Original article

HPLC-PDA method validated for the determination of hibalactone in *Hydrocotyle umbellata* subterraneous parts and its ultrasound-assisted extraction optimization

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ARTICLE INFO

ABSTRACT

In this work, we developed and validated a HPLC-PDA method for the quantification of hibalactone in *Hydrocotyle umbellata* L., Araliaceae, subterraneous parts extracts and optimized its ultrasound-assisted extraction. Chromatographic separations were carried out with an isocratic mobile phase of acetonitrile/methanol/water (10:65:25), a flow of 0.8 ml min^{-1}, detection at 290 nm and C18 column (250 × 4.6 mm, 5 μm). The method validation parameters were determined according to Brazilian legislation. The optimization of the hibalactone ultrasound-assisted extraction was performed using Box–Behnken design and response surface methodology. The HPLC method for hibalactone quantification proved to be selective, linear, precise, accurate and robust, being useful for the analysis of hibalactone in *H. umbellata* subterraneous parts extracts. The optimal ultrasound-assisted extraction conditions were obtained with solid-to-liquid ratio of 1:5 g ml^{-1}, ethanolic strength of 70% (v/v) and temperature of 65 °C. The results can provide support of the quality control and standardization of raw materials from *H. umbellata*.
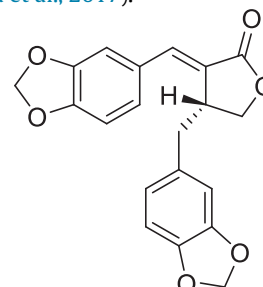
Introduction

Hydrocotyle umbellata L., Araliaceae, also known as acariçoba, is an herbaceous specie native from the American continent that grows mainly on moist soils (Lorenzi and Matos, 2002; Snedden and Steyer, 2013). Acariçoba is a plant with multiple applications in aquatic phytoremediation, cosmetic and medicinal fields (Reis et al., 1992; Sooknah and Wilkie, 2004; Rocha et al., 2011). This specie is also recognized in folk and Ayurvedic medicine (Indian) due to its anxiolytic effects (Ficher et al., 1994).

Acariçoba's active compounds reported include flavonoids, saponins, tannins, polyacetylenes, lignan and essential oils in leaves and subterraneous parts (Martins et al., 2008; Rojas et al., 2009; Oliveira et al., 2017). In a recent work, it was demonstrated that the lignan hibalactone, isolated from the subterraneous parts of

the plant, was the potential active compound responsible for the antinociceptive, anti-inflammatory and anxiolytic-like effects of the specie (Oliveira et al., 2017).

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Hibalactone (**1**), also known as savinin, is a dibenzylbutyrolactone lignan present primarily in woody plant species (Takaku et al., 2001). In addition to the activity regarding *H. umbellata*, hibalactone features a broad spectrum of biological activities, such as

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insecticidal properties (Matsubara, 1972), antimicrobial (Bastos et al., 1999), antitumoral (Chang et al., 2000), anti-estrogenic (Lee et al., 2005), anticholinesterasic and neuroprotective activity (Yoon et al., 2008; Jung et al., 2015).

Because hibalactone's medicinal potential, an appropriate analytical method for its determination is desired for the extracts' standardization of species like *H. umbellata*, as well as for its bioproducts. However, there is a lack of studies reported that address analytical methods for determination of hibalactone. Only a LC–MS/MS method was developed for its determination, however for biological material (Song et al., 2012). HPLC has been described as a method of choice for analysis of lignans in plant material, besides of being accessible to most laboratories (Willfor et al., 2006).

On the other hand, natural sources often do not provide commercially quantities of lignans (Calvo-Flores et al., 2015), which hinders the obtainment of raw materials from *H. umbellata* rich in hibalactone. Accordingly, an efficient extraction method and an investigation of the factors involved in the hibalactone extraction from the plant material are required.

