening

Phytochemical analysis of methanolic extract of *Ximenia americana* L. barks revealed the presence of condensed leucoanthocyanidins and proanthocyanidins as well. Also, were revealed the presence of reducing sugar and saponins. Other secondary metabolites (flavonoids, tannins gallic, cinnamic derivatives, alkaloids, triterpenes, monoterpenes and sesquiterpenes) were not found.

Determination of Chromatographic profile by High Performance Liquid Chromatography (HPLC-DAD) and quantification of commercially available markers in bark of *Ximenia americana* L. species

The highest value for HCRF was found in the experiment 6 - Table 2 - which the chromatogram can be seen below in Figure 4 and equal to 93,999999.

Table 2: Results for HCRF according to factorial design for the experiments.

Experiment	Number of peaks	Lower resolution value	(Tm – Ti) a	HCRF [b]
1	81	0	12,342	81.000.012,34
2	93	0	-6,76	92999993,02
3	89	0	-7,381	88999992,62
4	73	0	11,033	73000011,03
5	91	0	-5,497	90999994,50
6	94	0	-1	93999999,00
7	85	0	2,741	85000002,74
8	94	0	-6,082	93999993,20

Legend - [a]Difference between maximum acceptable value for the analysis time (t_m) and retention time for the last chromatographic peak (t_l); [b] Hierarchical Chromatographic Response Function; Bold: Greater result found.

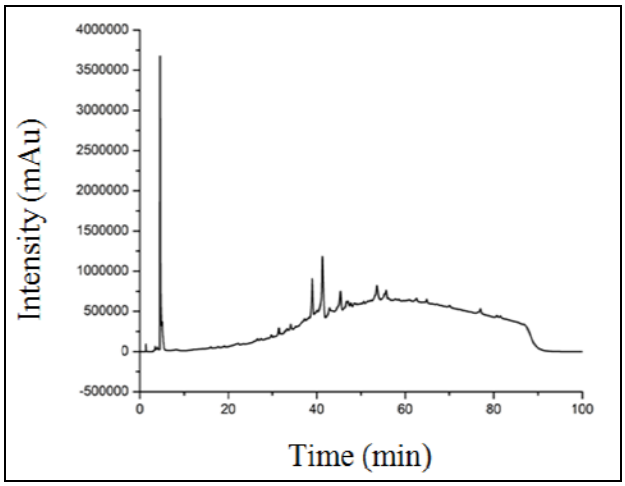

Fig 4: Best result for the chromatogram of the *Ximenia americana* L. species, according to the factorial design conducted.

Discussion

The particle size analysis allowed the classification of the vegetable powder as type coarse powder. All data in question corroborate findings of other authors raised in literature searches (Brasileiro *et al*., 2008) [5].

Due to the absence of pharmacopoeia monograph on the species, moisture to reference values used took into account a range corresponding to 8-14%. The value found, in turn, is within the prescribed limit. For the ash content, and the absence of a specific monograph, the value found was near to the minimum limits established by the

Brazilian Pharmacopoeia (2010) for other species.

The content for total polyphenols found (mean value) in terms of catechin, corroborates the findings of other authors raised by the literature (Brasileiro *et al*., 2008; LamienMeda *et al*., 2008) [5]. The same results works to the phytochemical analysis, corroborating to finds of Brasileiro *et al*. (2008) [5] studies.

The highest value for HCRF allowed the identification of secondary metabolite gallic acid, considering the similar absorbance peak (Figure 5). It is worth noting that this compound had been previously identified in other studies, which confirms our findings (Lamien -Meda *et al*., 2012;.. Le *et al*., 2012; Zhen *et al*., 2015) [12].

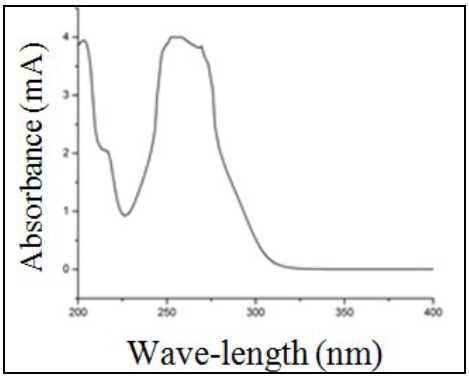

Fig 5: Absorbance spectrum for the gallic acid.

Although not identified (due to low resolution of the chromatogram), previous studies indicate the presence of sambunigrin compounds, □-glucogalina, quercetin and kaempferol (Le *et al*., 2012; Zhen *et al*., 2015.) [12]. Furthermore, it should emphasize the quantitative content - although this is not the aim of this research – of some of the compounds present in the species.

According to Lamien-Meda *et al*. (2008), values equal to 2230,00 ± 76,09 and 2086,67 ± 55,11 was obtained for the total phenolic content (in milligrams equivalent of gallic acid per 100 grams of sample - mg GAE / 100g) prepared extracts using methanol and acetone respectively as solvent. For the total flavonoid content (in milligrams equivalent of quercetin per 100 grams of sample - QE mg / 100g), using the same solvents were respectively equal to 30,95 ± 3,76 and 23,60 ± 1,75. However, one must emphasize here use only fruit (whereas this project we used only the husk) of the specie.

Generally speaking, and considering the findings, two points stand out: a) the official compendia (Brazilian Pharmacopeia) lack of data on the physicochemical

characterization of the studied species, which makes this study not only pioneer, but also a source of data which helps in standardization and identification of quality parameters process for *X. americana* L. stem and; b) There are also methodological limitations imposed by official compendia, such as lack of technical clarity in the texts as well as the fact that it methods of general nature analyzes, instead of taking into account the particularities of each species concerned, as the inter and intraspecific particularities of their secondary metabolites. Moreover, it has not been possible to develop and validate the method by HPLC-DAD for the species involved, despite the results that support and guide future designs based on a fingerprint approach.

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