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ISOLATION AND QUANTITATIVE HPLC-PDA ANALYSIS OF LUPEOL IN PHYTOPHARMACEUTICAL INTERMEDIATE PRODUCTS FROM *Vernonanthura ferruginea* (LESS.) H. ROB.

Ezequiane Machado Silva Oliveira*, Sabrine Lemes Freitas, Frederico Severino Martins, Renê Oliveira Couto, Mariana Viana Pinto, José Realino Paula, Edemilson Cardoso Conceição e Maria Teresa Freitas Bara
Faculdade de Farmácia, Universidade Federal de Goiás, Praça Universitária, s/n, Campus I UFG, 74605-220 Goiânia - GO, Brasil

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Prior to obtain a standardized dried extract from *V. ferruginea*, lupeol was first time isolated from leaves and used as chemical maker. An analytical method using HPLC-PDA for lupeol determination in *V. ferruginea* intermediate products was developed using a C8 reverse-phase column, acetonitrile-acetic acid (99.99:0.01, v/v) as mobile phase at 0.8 mL min^{-1}, oven temperature at 23-25 °C, sample injection volume at 30 μL and detection at 210 nm. The method presented linearity from 10 to 160 μg mL^{-1}, accuracy, precision, robustness and suitable sensitivity proving to be a useful tool to the obtainment process of lupeol standardized dried extracts of *V. ferruginea*.

Keywords: quality control; triterpene; standardized dried extract.

INTRODUCTION

Pharmaceutical companies have a special interest in herbal dried extracts since that its use in pharmaceutical formulations has advantages compared to the powdered plant and conventional fluid forms, such as greater chemical, physical-chemical and microbiological stabilities, easier standardization, higher concentration of active compounds and higher processing capacity in different types of solid dosage forms.[1] However, the chemical complexity inherent to phytopharmaceuticals turns its quality control a complex task.[2] Therefore, the establishment of validated analytical methods plays an important role on the quality assurance of both herbal raw material and intermediate products,[3] allowing at least its standardization on the content of active principles.

The *Vernonanthura ferruginea* (Less.) H. Rob., known as *Assa-peixe* in Brazil, is a plant widely used in folk medicine and it has showed antiulcer activity in preclinical studies.[4] In our laboratory, the same activity was observed to the spray dried extract of *V. ferruginea* leaves.[5] Despite the pharmacological potential of this plant, efforts to develop a technological product that aims to increase its effectiveness in therapeutic application has been not yet observed.

The lupeol is a pentacyclic triterpene commonly found in species of the Asteraceae family, mainly in the typical Vernoniinae.[6,7] It has been known that lupeol has a number of important bioactivities,[8] among them its gastroprotective effect.[9] The lupeol has also been identified as the active principle of *Vernonia polyanthes* (Less.) antiulcerogenic activity.[10] Although the literature reports several methods for the quantification of lupeol,[11-13] no studies of the isolation and quantification of lupeol on the *V. ferruginea* species have been reported.

In order to obtain a standardized dried extract of *V. ferruginea*, the aims of this work were to isolate a majority compound with an important biological activity to be used as the chemical marker for this species. And then, develop and validate an analytical method for quantitative determination of this compound in *V. ferruginea* intermediate phytopharmaceutical products.

