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PHYSICOCHEMICAL CHARACTERIZATION AND QUANTIFICATION OF TOTAL ANTHRAQUINONES OF *VISMIA GUIANENSIS* (AUBL.) CHOISY

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ABSTRACT

Vismia guianensis is a plant species of high occurrence in the Amazon and northeastern Brazil, which has been studied lately, because of its antimicrobial and anticancer properties. In order to make an herbal medicine, National Agency for Sanitary Surveillance of Brazil, recommends the production of a drug and plant extracts analysis report indicating: method, specifications and results obtained. The drug was characterized by particle size, amount of water and the total ash content; and plant extract through its density, pH and dry residue. The quantification of total anthraquinones and method validation was done by UV-VIS spectroscopy. The drug in this study produced a coarse powder whose determination of water is within the acceptable limits and the total ash content was 1.64%. The extractive solution showed a low pH, density of 0.89 mg/mL and 9.5% on yields from solids. HPLC found that the EtOAc fraction of the plant extract is consisted of phenolic compounds and anthraquinones. And the method of quantification of total anthraquinones, using emodin as external standard, was validated by UV-VIS spectroscopy at λ 290 nm, determining that in 1 g of extractive solution there are 42.85 mg of total anthraquinones. The results obtained served as reference for the quality control of drug and plant derivative of this species; and the validation of the quantification method showed a high performance.

INTRODUCTION: Herbal medicines are obtained with the exclusive use of active raw vegetables, whose efficacy and safety are validated through ethnopharmacological surveys, use of techno-scientific documentation or clinical evidence, not being considered herbal medicine which includes in its composition isolated synthetic or natural active substances, neither of these associations with plant extracts [1].

Herbal therapy is an accepted practice in the Health System since the interministerial action which promulgated the National Policy on Integrative and Complementary Practices (NPICP), ensuring the population a secure access and the rational use of herbal medicines [2]. And so, the Brazilian state provides some recommendations set out in the Resolution RDC nº 14 dated March 31, 2010, for the registration of domestic products.

In addition to the documentation, some reports are also necessary, including those concerning production and quality control. For when vegetable-derived or plant drug is used in herbal medicine as an active compound, it is necessary to submit an analysis report indicating the methods used, the specifications and results obtained [1]. This procedure ensures the efficacy of the medicine, the risks of its use, its reproducibility and the constancy of its quality.

This article presents the recommendations of the National Agency for Sanitary Surveillance of Brazil (ANVISA), and produced a report on quality control of the drug and herbal extract of *Vismia guianensis* (Aubl.) Choisy – Clusiaceae leaves, popularly known as "lacre" [3]. This species occurs in secondary vegetation forests in the states of Amazonas, Pará, Maranhão, Bahia and Minas Gerais [4].It is used primarily in the treatment of dermatosis [5] and presents itself as a potent laxative [3]. The aqueous extract of its fruits and seeds were lethal on a colon adenocarcinoma cell line (KM-12) [6] and the leaves are used as tonic [7]. It has antipyretic and anti-rheumatic properties [3] and its antimicrobial efficacy using the diffusion method disk against *staphylococcus aureus* has been proved [8].

MATERIAL AND METHODS:

Processing plant material: The botanical material was collected in Belém Metropolitan Area, (1°17'46" S; 48° 27' 58.02" W) collected in the end of March 2008. Species identification was confirmed by the Goeldi Museum herbarium (voucher specimen under the MG registration number: 2500133).

The fresh leaves were selected, washed under running water and subjected do drying in a circulating air oven at a temperature of 40±2°C. Then, they were crushed in stainless steel knives mill in order to obtain powdered plant material.

Determination of particle size distribution: About 25 g of powdered plant material were subjected to a series of sieves with mesh size opening (1700, 710, 355, 250, 180 and 125 µm) using a sieve shaker for 15 minutes. Particle size was analyzed in triplicate and measured by quantifying the percentage of powder retention according to the Brazilian Pharmacopoeia [9].

Determination of Water Content: Exactly 2g of powdered plant were transferred to a tared weighing bottle previously dried for 30 minutes under the same conditions to be adopted for the sample. The sample was submitted to a temperature of 105°C for 5 hours, until constant weight. The calculation of the percentage of water was obtained in triplicate in relation to the drug [9].

