Pharmacognosy

Eco-friendly extraction and simultaneous determination of two coumarins in *Justicia pectoralis* (Acanthaceae)

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Abstract

Justicia pectoralis (Acanthaceae) is employed in folk medicine for its analgesic, anti-inflammatory and sedative effects and to treat respiratory diseases. It is known for properties of its coumarins, 1,2-benzopyrone and umbelliferone. A green, simple, fast, and inexpensive ultrasound-assisted extractive (UAE) method for extracting umbelliferone and 1,2-benzopyrone from *Justicia pectoralis* was optimized. Additionally, a HPLC analytical method was developed and validated for the simultaneous determination of both coumarins. The Box-Behnken design and response surface methodology were used to evaluate the UAE process. Ethanol concentration, extraction time, plant-to-solvent ratio were the independent variables studied and the coumarin content was the dependent one. The HPLC-UV/VIS method was validated in terms of recovery, linearity, accuracy, precision and robustness, proving to be valuable for the quality control of *Justicia pectoralis* extract and in the development of its herbal products. Results show that the optimal UAE conditions were: ethanol concentration of 15% (w/w), extraction time of 34 min and plant-to-solvent ratio of 0.1 g/mL. The predicted values of coumarin contents (22.16 µg/mL - umbelliferone and 163.86 µg/mL - benzopyrone) were determined under the optimal UAE conditions and proved that UAE is an efficient and eco-friendly extractive process for the production of aerial part extracts from *Justicia pectoralis*.

Key words: benzopyrone, Box-Behnken design, response surface methodology, umbelliferone.

Resumo

Justicia pectoralis (Acanthaceae) é utilizada na medicina popular como analgésico, anti-inflamatório, sedativo e no tratamento de doenças respiratórias. É conhecida pelas propriedades de suas cumarinas (1,2-benzopirona e umbeliferona). Um método ecológico, simples, rápido e barato de extração assistida por ultrassom (EAU) destas cumarinas, a partir das partes aéreas de *Justicia pectoralis*, foi otimizado. Adicionalmente, foi desenvolvido e validado um método analítico por CLAE para a determinação simultânea das duas cumarinas. Um modelo Box-Behnken 3^3 e a metodologia de superfície de resposta foram utilizados para estimar as melhores condições da EAU. As variáveis independentes estudadas foram: concentração de etanol, tempo de extração e proporção droga/solvente. A concentração das cumarinas foi a variável dependente. O método analítico foi validado quanto aos parâmetros recuperação, linearidade, exatidão, precisão e robustez, demonstrando ser útil para o controle de qualidade do extrato de *Justicia pectoralis* e no desenvolvimento de seus fitoterápicos. Os resultados mostraram que as melhores condições da EAU foram: concentração de etanol de 15% (p/p), tempo de extração de 34 min e proporção droga/solvente de 0,1 g/mL. Os valores previstos pelo modelo para as concentrações de umbeliferona (22,16 µg/mL) e benzopirona (163,86 µg/mL) foram determinados nas condições otimizadas e provaram que a EAU é um processo extrativo eficiente e ecologicamente correto para a produção de extratos a partir das partes aéreas da *Justicia pectoralis.*

Palavras-chave: benzopirona, modelo Box-Behnken, metodologia de superfície de resposta, umbeliferona.

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Introduction

Justicia pectoralis Jacq. (Acanthaceae) is a herb native to Latin America that has been widely researched for its medicinal properties such as analgesic, anti-inflammatory, anti-asthmatic, antioxidant, and sedative effects (Lino *et al.* 1997; Parra *et al.* 2001; Cheng *et al.* 2004; Han *et al.* 2005; Kostova 2005; Chanfrau *et al.* 2008; Venâncio *et al.* 2011; Arcanjo *et al.* 2012; Leal *et al.* 2017). The main active compounds are the coumarins 1,2-benzopyrone and umbelliferone (Fig.1) found in higher concentration in its aerial parts (Angonese *et al.* 1992; Barros *et al.* 1997; Oliveira & Andrade 2000; Govín *et al.* 2003). Pre-clinical studies showed an anxiolytic-like effect of *J. pectoralis* extracts (Venâncio *et al.* 2011). Dry extract has been used as an active pharmaceutical ingredient in liquid and tablet formulation, but there are still practical limitations for its clinical application, such as the efficiency of extraction/drying methods and the obtainability of sensitive and accessible analytical methods for a quality control routine (Fonseca *et al.* 2010; Arteaga *et al.* 2011; Martín-Viaña *et al.* 2011; Chanfrau & Rodríguez 2014; Chanfrau *et al.* 2015).

Different HPLC analytical methods have been proposed and used in the quantification of coumarins from *J. pectoralis* (Chanfrau *et al.* 2008; Fonseca *et al.* 2010), but many have been performed from complex extractive processes that make the method more difficult, with high and costly solvent consumption. In addition, there are no validated methods proposed in the literature to quantify the two markers simultaneously. The efficiency and accuracy of an analytical method for evaluating bioproducts depends on the extraction method.