Classical methods, such as maceration, percolation, and reflux are usually reported in studies of hibalactone extraction (Lim et al., 2009; Cuca-Suarez et al., 2015; Jung et al., 2015). However, in recent years, some authors have employed the ultrasound-assisted extraction (UAE) to extract hibalactone from diverse plant species (Yoon et al., 2008; Jeong et al., 2014). Despite that, no study of optimization of its UAE has been reported yet. In the UAE, the cavitation phenomenon leads to the disruption of the cell wall, thus reducing the particle size and increasing mass transfer through the membrane, making it a fast and efficient method (Luque de Castro and Piegro-Capote, 2007; Wang et al., 2013).

The aim of the present study was to develop and validate a HPLC-PDA method for the quantification of hibalactone in *H. umbellata* subterraneous parts extracts and to optimize the UAE of this lignan in raw materials from *H. umbellata*.

Materials and methods

Chemicals and reagents

Hexane P.A. (Neon), dichloromethane (Chemis), ethyl acetate 99.5% P.A. (Neon) and methanol P.A. (Neon) were used in column chromatography (CC). $CDCl_3$ 99.8% was used for NMR analysis. The hibalactone used as standard in HPLC analysis was isolated in our laboratory for this pourpose. Methanol (Tedia, HPLC grade), acetonitrile (Tedia; HPLC grade) and water filtered through a Milli-Q apparatus (Millipore) were used in sample and mobile phase preparations for HPLC analysis.

Apparatus

Column chromatography was conducted on silica gel G60 (0.05–0.2 mm, Vetec, Brazil). Fractions were monitored by thin-layer chromatography (TLC) using silica gel F_{254} (Vetec) and CC silica gel 60G 0.05–0.2 mm (Vetec). To observe the chemical constituents in the TLC plates, UV light at 254 and 365 nm was used, which revealed vanillin–sulfuric acid solution followed by heating. ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectra, HSQC and HMBC data were obtained on a Brüker Avance III spectrometer. An ultrasonic device (USC 1800A, 40 kHz, Uniques) equipped with a digital timer and a temperature controller was used for UAE. Chromatographic separations were performed using a Zorbax Eclipse XDB-C18 reversed-phase column (250 × 4.6 mm, 5 μm). A Waters Alliance with e2695 separation module, 2998 photodiode array detector (PDA), and software Empower (version 2.0) were used. The

mobile phases were filtered through a 0.45 μm PVDF membrane (Merck) and degassed using an ultrasonic bath. Samples and analytical standard solutions were previously filtered through a 0.45 μm PTFE membrane (Millex).

Plant material

Hydrocotyle umbellata L., Araliaceae, subterraneous parts were collected from December 2013 to January 2014 in the Garden of Medicinal Plants of the Faculty of Pharmacy of the Federal University of Goiás, located at 16°40′33″S and 49°14′39″W at an altitude of 768 m above sea level. The authenticity of the plant material was verified by Dr. Heleno Dias Ferreira and Dr. José Realino de Paula, a voucher specimen was deposited in the Herbarium of University of Goiás under registration number UFG-22394. The material was washed with water, desiccated at 40 °C, and ground in a Willye mill. The powder was refrigerated to −6 °C and stored in containers able to provide protection from moisture and light.

Extraction, fractionation and isolation

The ethanolic extract was obtained by stirring maceration, employing 400 g of powdered material and 2 l of ethanol 95% (v/v) as extractor solvent, for 4 h. In order to obtain an efficient extractive process, the maceration was carried out more twice, thereby obtaining the crude ethanolic extract, which was concentrated on a rotary evaporator (MA-120, Tecnal) at 40 °C and after stored at −6 °C away from light. Crude extract (30 g) were dissolved in 250 ml of methanol–water solution (7:3). The mixture was subjected to liquid–liquid partition with increasingly polarity solvents (*i.e.*, hexane, DCM, and EtOAc) (Ferri, 1996). Hibalactone was isolated from the DCM fraction (1.5 g) according to Oliveira et al. (2017), affording 25 mg of pure substance. ^{1}H NMR, ^{13}C NMR, HMBC and HSQC spectral data were used to identified the isolated compound.