Determination of Total Ash Content: About 3g of powdered plant was transferred to a tared porcelain crucible. The sample was incinerated by gradually increasing the temperature up to a maximum of 600 ± 25°C. The crucible was cooled in a desiccator and weighed. The procedure was repeated until obtaining constant weight. The ash percentage was calculated in relation to dried drug in triplicate according to the Brazilian Pharmacopoeia [9].

Preparation of Plant Extracts and their fractions: The tincture was obtained by maceration where 500 g of the dried leaves powder remained in contact with 2500 mL of alcohol 70°GL in a closed stainless steel container, at room temperature and periodic agitation for 8 days [10]. Then, the plant derivative was filtered, concentrated on rotary evaporator at low pressure, and then subjected to a solid liquid partitioning with solvents: chloroform, ethyl acetate (EtOAc) and methanol, respectively.

Purity assays of Plant Extracts:

Determination of Apparent Density: The determination of the relative density was performed using a clean dry pycnometer with capacity of 5 mL. The pycnometer was previously calibrated and filled with the sample (hydroalcoholic tincture) at 20°C and weighed. The value of the apparent density was obtained in triplicate, by the sample weight through mass difference of full and empty pycnometer [10].

Determination of pH: Determination of pH was carried out in potentiometer previously calibrated with buffer solutions pH 4.0 and 7.0 and the results correspond to the mean of three determinations [9].

Determination of Dry Residue: A sample of 2 mL of the extract was transferred to a weighing bottle, evaporated to dryness in water bath and dried at 105°C for 3 hours. Then it was cooled in a desiccator

and weighed. The dry residue percentage was calculated in triplicate on the volume [9].

Chromatographic Profile: The sub-fractions A and B were obtained by preparative thin-layer chromatography (PTLC) from the ethyl acetate with ethyl acetate, methanol and water fraction (75:15:10) on normal silica gel. They were dissolved in 5 mg/mL methanol and then filtered through a 0.45 µm membrane filter (Millipore, Merck). Aliquots of 20 µL of both samples were analyzed in chromatograph (Merck Hitachi), model L-7455 LaChrom, equipped with collumn LiChrospher 100® RP-18 (250 x 3.5 µm) and stabilized at 25±1°C. The samples were eluted with acetonitrile/water at a flow rate of 1 mL/min, in gradients: 0min (10:90), 15 min (40:60) and 75 min (100:0) and maintained for 20 minutes [11].

Quantitative analysis of anthraquinones by UV-VIS spectrophotometry:

Preparation of Standard Solution: An reference solution of 5mg/mL emodin (Sigma®, 98.9% purity) was prepared in 70°GL alcohol (Impex), from which solutions were prepared in the following concentrations: 0.0076, 0.0083, 0.0090, 0.0100, 0.0111, 0.0125 and 0.0143 mg/mL, of which the five intermediate points were part of the calibration curve. Each analysis was repeated six times [12].

Method Validation: The method validation was performed according to the Resolution 899 [12] by ultraviolet-visible spectrophotometry with absorbance at λ=290 nm (Spectrum UV-VIS spectrophotometer / SP-2000UV), determining the parameters of selectivity, linearity, precision, accuracy and robustness. The calibration curve was obtained by linear regression and the robustness of the method suffered ANOVA variance followed by Tukey test.

Quantification of Total Anthraquinones: The validated method allowed the quantification of total anthraquinones present in the hydroalcoholic tincture of *V. guianensis* using the line equation obtained with the calibration curve. The ethyl acetate fraction at concentration of 0.01 mg/mL was subjected to spectrophotometer reading at λ 290 nm. And the data were analyzed using Microsoft Office Excel 2007® and Bioestat 5.0 software.

RESULTS AND DISCUSSION: The particle size determination of the powder from the leaves of *V. guianensis* (**Figure 1**) showed a diameter of 0.824 mm which is considered thick, according to the Brazilian Pharmacopoeia [9]. The particle size distribution of herbal drugs determines the contact surface available for interaction with the solvent used to obtain the plant derivative. In this case the procedure chosen to obtain the hydroalcoholic tincture was the maceration, since the goal was not the exhaustion of the drug but the concentration balance between drug and solvent.