Methods by solid liquid extraction with water by reflux process or with hydroalcoholic solution by percolation extraction are used for extraction involving the aerial parts of *J. pectoralis* (Lino *et al.* 1997; Chanfrau & Rodríguez 2014; Locklear *et al.* 2010; Chanfrau *et al.* 2013; Cameron *et al.* 2015). These methods have some limitations, such as high energy consumption, long extraction time (up to 4h for Soxhlet or days for percolation extractions) and the low yield of the process. However, faster extraction methods, such as ultrasound-assisted extraction (UAE), which enable higher levels of the markers of *J. pectoralis* to be obtained in a short time and with reduced solvent consumption, are not described in the literature. This method is based on the mechanical and cavitation forces caused by sound waves, and it leads to the reduction of particle size, breakdown of the plant cell wall and increased mass transfer through the membrane (Cárcel *et al.* 2012; Wang *et al.* 2013; Paz *et al.* 2015; Zhou *et al.* 2017), making it a cheap, fast, green and efficient alternative compared to conventional extraction techniques.

The objective of this study was to achieve the optimal eco-friendly extraction process for 1,2-benzopyrone and umbelliferone from the aerial parts of *J. pectoralis* through ultrasound-assisted extraction. Additionally, was validated a HPLC-UV/VIS method for simultaneous identification and quantification of 1,2-benzopyrone and umbelliferone in these extracts.

Materials and Methods

Plant material

The aerial parts (branches with leaves and flowers) of *J. pectoralis* were collected in the herb garden of the Goiânia Botanical Garden (latitude 16º19'36"S, longitude 48º57'10"W, altitude 1,017 m), located in Goiânia city, and at the Universidade Estadual de Goiás (UEG) (latitude 16º17'13.8"S, longitude 48º57'22.7"W, altitude 1,074 m), Anápolis city, both in Goiás state, Brazil. Voucher specimens are deposited in the Herbarium of the UEG (HUEG10764). The samples (8 kg fresh plant) were dried at 40 ºC for 24 h in an oven with circulating air and crushed in a knife mill. The loss during drying was of 8.76% ± 0.18 and the average diameter of powder particles was 0.425 mm (Brazil 2010).

General procedures

An ultrasonic device (USC 2800A, 40 kHz, 154W, Unique®) equipped with a digital timer and a temperature controller was used for the ultrasound-assisted extractions (UAE).
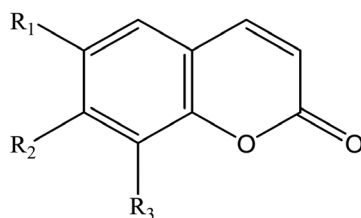
R1 = R2 = R3 = H: 1,2-benzopyrone (coumarin)
R1 = R3 = H, R2 = OH: umbelliferone (7-hidroxicoumarin)

Figure 1 – The main metabolites present in *J. pectoralis*.

A Varian HPLC ProStar with separation modules, 240 ternary pump, 310 automatic injector and 20599 UV detector, and software Star (Chromatography Workstation) were used. Column Ascentis® Supelco Analytical C18 (250 mm × 4.6 mm, 5 µm). RC -Vliesverstärkt membrane (0.45 µm, Sartorius Biolab Products). The analytical standards were 1,2-benzopyrone and umbelliferone (Sigma Aldrich, St. Louis, MO, USA). The samples and analytical standard solutions were previously filtered through a 0.45 µm O-45/15 MS membrane (Macherey-Nagel).

Method validation

The HPLC-UV/VIS chromatographic systems tested before validation method and the HPLC-UV-VIS system chosen includes an isocratic mobile phase with methanol/water (40:60), wavelength of 323 nm, and flow rate of 1 mL/min. The sample was prepared through ethanol extract obtained by UAE (ethanol - 20% (w/w), plant-to-solvent ratio of 0.066 g/mL and extraction time of 20 minutes). The prerogatives of the International Conference on the Harmonization (ICH) of Technical Requirements for the Registration of Pharmaceuticals for Human Use (ICH 2005) and Brazilian legislation (Brazil 2017) were followed for method validation.

Linearity: the calibration curves for linearity were determined by analysis at six concentration levels of the 1,2-benzopyrone standard (512.0, 409.6, 286.7, 172.0, 103.2 and 41.3 µg/mL) and six concentration levels of the umbelliferone standard (29.7, 23.7, 16.6, 9.9, 5.9 and 2.4 µg/mL) in mobile phase. The calibration curve was fitted by linear regression from the correlation between the peak areas and the concentration of the standards, with analysis of linear regression coefficients (R) and analysis of variance (ANOVA). The average calibration curve and the resulting equation of the standard linear regression were used to quantify coumarins in *J. pectoralis* extracts.

Limit of detection (LOD) and limit of quantification (LOQ): these were calculated according to Eqs. 1 and 2, based on the standard deviation (SD_b) of the intercept with the y-axis and the slope of the calibration curve (S), obtained from three equations of the linearity calibration curves.

$$LOD = \frac{SDb \times 3}{S} \qquad \text{(Eq. 1)}$$

$$LOQ = \frac{SDb \times 10}{S} \qquad \text{(Eq. 2)}$$

Selectivity: the coelution of analytes and other extract components was evaluated comparing the chromatograms of a blank (methanol/water, 40:60), sample solution and standards; and comparing UV absorption spectra of the analytes in the samples and standards.

Precision: the method's precision was considered at the levels of repeatability and intermediate precision. The repeatability (intra-day precision) was evaluated by analyzing six injections of the test concentration and the intermediate precision (inter-day precision) was evaluated by this same process, performed by two different analysts on different days. In both tests, the relative standard deviation (RSD) was used as the evaluation criterion, in agreement with ICH recommendations (ICH 2005).

Accuracy: 1,2-benzopyrone and umbelliferone reference standards were accurately weighed, and the sample solutions were prepared at three concentration levels corresponding to 80, 100 and 120% of the standard concentration in the linear range, with and without the addition of a known amount of the 1,2-benzopyrone standard (60.7 µg/mL) and umbelliferone standard (17.6 µg/mL). At each level, samples were prepared in triplicate and the recovery percentage was determined.