HPLC-PDA analysis

Preliminary tests for the HPLC-PDA method development

The HPLC-PDA analytical method for hibalactone quantification in the extracts was developed based on the previous work of Schmidt et al. (2006) for identifying this marker, by varying the composition of mobile phase (acetonitrile/methanol/water) and flows ranging from 0.5 to 1 ml min^{-1}. To evaluate the test-conditions, a hibalactone standard (200 μg ml^{-1} in methanol) and ethanolic extract of *H. umbellata* subterraneous parts were analyzed. The injection volume was 10 μl and the experiments were performed in triplicate. The conditions that provided the best hibalactone separation from ethanolic extract of the plant were employed for the proposition of this method.

Instrumentation and chromatographic conditions

The HPLC system consisted of a Waters Alliance with e2695 separation module and 2998 photodiode array detector (PDA). Data acquisition was performed by using the Empower software (version 2.0). Chromatographic separations were carried out using a Zorbax Eclipse XDB-C18 reversed-phase column (250 × 4.6 mm, 5 μm). The temperature column was kept as 25 °C and the injection volume was 10 μl. Satisfactory separation was obtained with an isocratic mobile phase with acetonitrile/methanol/water (10:65:25) at a flow rate of 0.8 ml min^{-1}. The detection wavelength was 290 nm. The mobile phases were previously filtered through a 0.45 μm PVDF membrane (Merck) and degassed using an ultrasonic bath.

Standard preparation

To prepare standard solutions, 8.15 mg of isolated hibalactone were dissolved in methanol and then diluted to 10, 25, 50, 100, 150

and $200\,\mu g\,ml^{-1}$. Prior to injection in HPLC system, the solutions were filtered through a 0.45 μm PTFE membrane (Millex).

Sample preparation

To evaluate the development and validation of the method, 2 g of the powdered dried *H. umbellata* subterraneous parts was ultrasonic extracted with 20 ml of ethanol 80% (v/v) for 30 min at 25 °C. Prior to injection in HPLC system, the solution was filtered through a 0.45 μm PTFE membrane (Millex).

System suitability test

The system suitability parameters for hibalactone peak (tailing factor – TF, resolution – R_s and number of theoretical plates – N) were evaluated according to prerogatives of the United States Pharmacopeia and Food and Drug Administration (US-FDA, 2001) and Ribani et al. (2004), and expressed as mean values from six determinations ($\pm$SD).

HPLC-PDA method validation

The method was validated according to Agência Nacional de Vigilância Sanitária (Anvisa – Brazilian National Health Surveillance Agency) guidelines (Anvisa, 2003). The validation procedure included an evaluation of the following parameters: selectivity, linearity, limit of detection and limit of quantification, precision, accuracy and robustness.

Selectivity

The selectivity of the method was assessed comparing the chromatograms of the standard solution, sample solution, mobile phase and the blank (methanol). The spectral similarity of hibalactone peaks in the standard and sample was also evaluated by comparing the UV spectra in the wavelength range of 190–400 nm.

Linearity

The linearity was determined by the calibration curves obtained from HPLC analysis at six concentration levels of the hibalactone standard (10, 25, 50, 100, 150 and $200\,\mu g\,ml^{-1}$) in methanol. Each point was performed in triplicate, and the calibration curve was fitted by linear regression from the correlation between the peak areas and the concentration of the standard. The linear regression coefficients (r) and analysis of variance (ANOVA) were calculated.

Limit of detection and limit of quantification

The limit of detection (LD) and limit of quantification (LQ) were calculated based on the standard deviation (SDb) of the intercept with the y-axis and the slope of the calibration curve (S) according to Eqs. (1) and (2):

$$LD = \frac{SDb \times 3}{S}, \tag{1}$$

$$LQ = \frac{SDb \times 10}{S}. \tag{2}$$

Precision

The precision was evaluated in terms of repeatability (intra-day) and intermediate precision (inter-day), using the relative standard deviation (RSD) as criteria. The repeatability was verified from three replicates of the high, mean and low concentrations of the standard linear range, comprising a total of nine injections of the test concentrations. The intermediate precision was evaluated by this same process, performed on a different day by different analyst.