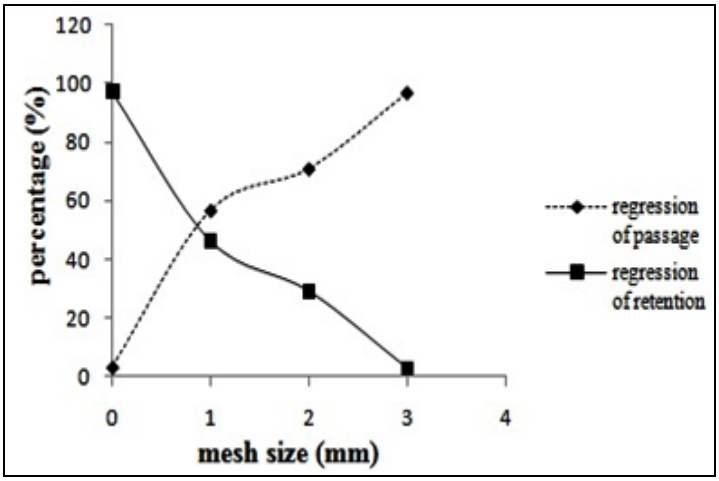

FIGURE 1: DETERMINATION OF PARTICLE SIZE DISTRIBUTION OF *V. GUIANENSIS* (AUBL.) CHOISY LEAVES POWDER

The gravimetric method of drying showed that the plant drug I within the limit set by different pharmacopoeias, which is 8-14% of moisture loss. The result shown in **Table 1** indicates that the drying of the vegetable raw material was efficient and the powder remained in a good state of preservation [10]. The determination of total ash content allowed to quantify the non-volatile inorganic residue present in the plant drug, which consisted of carbonates, chlorides and various oxides [13]. At temperature of 600 °C the ash content found was of 1.64% which corresponds to minerals and/or impurities in the sample [14].

The purity test of the plant extract (Table 1) showed that the density is within the range that varies from 0.87 to 0.98 g/mL, corresponding to the density of tincture sat 20°C when using the pycnometer method [15]. Among other factors the presence of phenolic compounds and anthraquinones in the tincture determined its acidic character [16].

The hydroalcoholic extract showed 9.5% of yield in solids, the determination of the dry residue is a fundamental and primary parameter when reaching the efficacy of an herbal formulation, because this assay involves the quantification of substances extracted from the plant by removing the extracting solvent. The fluid extract used to produce solid formulations needs to pass through the drying step, which influences these formulations state of integrity and stability.

The spray drying method is constantly used when drying plant extractive solutions, however due to the cost of the process, the spray dryer must operate with the maximum possible solids content, allowing a proper use of the heat [17]. Low concentrations of solids need large amount of solvent to be eliminated or require the addition of drying adjuvants, in order to form larger particles and to optimize the process yield [18].

TABLE 1: DETERMINATION OF WATER LOSS AND TOTAL ASH CONTENT OF THE PLANT DRUG AND THE PURITY ASSAYS OF VISMIA GUIANENSIS (AUBL.) CHOISY HERBAL DERIVATIVE

Tests		Determinations	Standard Deviation
Loss of water	3	11.60%	0.12
Total ash content	3	1.64%	0.25
Apparent density	3	0.89 g/mL	0.0025
pH	3	5.69	0.09
Solid yield	3	9.5 %	0.02

The results of herbal derivative purity assays (**Table 1**) as well as factors like radiation, light, air, pH and the presence of other chemical substances influence the stability of the formulations. Therefore, these factors are relevant to the obtainment of an herbal formulation, since they influence the choice of suitable pharmaceutical insumes for the desired product, ensuring its quality and efficacy.