Robustness: this was evaluated by analyzing the results of the coumarin content obtained from the changed in the column lot, injection volume and the flow. The results were evaluated by RSD calculation, with samples in triplicate.

Extract preparation

The extraction conditions for optimization of the UAE process for coumarins from *J. pectoralis* included ethanol as a green solvent (Prat *et al.* 2016), and the experiments were set out in a Box-Behnken design. The variables studied, which affect extraction efficiency, were chosen from literature data (Chanfrau *et al.* 2008; Wang *et al.* 2013) and our previous experiments (data not showed), which indicated increased coumarin extractions with low ethanol concentrations. The three variables investigated at three levels (3^3) were: ethanol concentrations of 15, 30, and 45% (w/w, x_1); extraction times of 20, 40, and 60 min (x_2), and plant-to-solvent ratios of 0.10, 0.07, and 0.05 g/mL (x_3). The response variables were the 1,2-benzopyrone and umbelliferone contents achieved by HPLC-UV/Vis validated method. The complete process was carried out in randomly arranged order, consisting of 15 combinations,

including 3 replicates at the central point. Results were adjusted to a second-order polynomial regression model according to the following equation (Eq. 3).

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i \, x_i + \sum_{i=1}^{k} \beta_{ii} \, x_i^2 + \sum \sum \beta_{ij} \, x_i x_j$$

(Eq. 3)

Where: y is the predicted response quantified by an extraction recovery; x_i and x_j represent the levels of the independent variables; β_0 is the model constant; β_i is the linear coefficient; β_{ii} is the quadratic coefficient; and β_{ij} is the cross-product coefficient (Said & Amin 2015).

Response surface methodology (RSM) was built to express the response effects of the three independent variables on the 1,2-benzopyrone and umbelliferone content, to verify the predictive capability of the model and to set the optimal extraction conditions within the evaluated intervals. Statistica software (version 12.0) was used to analyze the experimental results, where two-way linear and quadratic interactions were included, considering only the factors with $p < 0.05$ as significant.

Coumarin contents achieved by the optimized UAE conditions were compared by ones achieved by percolation process. To obtain the percolated extract, 500 g of plant material were exhausting percolated in approximately 2.5 L ethanol 20% (w/w) and concentrated in a Buchi® rotary evaporator (R-220SE model) under vacuum (40 ºC), until solid content of 2.5% (w/w).

Results and Discussion

Method validation

The UV/VIS scanning spectrum (200–800 nm) of 1,2-benzopyrone and umbelliferone reference standards showed 323 nm as the best wavelength for the analysis of both coumarins.

The comparison of the concentrated liquid extract (CLE), coumarin standards (CS), and diluent chromatographic profiles (Fig. a,b) confirmed the spectral similarity of coumarin peaks at CLE and CS, as well the selectivity of the method. Peaks of interfering substances in the blank were not observed at the retention time of coumarins (Fig. 2c). Different conditions for the mobile phase and flow were tested according to the literature and the best condition chosen for method validation was 40/60 methanol/water for mobile phase and 1 ml/min for flow. This method showed system suitability results in accordance

with ICH and Brazilian regulations (ICH 2005; Brazil 2017) (Tab. 1).

The method was linear in the analyzed interval and the representative linear equation was y = 22473x - 13376 (N = 6; R^2 = 0.9999; RSD = 1.00%) for 1,2-benzopyrone and y = 36778x – 14097 (N=6, R^2 = 0.9999, RSD = 1.00%) for umbelliferone. The slope of the 1,2-benzopyrone and umbelliferone calibration curves showed a relative standard deviation (RSD%) of 1.00%. This value is within the limits set by ICH and Brazilian regulations (ICH 2005; Brazil 2017), which should not exceed 5%. However, a correlation coefficient value (R^2) close to unit is not enough to confirm the linear correlation; thus, it is necessary to apply a lack-of-fit test to evaluate the variance of the residual values (Hadad *et al.* 2009). In the ANOVA evaluation for the 1,2-benzopyrone and umbelliferone linearity, the calculated *F* value for the lack-of-fit was smaller than the tabulated *F* value, with a confidence level of 95% ($p = 0.05$), not showing lack-of-fit for linear regression (Tabs. 2; 3).

The limits of detection (LOD) in the indicated experimental conditions were 5.81 µg/mL (0.00581 mg/mL) for 1,2-benzopyrone and 0.35 µg/mL (0.00035 mg/mL) for umbelliferone. The limits of quantification (LOQ) under the indicated experimental conditions were 19.37 µg/mL (0.01937 mg/mL) for 1,2-benzopyrone and 1.799 µg/mL (0.001799 mg/mL) for umbelliferone.

In the intra-day precision analysis, on day 1 (analyst 1) the RSD results were 2.77% for 1,2-benzopyrone and 3.61% for umbelliferone; on day 2 (analyst 2) the RSD results were 5.91% for 1,2-benzopyrone and 4.65% for umbelliferone. In the inter-day precision analysis, the RSDs were 5.35% for 1,2-benzopyrone and 6.55% for umbelliferone. Table 4 shows the results obtained for the method's precision, at the levels of repeatability and intermediate precision. In both tests, the RSD for markers was carried out with six determinations.