*e-mail: ezequimachado@yahoo.com.br

RESULTS AND DISCUSSION

Isolation and identification of lupeol

The phytochemical investigation of *V. ferruginea* by chromatographic fractionation of the leaves chloroform extract allowed the isolation of lupeol (Figure 1). In the ^{1}H NMR ($CDCl_3$, 500 MHz) spectrum of isolated compound were found some signs that characterize the lupeol molecule, especially at δ 4.56 (1H, d, H_A-29) and δ 4.68 (1H, d, H_B-29) corresponding to the two geminis olefinic hydrogens, the double-doublet at δ 3.18 (1H, dd, H-3) for the carbinolic hydrogen and accumulation of signals in the diamagnetic region characteristic of terpenes (δ 0.7 to δ 2.0). These signs, combined with the methyl signal at δ 1.68 ppm (3H, s, H-30) confirm presence of isopropenil, thus proving that it is a lupane pentacyclic triterpene.[7,14,15] The infrared spectrum showed typical bands of lupeol at 3380 cm^{-1} attributed to the axial strain of alcoholic OH, at 2921 cm^{-1} attributed to the axial deformation of aliphatic CH and at 1639 cm^{-1} attributed to the axial deformation of methylene groups.[7,14]

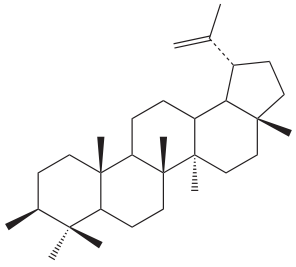

Figure 1. *Chemical structure of lupeol*

The TLC analysis of the isolated compound revealed a violet spot with retention factor of 0.5 as observed for the standard lupeol. In the HPLC chromatogram of the sample was also observed the presence of a peak with the same retention time of standard lupeol. The addition of these data to the spectral data allowed the identification of lupeol for the first time in the leaves of *V. ferruginea*. This finding may be related, at least partially, with the antiulcer potential previously described to this specie,[4,5] once the gastroprotective effect of lupeol has been proven.[9,10]

Development of HPLC-PDA method for lupeol quantification

The *n*-hexane was chosen as the extracting solvent because it showed greater selectivity for the analyte and lesser ability to extract polar interfering compounds. However, peak distortion is reported when using *n*-hexane as the injection solvent on reverse-phase columns.[16] Thus, the authors chosen to evaporate this solvent in sample and then to dissolve the resulting residue in acetonitrile prior injection. The extraction procedure allowed the depletion of lupeol in powdered leaves sample employing ultrasound bath. This is advantageous when compared to the convencional extraction methods that employed high temperatures, since them showed disadvantages such as the loss of compounds owing to hydrolysis, oxidation and ionization during extraction as well as the long extraction time.[17] Moreover ultrasound-assisted extraction is a simple, efficient and inexpensive technique.[18] The ultrasonic cavitation creates shear forces that break cell walls mechanically and improve material transfer. Furthermore, there is no chemical involvement in the ultrasound-assisted extraction, which can prevent possible chemical degradation of target compounds.[19]

The chromatographic methods, especially HPLC, are the most widely used method for quantitative analysis of phytopharmaceuticals, mainly due to its higher separation capability and detection sensitivity being more specialized in analyzing complex mixtures.[20] Because lupeol lack chromophores, the sensitivity of ultraviolet detection is limited and dependent on the mobile phase.[11] Acetonitrile as the mobile phase enabled sensitive detection at 210 nm. The acidification of the mobile phase is widely used. [21] In this work, the addition of acid to the mobile phase aimed to lower the pH and suppress the ionization of the hydroxyl group of the lupeol. Thus, under the molecular form the retention and the separation of lupeol was therefore based on their hydrophobicity and allowed a better interaction with the stationary phase.[22] Furthermore, it has been observed that the peak of lupeol obtained using non-acidified acetonitrile showed tail and broad-based, affecting its symmetry and resolution (data not shown). The resolution of some triterpenes is complex due to similarities in structure and polarity.[12] It has been observed that using a low mobile phase flow rate (0.8 mL min^{-1}), a low column temperature (23-25 °C), and the slightly acidic mobile phase the better separation for the studied compound peak was observed. The similar set condition of these parameters, but using C18 column shows satisfactory results in separation of three triterpene mixture (lupeol, α- and β-amirin) in epicuticular wax of *Brassica oleracea* L.[11] However, when we used a C18 column in our laboratory the time spent in the chromatographic run has been increased.