The quality control of herbal medicine begins with the botanical characterization of the plant material and then by the definition of which marker substances must be present to ensure the identity of the plant. In the studied species, literature cites various types of secondary metabolites as part of its constitution [11, 19-24]. And the major component of *V*. *guianensis* leaves is the class of anthraquinones, whose basic skeleton is the emodin [11]. After fractionation of the hydroalcoholic extract obtained from the leaves of *V. guianensis*, the EtOAc fraction, which was best demonstrated by a parting bead analytical thin layer chromatography (TLC). Since recent studies, Lins *et al.,* [25], stated that the fraction obtained from the ethyl acetate solvent, is of utmost importance due to its high content of phenolic compounds, showing high activity against DPPH and ABTS. This fraction was used in a PTLC obtain the sub-fractions A and B which were analyzed by high performance liquid chromatography (HPLC), tracing the composition profile of *V. guianensis* hydroalcoholic extract; showed the same retention time for phenolic compounds, a derivative of xanthone and different types of anthraquinones [8].

The tincture extracted with a 70% hydroalcoholic solution, showed different absorption maxima (**Figure 2**). However, its fraction, extracted with ethyl acetate, free of some components such as fats, waxes, carotenoids and chlorophyll, showed only absorption maxima similar to that of emodin (λ 290 nm). Wavelength used for the validation of the method of quantification of total anthraquinones emodin with equivalent, outer marker of *V. guianensis*.

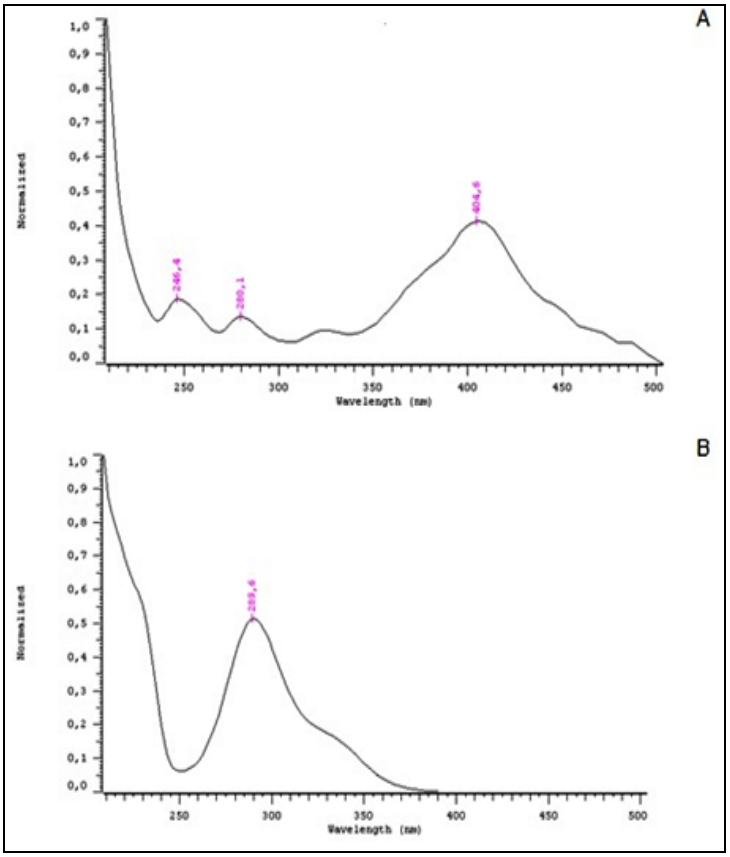

FIGURE 2: SCANNING IN THE RANGE OF Λ 240 A 500 nm, MAXIMUM ABSORPTION OF THE HYDROALCOHOLIC TINCTURE (246, 4; 280 E 404,6 nm) (A) AND THE MAXIMUM ABSORPTION OF THE ETHYL ACETATE FRACTION (290 nm) (B)

The validation of the method of quantification by UV-VIS spectroscopy, an accessible tool to most laboratories, aimed at quantifying the total anthraquinones with emodina equivalent, to determine such an active ingredient in pharmaceutical products. In accordance with the requirements of ANVISA [12], this validation method is part of category I tests, whose purpose is: Quantitative tests for determining the active ingredient in pharmaceutical products or raw materials.

Therefore the required parameters were selectivity, linearity, range, precision (repeatability), accuracy and robustness.