Table 5 data show the results of accuracy by the recovery test. The recovery ranged from 86.36% to 97.81% for 1,2-benzopyrone, with an average of 94.07% (RSD = 3.65%), and from 92.44% to 99.48% for umbelliferone, with an average of 94.93% (RSD = 4.81%). The recovery test measures the amount of the substance of interest present or added in the analytical portion of the test material that is extracted and capable of being measured (Thompson *et al.* 1999). The
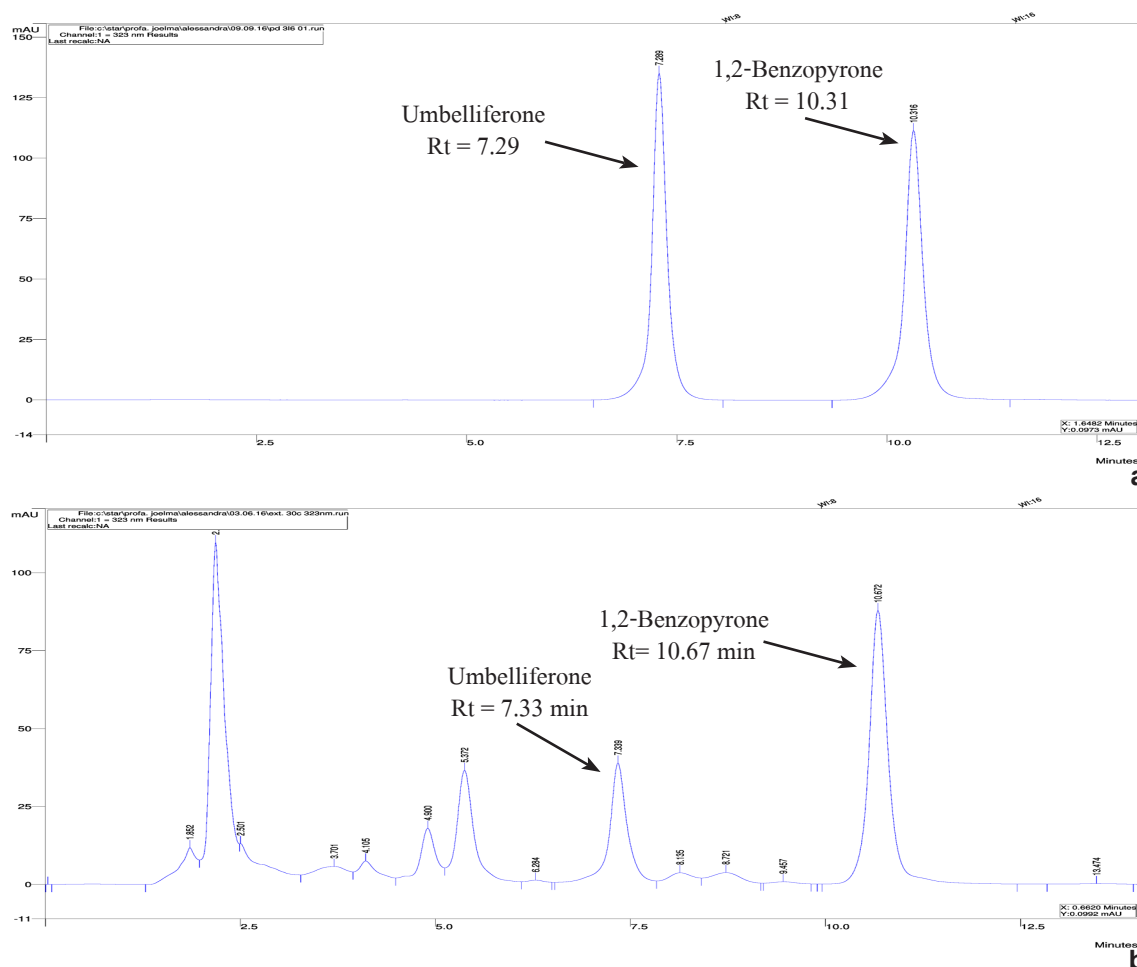
Figure 2 – a-b. HPLC-UV chromatographic profiles – a. coumarin standards; b. concentrated liquid extract from *Justicia pectoralis* aerial parts. Chromatographic conditions: C18 column (250 × 4.6 mm, 5 μm); mobile phase methanol/water (40:60); flow rate of 1.0 ml/min, λ = 323 nm; injection volume of 20 μL.

acceptable intervals may range from 50 to 120% with an accuracy of ± 15% (Ribani *et al.* 2004).

The statistical analysis of robustness confirmed no significant difference between the results obtained in the different analytical conditions for the method. The RSDs for coumarin concentrations were 6.74% for 1,2- benzopyrone and 6.60% for umbelliferone under the flow changed at 0.1 mL/min.; when the injection volume was changed at 1μL the RSDs were 4.31% for 1,2- benzopyrone and 4.74% for umbelliferone, and when column lot was changed the results were 2.06% for 1,2- benzopyrone and 2.43% for umbelliferone. These results showed the robustness in accordance with recommends of guidelines for registering herbal medicines and for notifying traditional herbal products (Brazil 2014). These

guidelines recommend defined that admissible the maximum for RSD values in the analytical validation should be defined by the kind of method, matrix complexity, analyte concentration and purpose of the method, but the RSD should not exceed 15%. The demonstration of robustness is critical in the transference of the analytical process to other laboratories (Ribani *et al.* 2004).