Validation of HPLC-PDA method for lupeol quantification

It can be observed that all the system suitability parameters were in accordance with the literature specifications (Table 1).[23] Thus, the HPLC system and procedure showed to be capable of providing data of acceptable quality. Performing the selectivity test, it was found, for all sample, that there was no compound interfering with the retention time of lupeol. Furthermore, well resolved peaks indicate the specificity of the method. Figure 2a-d shows the representative chromatogram of lupeol standard and *V. ferruginea* sample. The retention time of lupeol was about of 17 min.

Table 1. System suitability parameters values to standard of lupeol and sample of powdered leaves of *V. ferruginea* and recommendations

Parameter	Standard[a]	Sample	Recommendations[23]
Repeatability	0.5 %	-	*RSD* ≤ 1% for N ≥ 5 is desirable
Tailing factor (*T*)	1.10	1.10	*T* of ≤ 2
Theoretical plates (*N*)	15842	18109	In general should be > 2000

[a] Solution at 40 μg mL^{-1}.

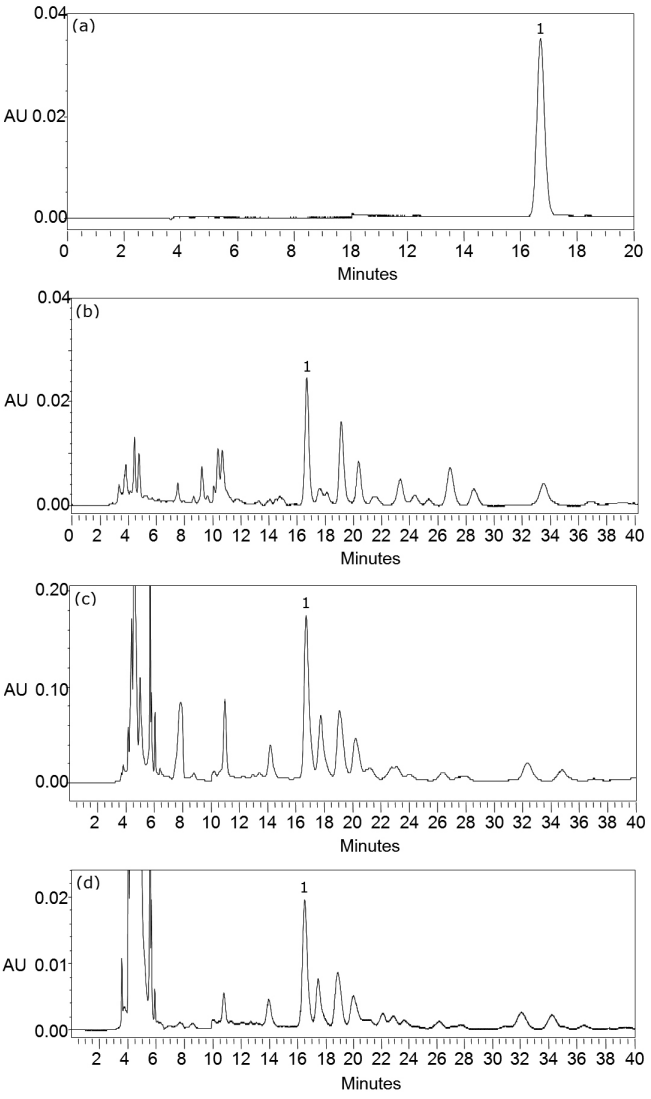

Figure 2. *HPLC-PDA chromatograms of (a) lupeol; (b) powdered plant material; (c) hydroalcoholic extract; and (d) dried extract obtained at 210 nm. [1]Peak of lupeol. Chromatographic conditions: column C8, 250 x 4.6 mm, 5 μm, 23-25 °C/MP:ACN acidified with 0.01% (v/v) acetic acid/flow rate: 0.8 mL min^{-1}/injection vol: 30 μL*