The validation began with the determination of the selectivity among the ethyl acetate fraction, the reference standard and the solvent at λ 240-400 nm. Only an absorption maximum was visualized at 290 nm (**Figure 3**), confirming that at this wavelength it is possible to quantify specifically the emodina fraction even in the presence of other components such as impurities, degradation products and matrix components.

Furthermore, the solvent used in the dissolution of the samples did not interfere, because it showed no absorption maxima in this range of reading.

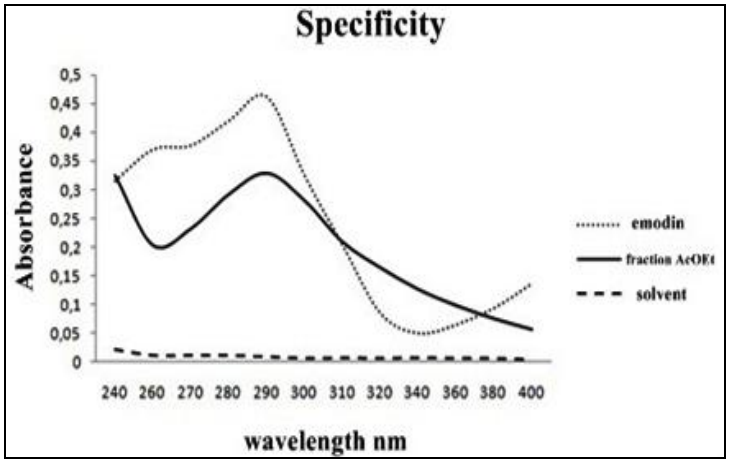

FIGURE 3: SELECTIVITY WITH SCANNING SPECTROPHOTOMETER Λ 240 TO 400 nm OF EXTERNAL STANDARD EMODIN, FROM THE ETHYL ACETATE FRACTION AND THE SOLVENT (ALCOHOL 70°GL).

The spectrophotometric method shows linearity, because the results were directly proportional to the concentration of the sample analyte at 290 nm, corresponding to a correlation coefficient of 0.9955, where the acceptable minimum is 0.99 [12].

And to ensure the adequacy of the linear fit to the calibration curve, the residual analysis showed that they are normally distributed, indicating that the curve is well fitted (**Figure 4**), showing errors with uniform distribution, zero mean, constant variance (homoscedasticity) and absence of atypical samples.

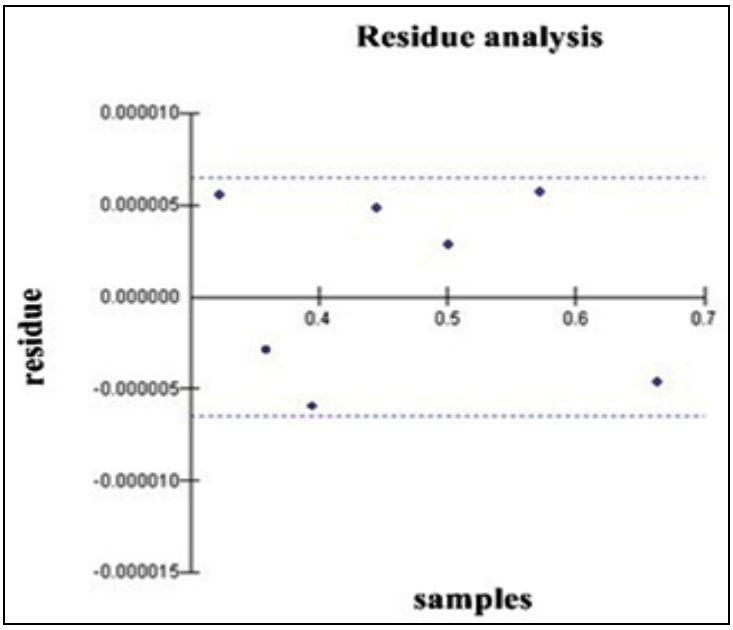

FIGURE 4: RESIDUE ANALYSIS OF THE LINEARITY SHOWING NORMALLY DISTRIBUTED RESIDUE AND HOMOSCEDASTICITY.