The literature methods were not so able to quantify both important coumarins in the same analysis, determining only 1,2-benzopyrone content (Chanfrau *et al.* 2008), or were not so simple, cheaper and useful for quality control application it necessary a more complex mobile phase in a gradient system and using a high flow (Fonseca *et al.* 2010). The HPLC-UV/VIS analytical method developed for coumarin quantification met the

Table 1 – Mean (± SD) of the system's suitability parameters for the validated chromatographic method, obtained from six coumarin determinations in the ethanol extract of *J. pectoralis*.

	System suitability		
	Tailing factor (TF)	**Resolution (Rs)**	**Theoretical plates (N)**
Sample umbelliferone peak	0	2.97 (± 0.05)	5157.8 (± 259.37)
Sample 1,2-benzopyrone peak	1.03 (± 0.01)	2.16 (± 0.05)	6220 (± 241.65)
Literature recommendations (ICH 2005)	≤ 2.00	≥ 2.00	> 2000.00

validation criteria established in the ICH guidelines and Brazilian regulations; the method demonstrated to be fast, not complex and able to determine the two most important compounds in aerial parts of *J. pectoralis*, simultaneously and with individual evaluation.

Evaluation of extraction parameters for coumarins content

The present study was performed to obtain an optimized extraction method of coumarins (1,2-benzpyrone/umbelliferone) in ethanol solution from the aerial parts of *Justicia pectoralis*. The ethanol was chosen because it is an agro-solvent with chemical and physical stability, low volatility, ease of use, and with the possibility of reuse. Promoting this way, safety and environmental protection, and process sustainability (Prat *et al.* 2016; Chemat *et al.* 2019). These characteristics define ethanol as a green solvent more suitable for use in friendly processes than methanol, a toxic solvent commonly used in

extractive process. The use reduction of hazardous solvents is also one of the priorities of international environmental policies and legislations for 2010–2050 periods (Bubalo *et al.* 2018).

Two extraction methods were used, extraction percolation and ultrasound assisted extraction, both with the ethanol solution as the solvent. This option was defined from previous tests that indicated better solubility of umbelliferone in low concentration ethanol solutions. This option was defined from previous tests that indicated better solubility of umbelliferone, in addition to the low toxicity of ethanol, which is the only solvent other than water allowed in medicinal plant extractions by national and international regulatory agencies.

The coumarin content of extracts from 15 experiments generated by the Box-Behnken design and multiple linear regression analysis using the quadratic polynomial model are showed in Tables 6 to 8. The results for 1,2-benzopyrone contents were demonstrated to be significant ($p = 0.000084$),

Table 2 – ANOVA data for umbelliferone and 1,2-benzopyrone linearity.

	DF	*SS*	*MS*	*F*	*F tab*
Umbelliferone	1	2.27107E+12	2.27107E+12	30942.13704	7.65878E-22
Residual	16	1026008626	64125539.1		
Lack-of- Fit	4	145239380.3	36309845.07	0.494701811	0.74009069
Pure error	12	880769245.3	73397437.11		
	DF	*SS*	*MS*	*F*	*F tab*
1,2-Benzopyrone	1	2.52336E+14	2.52336E+14	221259.0286	5.73934E-27
Residual	16	28011982530	1750748908		
Lack-of- Fit	4	14326544369	3581636092	3.140537599	0.055327445
Pure error	12	13685438161	1140453180		

DF = Degrees of freedom; SS = Sum of Squares; MS = Mean Squares; F = calculated F value; Ftab = tabulated F value.

Table 3 – Summary of the calibration curve parameters of umbelliferone and 1,2-benzopyrone.

	Umbelliferone	1,2-Benzopyrone
Linear range (µg/mL)	29.7 - 2.4	512.0 - 41.3
Limit of detection (µg/mL)	0.3539	5.8110
Limit of quantification (µg/mL)	1.1799	19.3700
Linear regression data*		
N	6	6
Slope (a)	36778	22473
Standard deviation of slope	210.01	125.19
Relative standard deviation of slope (%)	0.57	0.55
y-axis intercept (b)	14097	133760
Linear correlation coefficient (r)	1.0000	1.0000

* $y = ax + b$, where x is the compound concentration and y is the peak area.

and R^2 and R^2 adj values were 0.997 and 0.99, respectively. The results for umbelliferone contents were significant too ($p = 0.000011$), and R^2 and R^2 adj values were 0.999 and 0.99, respectively. These confirm the model's suitability. The lack-of-fit was not significant ($p > 0.05$), which indicates the suitability of the model to accurately predict the variation. The data from the analysis of variance (ANOVA) for the quadratic polynomial regression model for umbelliferone content showed a significant primary linear effect ($p < 0.05$) of ethanol concentration (x_1), time (x_2) and plant-to-solvent ratio (x_3) and a significant primary quadratic effect ($p < 0.05$) of ethanol concentration (x_1^2) and of plant-to-solvent ratio (x_3^2) on the umbelliferone content. The second-order interactions were significant ($p < 0.1$) for ethanol concentration(x_1)/time(x_2^2), ethanol concentration(x_1^2)/time(x_2), ethanol concentration(x_1)/plant-to-solvent ratio(x_3), ethanol concentration(x_1^2)/plant-to-solvent ratio(x_3) and time(x_2)/plant-to-solvent ratio(x_3). The data for 1,2-benzopyrone content also showed a significant primary linear effect ($p < 0.05$) of the plant-to-solvent ratio (x_3) on the 1,2-benzopyrone content. The second order interactions were significant ($p <$ 0.05) for ethanol concentration(x_1)/time(x_2), ethanol concentration(x_1)/plant-to-solvent ratio(x_3^2) and time(x_2)/plant-to-solvent ratio(x_3^2).