The Table 2 resumes the important parameters values obtained from method validation. The lupeol calibration curves showed a linear response obtaining correlation coefficients (*r*) greater than 0.999. The equation to estimate lupeol contents was defined. The LOD and LOQ showed that the present method has adequate sensitivity to detect lupeol in the prepared sample, which showed concentrations greater than 30 μg mL^{-1}. The RSD values obtained for both repeatability and intermediate precision ranged from 0.48 to 3.91%. Therefore, the precision of the proposed analytical method was assured to the lupeol quantification in *V. ferruginea* phytopharmaceutical intermediate products. The recovery results are very close to 100% with small standard deviations, which prove the suitability and accuracy of the proposed method. Despite using acetonitrile and column chromatography purchased from different manufacturers, the lupeol

contents obtained on each performed set of conditions present RSD lower than 5%. Thus, the method was considered robust, remaining unaffected by deliberate variations in some relevant parameters which may reflect normal day to day variation, especially considering that in the routine laboratory materials are constantly being purchased from different manufacturers. However, we have observed that temperatures above 25 ºC occasionally hindered the peak's resolution (data not shown), so it is suggested that the range from 23-25 ºC should be strictly employed.

Table 2. Validation parameters values obtained from HPLC-PDA method for the determination of lupeol in *V. ferruginea*

	Parameter	Results
Linearity	Linearity range (μg.mL^{-1})	10-160
	Slope (*a*)	17693 ± 24 [a]
	Intercept (*b*)	2149 ± 1648 [a]
	$Y = ax + b$	$Y = 17693x + 2149$
	Correlation coefficient (*r*)	1.0000
Sensitivity	Limit of deteccion (*LOD*, μg mL^{-1})	0.38
	Limit of quantification (*LOQ*, μg mL^{-1})	0.98
Precision		
P_M	Repeatability (% recovery) / *RSD* (%)	0.17 ± 0.006 [a] / 3.91
	Intermediate precision (% recovery) / *RSD* (%)	0.17 ± 0.006 [a] / 3.31
H_E	Repeatability (% recovery) / *RSD* (%)	4.31± 0.021 [a] / 0.48
D_E	Repeatability (% recovery) / *RSD* (%)	2.47 ± 0.040 [a] / 1.66
Accuracy	Recovery 80%	101 ± 3 [b]
	Recovery 100%	100 ± 3 [b]
	Recovery 120%	96 ± 1 [b]
Robustness	Changing column mark (% recovery) / *RSD* (%)	0.16 ± 0.002 [a] / 3.38
	Changing acetonitrile mark (% recovery) / *RSD* (%)	0.17 ± 0.003 [a] / 0.32

[a] Data expressed as *mean* ± *SD* (standard deviation); [b] Data expressed as *mean* ± $CI_{95\%}$ (confidence interval); P_M : powdered plant material; H_E: hydroalcoholic extract and D_E: dried extract.

The lupeol content means obtained for sample powdered plant material, hydroalcoholic extract, and dried extract are shown in the Table 3.

Table 3. Quantitative results of lupeol for samples

Sample	Lupeol content (% w/w)	CV (%)*
P_M	0.17	3.91
H_E	4.31[a]	0.48
D_E	2.47	1.66

P_M : powdered plant material; H_E: hydroalcoholic extract and D_E: dried extract; *Coefficient of variantion (n = 6); [a] % w/w of dry residue in the hydroalcoholic extract.

A HPLC method for the simultaneous determination of β-sitosterol and lupeol in *Vernonia cinerea* Linn. was proposed.[13] However, the method proposed in our work showed to be more sensitive and better applicable to various concentrations of lupeol in the sample, due to the lower limit of quantification and greater amplitude of the linear range. In addition, the system suitability parameters were checked for both the sample and for the standard, ensuring a more efficient separation of the components of the complex matrix.

The results allowed us to conclude that the proposed method can be successfully used as tool for quality control of lupeol standardized dried extracts of *V. ferruginea* in various steps of the obtainment process, such as the powdered plant material, hydroalcoholic extract and dried extract.