Accuracy

The accuracy was determined by recovery analysis. Sample solutions were prepared, in triplicate, at three concentration levels

Table 1
Levels of variables for the Box–Behnken (3^3) experimental design.

Symbols	Independent variables	Levels		
		−1	0	+1
X_1	Solid-to-liquid ratio (g ml^{-1})	1:10	1:7.5	1:5
X_2	Ethanolic strength % (v/v)	50	60	70
X_3	Extraction temperature (°C)	45	55	65

corresponding to 50%, 100% and 150% of the standard concentration in the linear range, with and without the addition of a known amount of the hibalactone standard ($42.60\,\mu g\,ml^{-1}$). The accuracy was calculated for each level through the ratio between the average experimental concentration and theoretical concentration of the added standard according to Eq. (3).

$$\text{Accuracy} = \frac{\text{Experimental concentration}}{\text{Theoretical concentration}} \times 100. \tag{3}$$

Robustness

The robustness of the method was evaluated by varying the column oven temperature of 25–26 °C and 27 °C; the composition of mobile phase of acetonitrile/methanol/water (10:65:25) to (5:70:25) and (10:60:30); and the Zorbax Eclipse XDB-C18 reversed-phase column dimensions of (250×4.6 mm, 5 μm) to (150×4.6 mm, 5 μm). Results were compared with that obtained with the original set of chromatographic conditions. The injections were performed in triplicate and the data were evaluated by the RSD calculation.

Ultrasound-assisted extraction

Previous assays for UAE optimization

Initially, the UAE were conducted with a solid-to-liquid ratio (*SLR*) of 1:30–1:10 g ml^{-1}, ethanolic strength (*ES*) of 65–95% (v/v), extraction temperature (*ET*) of 25–55 °C and extraction time of 15–45 min. The experiments were carried out following a Box–Behnken design associated with the response surface methodology (RSM) with four factors and three levels. The conditions that provided the highest concentrations of hibalactone, quantified by HPLC-PDA, formed the basis for optimization design.

Experimental design

The optimization of the hibalactone UAE of *H. umbellata* was performed from the experiments set out in the Box–Behnken design, as shown in Table 1. Three factors that affect extraction efficiency in three levels (3^3) were investigated: *SLR* of 1:10, 1:7.5 and 1:5 g ml^{-1} (X_1); *ES* of 50, 60 and 70% (v/v, X_2); *ET* of 45, 55 and 65 °C (X_3). The extraction time was fixed at 15 min. The complete design was carried out in random order and consisted of seventeen combinations, including five replicates at the central point to estimate the pure error and the adequacy of the fitted model (Lundstedt et al., 1998). A second-order polynomial regression model was used to express the hibalactone content as a function of the independent variables (Eq. (4)).

$$y = \beta_o + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j, \tag{4}$$

where y is the predicted response (hibalactone content), β_0 is a constant, and β_i, β_{ii} and β_{ij} are the linear, quadratic and interactive coefficients of the model, respectively. Accordingly, x_i and x_j represent the levels of the independent variables.

The influence of the independent variables on the hibalactone content was analyzed by RSM. The statistical analysis were conducted using Statistic software version 7.0 (StatSoft, 2004) and the effects were considered significant for a $p < 0.05$.

Table 2
Mean (±SD) of the system suitability parameters for chromatographic system, obtained from six hibalactone determinations in the standard and sample solution.