Figure 3a-c present the response surface and contour plots for the influences of UAE parameters on coumarin content. As shown in Figure 3a-b, the maximum extraction of umbelliferone was obtained in plant-to-solvent (x_3) range between 0.07 and 0.1 g mL^{-1}, at any of the ethanol concentrations(x_1) and times(x_2), guiding that the maximum extraction point is outside the experimental limit, but due to high intumescence of the plant material this test is not viable.

In Figure 3c, the maximum extraction of umbelliferone was obtained in low ethanol concentration(x_1) at any of the times(x_2). Umbelliferone is slightly soluble in water, and is freely soluble in ethanol, chloroform, acetic acid and dilute alkaline solution (Macrae & Towers 1984; Vriest *et al.* 1988), which may explain the efficiency of its extraction in ethanol solution. Hydroethanolic solutions are widely used in extractive processes precisely because of their extraction efficiency and low toxicity compared to other organic solvents.

Table 4 – Precision studies for coumarin quantification in the concentrated liquid extract from *Justicia pectoralis* aerial parts.

Coumarins	% Assay (Day-1, Analyst-1)	% RSD of Assay (N = 6)	% Assay (Day-2, Analyst-2)	% RSD of Assay (N = 6)	% RSD Inter -day
1,2-Benzopyrone	0.778	2.77	0.732	5.91	5.35
Umbelliferone	0.096	3.61	0.087	4.65	6.55

Table 5 – Accuracy studies for 1,2-Benzopyrone and Umbelliferone quantification in a concentrated liquid extract from *Justicia pectoralis* aerial parts.

Coumarins	Levels (%)	Amount recovered (mg/mL)	% Recovery	Mean % recovery	% RSD
1,2-Benzopyrone	Low	16.00	95.46		
	Medium	20.00	95.70	94.07	3.65
	High	24.00	91.06		
Umbelliferone	Low	16.00	99.30		
	Medium	20.00	95.57	94.93	4.81
	High	24.00	89.92		

Figure 4a shows that the maximum extraction of 1,2-benzopyrone was obtained in the low ethanol concentration(x_1) at low times(x_2) of the experiment. This effect may be related with the coumarin degradation when the sample was kept longer in the ultrasound bath. Studies about ultrasonically assisted extraction have shown that extraction rates do not increase significantly with increasing extraction times, often causing degradation of various metabolites (Vinatoru 2001; Chanfrau *et al.* 2016). These results indicate the need of the further studies to evaluate possible degradation products can take place during the process.

Evidencing the quadratic effect identified by the model, a lower concentration of ethanol decreased 1,2-benzopyrone extraction when the time of extraction was more than 35 min.

Figure 4b-c shows that the greatest 1,2-benzopyrone contents were observed at

Table 6 – Experimental design (Box-Behnken 3^3) used to analyze the coumarin content (dependent variable) of UAE of *J. pectoralis*, and the process variables X_1, X_2, and X_3.

Run number	X_1 (%)	X_2 (min)	X_3 (g/mL)	Umbelliferone (µg/mL)	1,2-Benzopyrone (µg/mL)
1	15	20	0.07	14.00	166.78
2	45	20	0.07	10.41	105.63
3	15	60	0.07	16.81	109.34
4	45	60	0.07	12.34	118.24
5	15	40	0.10	22.58	161.04
6	45	40	0.10	17.95	171.47
7	15	40	0.05	11.41	84.85
8	45	40	0.05	9.81	86.46
9	30	20	0.10	17.38	150.69
10	30	60	0.10	21.62	163.11
11	30	20	0.05	7.81	71.51
12	30	60	0.05	9.94	85.52
13	30	40	0.07	12.34	111.61
14	30	40	0.07	12.59	110.67
15	30	40	0.07	12.47	106.48

X_1 = ethanol; X_2 = time; X_3 = plant-to-solvent ratio.

Table 7 – Analysis of variance (ANOVA) for the quadratic polynomial regression model for umbelliferone content.

Factor	Sum of squares	Degrees of freedom	Mean squares	F value	p
X_1	24.9761	1	24.9761	1597.62	0.000625*
X_2	12.5611	1	12.5611	803.48	0.001242*
X_3	179.2208	1	179.2208	11464.02	0.000087*
X_1^2	3.1250	1	3.1250	199.89	0.004965*
X_3^2	2.1819	1	2.1819	139.57	0.007089*
$X_1 X_2^2$	0.4186	1	0.4186	26.78	0.035376*
$X_1^2 x_2$	0.3321	1	0.3321	21.24	0.043990*
$X_1^2 X_2^2$	0.0985	1	0.0985	6.30	0.128760
$X_1 X_3$	2.2952	1	2.2952	146.82	0.006742*
$X_1^2 X_3$	0.4705	1	0.4705	30.09	0.031661*
$X_2 X_3$	1.1130	1	1.1130	71.20	0.013757*
Lack-of-fit	0.193	1	0.1936	12.38	0.072124*
Pure error	0.031	2	0.0156		
Sum of squares total	268.3766	14			

X_1 = ethanol; X_2 = time; X_3 = plant-to-solvent ratio *p < 0.05.

Table 8 – Analysis of variance (ANOVA) for the quadratic polynomial regression model for 1,2-benzopyrone content.