EXPERIMENTAL

Standard and chemicals

Lupeol (94%) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile (J.T. Baker, Phillipsburg, NJ, USA and Burdick & Jackson, Muskegon, MI, USA), methanol (J.T. Baker) and glacial acetic acid (Vetec Química Fina Ltda., Duque de Caxias, RJ, Brazil) were of HPLC grade. Additionally, *n*-hexane (Quimis, Diadema, SP, Brazil) and ultrapure water from a Milli-Q system (Millipore, Bedford, MA, USA) were used. All other chemicals were of reagent grade and were used without further purification.

Plant material, hydroalcoholic and dried extracts

The leaves of *V. ferruginea* were collected in February 2010 in Goiânia, GO, Brazil (altitude 768 m; 16º 40' 33.3" S; 14' 39.5" W) and identified by Dr. J. A. Rizzo (Conservation Unity/ICB/UFG) and a voucher specimen (number UFG-43196) has been deposited in the Herbarium of the Universidade Federal de Goiás. The leaves were air dried (40 °C; forced air ventilation; 5 d) and ground in a knives mill.

The hydroalcoholic extract was obtained by exhaustive percolation of the 1 kg of *V. ferruginea* leaves powder, using as solvent ethanol at 95% (v/v). The collected extract was evaporated at 40 ± 2 ºC using a rotary evaporator. In total, 2 L of concentrated extract were obtained.

The dried extract was obtained in a laboratory-scale spray dryer from the hydroalcoholic extract using 20% (w/w of dry residue in the hydroalcoholic extract) of colloidal silicon dioxide as drying adjuvant.

LC isolation and identification of the triterpene lupeol

The dried leaves (100 g) of *V. ferruginea* were macerated three times at room temperature for 24 h with chloroform (500 mL each time). The combined extracts were filtered and evaporated to dryness. Part of the resulting residue (2.5 g) was chromatographed on a silica gel column (30 x 3 cm i.d.; 100 g) eluted first with *n*- hexane (fraction A), after with chloroform (fraction B) and then methanol (fraction C). Fraction C (0.58 g) was rechromatographed on a silica gel column (30 x 2 cm i.d.; 40 g) eluted with a mixture dichloromethane : petroleum ether (7:3) resulting 54 fractions of 10 mL each. The fractions 18 to 36 were reduced to two groups, C1 and C2 (0.07 and 0.04 g, respectively), and rechromatographed on a silica gel column (30 x 1 cm i.d.; 10 g) eluted with *n*-hexane, gradually increasing the polarity with ethyl acetate to *n*-hexane:ethyl acetate (1:1). Were collected 55 fraction of 5 mL for each group. The fractions 14 to 46 (C1 group) and the fractions 14 to 42 (C2 group) were reduced to 4 group each (C1.1 to C1.4) and (C2.1 to C2.4). The residues of each group was applied to a SPE cartridge and eluted with volumes (10 mL each) first of n-hexane, after of chloroform and then chloroform : methanol (1 : 1).

The isolation of Lupeol was monitored in the fractions by TLC on silica gel 60 F254 layers using *n*-hexane:ethyl acetate (85:15) as mobile phase. The separated components were detected by spraying the layers with sulfuric vanillin reagent.[24] The isolated compound was characterized by ^{1}H- NMR in Brüker (Madison, Wisconsin, USA) model Avance III - 500 (^{1}H, 500 MHz) spectrometer using

deuterochloroform as solvent and tetramethylsilane (TMS) as internal standard. Also was performed IR spectroscopy in a Perkin-Elmer (Norwalk, Connecticut, USA) model Spectrum BX II spectrometer. The spectral data was compared with the literature.[7,14,15]

Development of HPLC-PDA lupeol quantification method

Sample and standard preparation

Sample A: The powdered plant material (1 g) was extracted three times by sonication (15 min each) using *n*-hexane as solvent. In the first two extractions were used 20 mL of solvent and 10 mL in the third. The extracts were combined, filtered and transferred to 50 mL volumetric flask. *N*-hexane was added to volume.