	System suitability		
	Tailing factor (TF)	Resolution (R_s)	Theoretical plates (N)
Standard (200 μg ml^{-1})	1.25 (±1.21)	–	7073.245 (±0.91)
Sample hibalactone peak	1.31 (±0.35)	2.92 (±2.65)	6870.248 (±0.92)
Literature specifications(US-FDA, 2001; Ribani et al., 2004)	≤2	≥2	>2000

Results and discussion

Characterization of compound isolated

The ^{1}H and ^{13}C NMR data of the compound showed characteristic signals of lignans in good agreement with literature (Feliciano et al., 1987; Lim et al., 2009; Oliveira et al., 2017). ^{1}H NMR spectrum indicates the existence of two methylenedioxy groups attached to the aromatic rings A and B (δ 6.04 and 5.93, respectively). ^{13}C NMR spectrum suggest the presence of a dibenzylbutyrolactone by the signals for unhydrogenated carbon (δc 172.7), typical for a lactone five-membered, and a carbon oxymethylene (δc 69.5) (Supplementary material).

^{1}H NMR (500 MHz, CDCl$_3$) δ: 7.04 (1H, d, J = 1.66 Hz, H-2), 6.64 (1H, dd, J = 1.74 Hz, H-5), 7.08 (1H, dd, J = 1.74 Hz, H-6), 7.50 (1H, d, J = 1.93 Hz, H-7), 6.04 (-OCH$_2$O, s, H-10), 6.88 (1H, d, J = 8.05 H-2′), 6.73 (1H, d, J = 7.82 Hz, H-5′), 6.67 (1H, d, J = 1.66 Hz, H-6′), 2.59 (1H, dd, J = 10.07 Hz, H-7′), 3.74 (1H, m, H-8′), 4.26 (1H, m, H-9′), 5.93 (-OCH$_2$O, dd, J = 1.45 Hz, H-10′) (Fig. A1); 2D NMR (HSQC/HMBC – 125 MHz, CDCl$_3$) δ: 126.1 (C-1), 108.3 (C2-H), 148.96 (C-3), 148.7 (C-4), 109.4 (C5-H), 126.2 (C6-H), 137.29 (C7-H), 126.0 (C-8), 172.7 (C-9), 101.83 (CH$_2$), 131.3 (C-1′), 108.8 (C2′-H), 149.17 (C-3′), 146.9 (C-4′), 108.3 (C5′-H), 122.4 (C6′-H), 37.9 (C7′-2H), 40.5 (C8′-H), 69.5 (C9′-2H), 101.03 (C10′-2H) (Fig. A2).

HPLC-PDA method development

In order to validate a versatile and efficient method for the quantification of hibalactone (**1**) in *H. umbellata* subterranean parts extracts preliminary tests were performed to select adequate and optimum conditions. Initially, acetonitrile/methanol in different ratios were tried at a flow of 0.5 ml min^{-1}, but no separated peaks were found. An addition of water on the mobile phase composition and its increase until the final proportion of acetonitrile/methanol/water (10:65:25) contributed significantly for separation of hibalactone with a retention time of 12 min.

This may due to the weakly polar character of hibalactone molecule, being preferentially attracted to the stationary phase, making its elution delayed in a reversed-phase stationary phase (C18) and thus favoring its separation. However, increasing the flow to 1 ml min^{-1} it reduced peak resolution. The balance reached between the retention time and peak resolution was found with the flow of 0.8 ml min^{-1}.

Then, the mobile phase of acetonitrile/methanol/water (10:65:25) at a flow rate of 0.8 ml min^{-1} afforded the best separation of hibalactone, with a retention time of 7.35 min. These chromatographic conditions showed the system suitability parameters for an hibalactone peak (tailing factor – TF, resolution – R_s, and number of theoretical plates – N) according to United States Pharmacopeia and Food and Drug Administration (US-FDA, 2001) and Ribani et al. (2004) (Table 2).

HPLC-PDA method validation

The chromatographic profile and UV spectrum of hibalactone obtained from the HPLC-PDA analysis of hibalactone standard (200 μg ml^{-1}), sample extract and methanol are shown in Fig. 1A–C,

respectively. These chromatographic profiles revealed no interfering substances at the retention time of hibalactone. In addition, the UV spectrum of hibalactone in the sample was found to be identical to the standard, demonstrating the selectivity of the method.