Factor	Sum of squares	Degrees of freedom	Mean squares	F value	p
X_1	39.68	1	39.68	5.319	0.147508
X_1^2	1.65	1	1.65	0.221	0.684861
X_2	3.22	1	3.22	0.432	0.578452
X_3	11268.90	1	11268.90	1510.692	0.000661*
$X_1 X_2$	1226.75	1	1226.75	164.456	0.006026*
$X_1 X_3^2$	516.65	1	516.65	69.261	0.014133*
$X_1^2 X_3^2$	1.83	1	1.83	0.246	0.669163
$X_2 X_3^2$	634.75	1	634.75	85.093	0.011549*
$X_2^2 X_3$	2.45	1	2.45	0.329	0.624220
$X_2^2 X_3^2$	38.99	1	38.99	5.227	0.149562
Lack-of-fit	20.08	2	10.04	1.346	0.426266
Pure error	14.92	2	7.46		
Sum of squares total	15885.45	14			

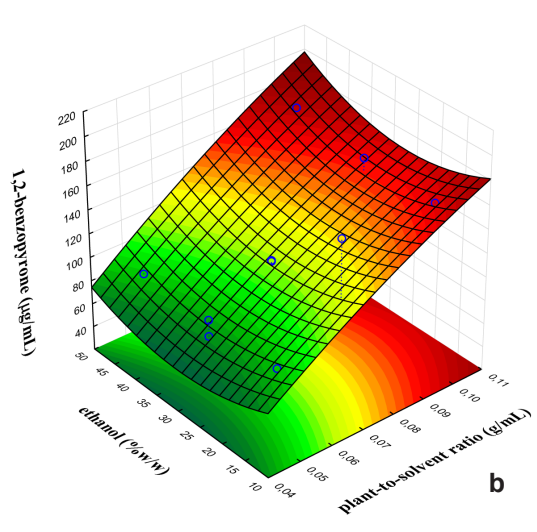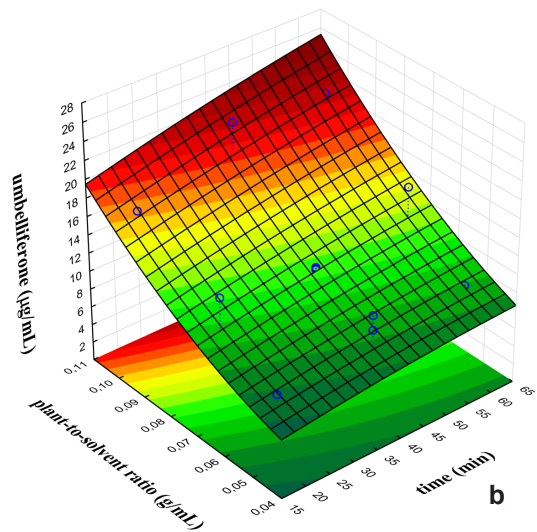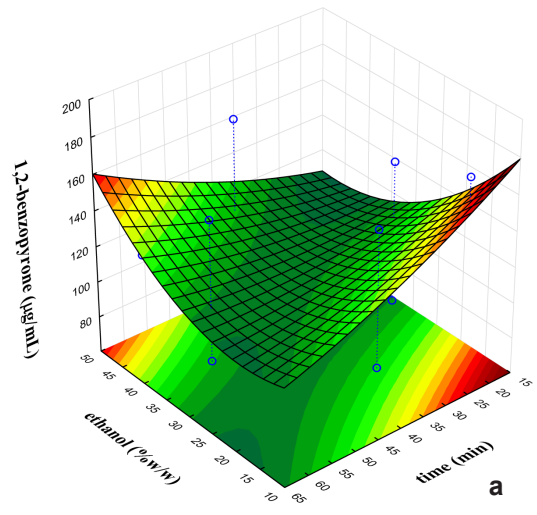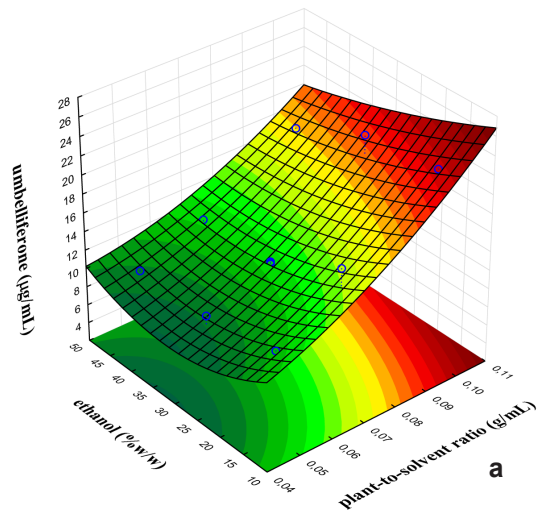X_1 = ethanol; X_2 = time; X_3 = plant-to-solvent ratio *p < 0.05.
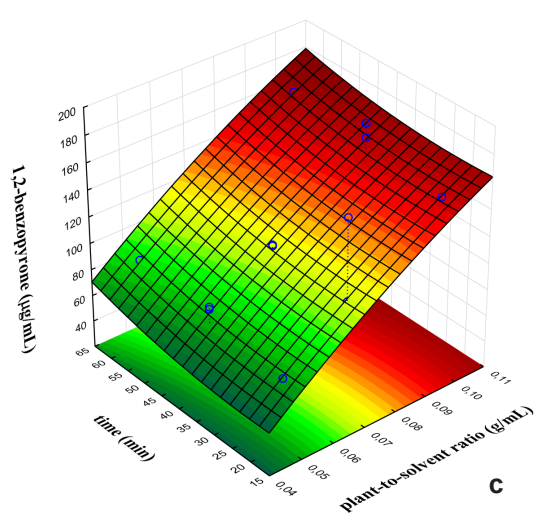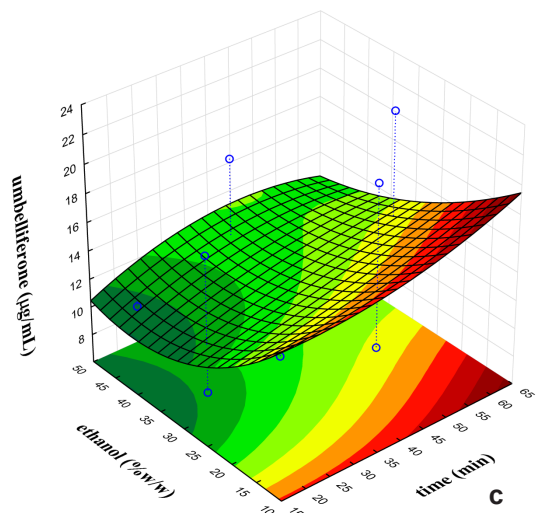
Figure 3 – a-c. Response surface for umbelliferone content from UAE experiments on *J. pectoralis*.