The limits of quantification top and bottom of the analytical method arising from linearity are between the percentage limits of 80% 120%, whose concentration range varied from 0.0076 to 0.0143 mg/mL.

The precision data (repeatability and intermediate precision) and accuracy of the method are shown in **Table 2**. The repeatability and accuracy was checked in one day, by the same analyst and with the same instrumentation; contemplating the linear range of the method with three concentrations low, medium and high, in replicates of three the standard emodin, with six repetitions.

The intermediate precision was performed considering the same parameters of repeatability; however it was performed by a different analyst on 2 different days. The results of this analysis are between 1.19 and 4.92% for precision and 95.46 to 101.6% for the values of accuracy, and are within parameters regulated by the Guide for the validation of analytical and bioanalytical methods [12].

TABLE 2: REPEATABILITY AND INTERMEDIATE PRECISION CONTAINING THE RELATIVE STANDARD DEVIATION AND ACCURACY OF THE CONCENTRATIONS OF 0, 0125; 0, 010 E 0, 0083 mg/ml.

Assays	Theoretical concentration (mg/mL)	ŋ	C (mg/mL)	SD	RSD (%)	A (%)
Repeatability	0.0083	6	0.0840	0.0039	4.6	101.2
	0.010	6	0.0980	0.0001	1.24	98
	0.0125	6	0.0127	0.0006	4.92	101.6
Intermediate precision	0.0083	6	0.0084	0,0001	1.19	101.2
	0.010	6	0.0099	0.0002	2.1	99.33
	0.0125	6	0.01193	0.0002	2.42	95.46

ŋ= number of determinations, C= mean concentration, SD= standard deviation, RSD= relative standard deviation and A= accuracy.

The values of the limit of detection and quantification, were obtained according to the equations contained in the Guide for the validation [12], were 0.005 mg/mL and 0.016 mg/mL respectively, confirming that the method has high sensitivity to detect and quantify the standard, without changing the equipment intrinsic factors.

The validation of the method of quantification of total anthraquinones was performed by UV-VIS spectrophotometry; some parameters had to be evaluated, because any change could result in variation in the response of the method. So it was necessary to evaluate the change in pH of the solution and the solvent different manufacturers [12].

The method showed robustness at different pH values (4.3, 5.6 and 6.3) and at different solvent (alcohol 70 °GL: A, B and C) manufacturers. Even in the presence of small and deliberate variations of the analytical parameters, it was able to be resistant to them [12], as the absorption maximum did not change, remaining at 290 nm, as specified during the selectivity. These two factors have undergone ANOVA variance analysis followed by Tukey test. And the method showed there was no statistically significant difference once $p<0.05$.

After the validation of the method of quantification it was possible to determine the total content of anthraquinones for the ethyl acetate fraction from the hydroalcoholic extract obtained from the leaves of *V. guianensis*. Where exactly 1 g of dry hydroalcoholic extract through a solid-liquid partition provides 54.4 mg of ethyl acetate fraction from which 42.85 mg are of total anthraquinones with emodin correspondent, whose values can be seen in **Table 3**.

TABLE 3: QUANTIFICATION OF TOTAL ANTHRAQUINONES WITH EMODIN EQUIVALENT IN THE PLANT DRUG (I), IN THE DRY TINCTURE (II) AND IN THE ETHYL ACETATE FRACTION (III) FROM THE HYDROALCOHOLIC TINCTURE OF *VISMIA GUIANENSIS* (AUBL.) CHOISY LEAVES

Material	Plant drug	Dry tincture	Ethyl acetate fraction	Total anthraquinones
Weight	2.32 g	1 g	54.4 mg	42.85 mg
I	100%	-	-	1.85%
I	-	100%		4.285%
III	-	-	100%	78.77%

CONCLUSION: The parameters set for the dried and pulverized leaves and for the hydroalcoholic extract of leaves of *V. guianensis* determined the reference results for the composition of the analysis report of these species as drug and plant derivative. The validation of the quantification method performed according to the Specific Resolution No. 899, from the National Agency [12], proved to be selective, linear, repeatable and robust, and therefore can be used to quantify total anthraquinones totals with emodin equivalent in pharmaceutical products.

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