The results of the method linearity are presented in Table 3 and its calibration curve is shown in Fig. 2. The hibalactone calibration curve showed a linear response within the range of 10–200 μg ml^{-1} and the linear equation was: $y = 23,517x + 208,526$. The RSD% for the slope of the hibalactone calibration curve was 1.16%, which is in accordance with limits set by the specifications (RSD < 5%) (Anvisa, 2003). The analytical curve presented a linear correlation higher than 0.99, which is an evidence of a fit of the data to the regression line.

The linearity data were also evaluated by ANOVA test (Table 4), which showed that the calculated F value for the model was higher than the tabulated F value for a confidence level of 95%, demonstrating that the model was suitable to predict the data.

The LD value was determined to be 0.035 μg ml^{-1}, which represents the lowest amount of analyte detectable in the sample but not necessarily quantified (Ribani et al., 2004). Concerning the LQ value, it was determined to be 0.116 μg ml^{-1}, which represents the lowest measurable concentration of analyte in the sample (Ribani et al., 2004). The experiments were performed in a range above the limits and, thus, the concentration values obtained for hibalactone were appropriated.

Concerning the precision of the method, the RSD values were less than 5% among triplicates of low, medium and high concentrations, as recommended by specifications (Anvisa, 2003) (Table 5). The precision at repeatability level indicates the correlation between the results of the method performed under the same conditions within a period. Whereas the intermediate precision indicates that although with different analysts on different days, the method can provide the same results (Anvisa, 2003).

The accuracy of the method afforded recovery ranging from 97.67% to 103.28% with an average of 100.34% and RSD of 2.07% (Table 6). These data are in agreement with the acceptable recovery intervals for tests in complex matrices (80–120%), such as natural products (Betz et al., 2011). The recovery test quantifies the amount of analyte added in the test material that is extracted and capable of being quantified (Thompson et al., 1999).

Concerning the robustness, variations in the column oven temperature, mobile phase composition, and column dimension resulted in RSD values below 5% for peak area and hibalactone content (Table 7), demonstrating the ability of the method to remain unaffected by the these tested variations, in addition to contribute to the transference of the analytical process to other laboratories (Fucina et al., 2012).

Previous assays for UAE optimization

The screening of the main factors affecting the hibalactone extraction efficiency from the plant material indicated the following influences on the hibalactone content: significant linear effect (p = 0.050) of *ET* in the range of 25–55 °C and significant linear effect (p = 0.001) and quadratic effect (p = 0.0028) of *SLR* in the range of 1:30–1:10 g ml^{-1}. No significant interactions were found among the factors evaluated. The Response Surface Quadratic
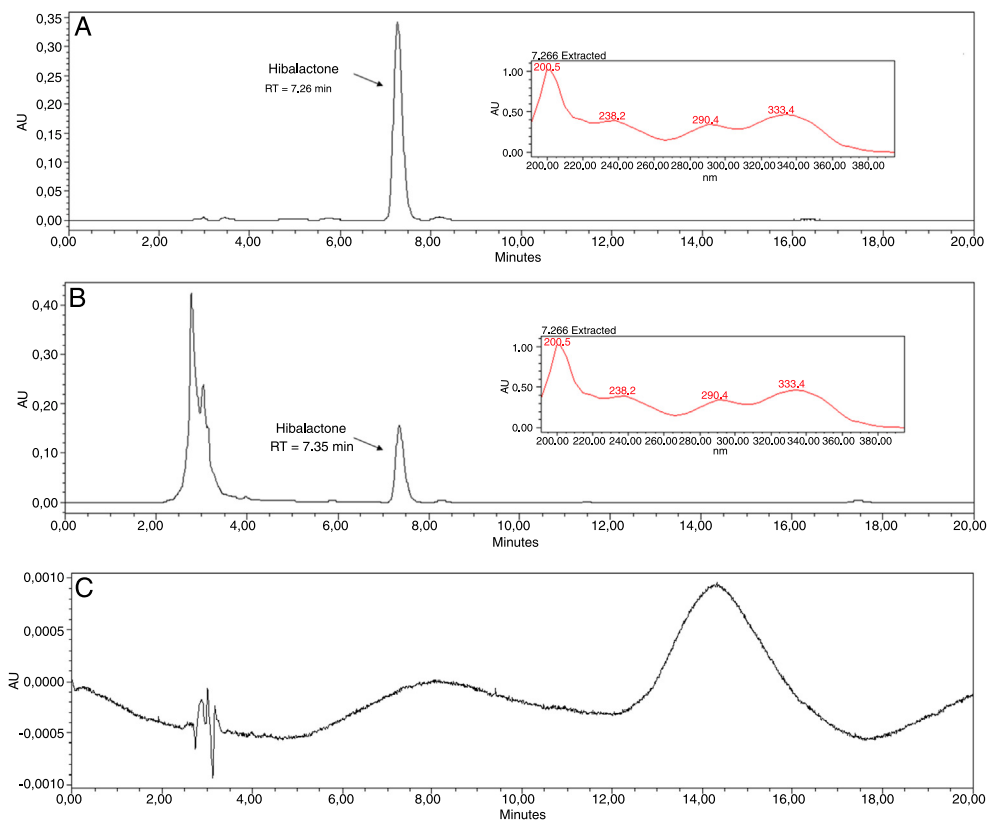
Fig. 1. HPLC-PDA chromatographic profiles of: hibalactone standard (A); sample extract (B); and methanol (C), followed by UV spectra of hibalactone (190–400 nm). Chromatographic conditions: C18 reversed-phase column (250 × 4.6 mm, 5 μm); mobile phase acetonitrile/methanol/water (10:65:25); flow rate of 0.8 ml min^{-1}; column temperature of 25 °C; λ = 290 nm; injection volume of 10 μl.