Sample B: The hydroalcoholic extract (1.5 mL), ethanol (3.5 mL) and water (5.0 mL) were shaken for 1 min in a separating funnel. The mixture was extracted four times with 10 mL *n*-hexane. The lower phases were discarded and the resulting hexanic solutions were filtered and transferred to a 50 mL volumetric flask. *N*-hexane was added to volume.

Sample C: 4 mg (dry basis) of dried extract, accurately weighted, was dissolved and then diluted with *n*-hexane in volumetric flasks to 25 mL.

Sample preparation: 5 mL of each sample (A, B and C) were transferred to round-bottom flasks and evaporated at 40 °**C** in a rotary evaporator. The dry residues were resuspended with 5 mL acetonitrile using an ultrasonic bath at room temperature (22 ± 1 °**C**).

Standard preparation: Ten mg of lupeol was dissolved in acetonitrile and then diluted to 10.0, 20.0, 40.0, 80.0 and 160.0 μg mL^{-1} to construction of calibration curve.

HPLC-PDA conditions

The HPLC analyses were carried out using a Waters LC system (Milford, Massachusetts, USA) comprising a quaternary pump, an on-line degasser, an autosampler and a photodiode array detector model 2998. Enpower 2.0 software was used for the control of the HPLC equipment and for the acquisition and treatment of data. Chromatographic separation was carried out with a Luna C8 reverse-phase column (250 x 4.6 mm, 5 μm) purchased from Phenomenex (Phenomenex Inc., Torrance, CA, USA). A similar column purchased from Waters was used to test the robustness. The mobile phase was composed of acetonitrile-acetic acid (99.99:0.01, v/v) at flow rate of 0.8 mL min^{-1}. The injection volume was set to 30 μL, the temperature at 23-25 °**C** and the run time at 38 min.

Single laboratoty validation of HPLC-PDA lupeol quantification method

A single laboratory validation study was performed following United States Pharmacopeia guidelines.[25] Some parameters of system performance such as theoretical plates (N), tailing factor (T), and standard repeatability (RSD) were determined following Food and Drug Administration (FDA) guidelines.[23]

The selectivity of the method was evaluated by analysis of the sample solution chromatogram obtained in the range from 190 to 400 nm compared to that of the lupeol solution (40 μg mL^{-1}). In order to verify the interference of the matrix constituents, a blank experiment was also carried out by injecting acetonitrile.

The linearity was checked by injecting (in triplicate) of 5 different concentrations (10-160 μg mL^{-1}) of the standard solutions in the HPLC system. The curves were constructed using the peak areas *versus* nominal concentrations of lupeol. The linear least-square regression analysis was performed to obtain the correlation coefficient (*r*) and the general standard curve equation.

Both limits of detection (LOD) and quantification (LOQ) were determined by linear regression of the three analytical curves obtained as described above. LOD and LOQ were calculated according to USP.[25]

To evaluate the repeatability and intermediate precision, sample at three different concentrations corresponding to 80, 100 and 120% of the test concentration were prepared (in triplicate for each concentration) and assayed on the same day and on 2 consecutive days (by 2 different analysts), calculating the lupeol content and its RSD.

The accuracy of the method was studied using the method of standard addition. The standard lupeol solution was added to the sample at three different concentrations corresponding to 80, 100 and 120% of the test concentration (in triplicate for each concentration). The lupeol content was determined and the accuracy calculated as percent recovery together with its confidence interval 95%.

Finally, the method robustness was evaluated by the use of acetonitrile and column chromatography purchased from different manufacturers. The results were compared with that obtained from the original material by calculating the RSD.

The proposed method was then co-validated for the hydroalcoholic and the dry extracts by the testing of selectivity and precision.

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