Figure 4 – a-c. Response surface for 1,2-benzopyrone content from UAE experiments on *J. pectoralis*.

plant-to-solvent(x_3) ratios between 0.07 and 0.1 g mL^{-1}, to any of the ethanol concentrations(x_1) and times(x_2) of the experiment, similar results were showed by Fonseca *et al.* (2010), confirming that the parameter plant-to-solvent ratio (x_3) as the most dominant factor influencing 1,2-benzopyrone and umbelliferone extraction.

Analyzed in the response surface graphs (Figs. 3a-b; 4b-c), we verified that the maximum point for the parameter plant-to-solvent ratio (x_3) is outside the experimental area. In these cases, increased levels should be used in new experimental designs to obtain the optimal value. However, this is not feasible experimentally, due to the high intumescence of the plant material, the same limitation was observed by Xinsheng *et al.* (2018).

Figure 5 shows the correlation between the data predicted by the model and the experimental data observed, showing high correlation between both.

The best conditions for UAE of coumarins from aerial parts of *J. pectoralis* in the investigated ranges and obtained from the RSM general optimization function were ethanol concentration of 15% (w/w), extraction time of 34 min, and plant-to-solvent ratio of 0.1 g/mL. Under these conditions, the value predicted for 1,2-benzopyrone content was 163.86 µg/mL and 22.16 µg/mL for umbelliferone. These conditions were validated in independent experiments conducted in triplicate, obtaining an average of 1,2-benzopyrone content of 169.36 µg/mL and 19.77 µg/mL for umbelliferone content. This corresponds to 103.3% of the predicted value for 1,2-benzopyrone and 89.2% for umbelliferone, showing the validity of the model in predicting the phenomenon studied.

The coumarin contents for samples obtained by exhaustive percolation of plant material were 166.78 µg/mL for 1,2-benzopyrone and 16.27 µg/mL for umbelliferone. Others studies demonstrated good extraction yields for coumarins from *J. pectoralis* in hydroalcoholic solution with low ethanol content (30%), but did not define the best ethanol concentration (Chanfrau *et al.* 2016); comparative studies showed the highest yields were obtained using ethanol 50% as the solvent (maceration) front of the ethanol 95% and supercritical CO$_2$ extraction (Molnar *et al.* 2017). The greater efficiency of the UAE was confirmed, showing that is an eco-friendly process because the solvent used is minimal (< 25 mL/sample),
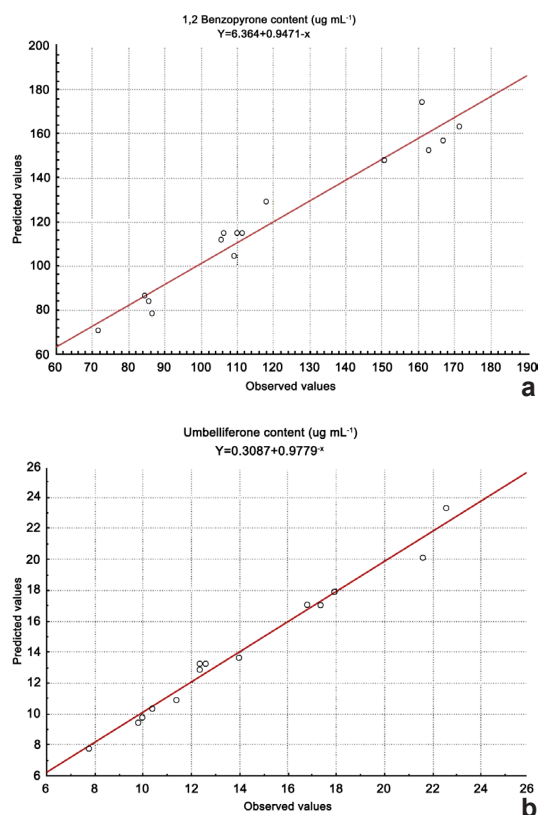

Figure 5 – a-b. Correlation between experimental values (observed values) and predicted values, and predictive equation, estimated using statistical model – a. for the 1,2-benzopyrone content; b. for the umbelliferone content.

with little energy cost in a short time. Therefore, as verified in other studies (Paula *et al.* 2016) UAE can be safely used as an inexpensive and simple extractive method.

Acknowledgments

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