Table 3
Summary of the calibration curve parameters of hibalactone.

Validation parameters	Hibalactone
Linear range (μg ml^{-1})	200–10
Limit of detection (μg ml^{-1})	0.035
Limit of quantification (μg ml^{-1})	0.116
Linear regression data	
N	6
Slope (*a*)	23,516.79
Standard deviation of slope	274.4236
Relative standard deviation of slope (%)	1.16
y-Axis intercept (*b*)	208,526
Linear correlation coefficient (*r*)	1

Fig. 2. Hibalactone calibration curve in the concentration range of 10–200 μg ml^{-1}.

Model generated from these data was not suitable for determination of extraction optimal conditions due to a significant lack-of-fit ($p = 0.0006$), thus new experiments with different levels of factors were conducted. As the extraction time in the range of 15–45 min showed no influence on the hibalactone extraction ($p = 0.970$), we decided to keep it constant at 15 min, according to the main advantages of UAE that it does not require high times for extraction (Luque de Castro and Piegro-Capote, 2007).

Effect of UAE factors on the hibalactone content

The experimental design of hibalactone UAE was carried out following a Box–Behnken design associated with RSM. The use of these statistical tools allows finding the best set of factors that produce the optimum response with fewer test runs in comparison with other factorial models (Bezerra et al., 2008; Grosso et al., 2014). Table 8 presents the data of hibalactone contents from the seventeen experiments generated by Box–Behnken design.
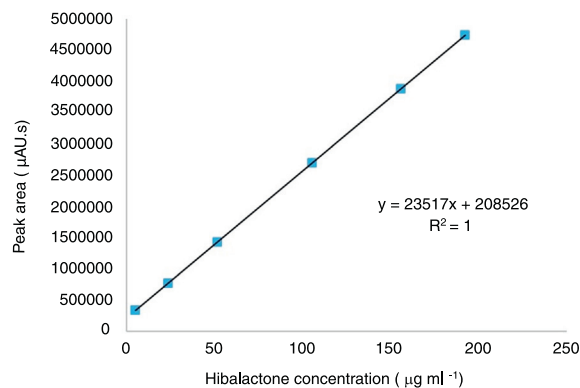
The concentrations of hibalactone varied between 0.0859 and 0.2124 mg ml^{-1}. The conditions for experiment 12 provided the extraction of hibalactone with the highest efficiency, which were further optimized by RSM.

The influence of extraction factors with the response investigated (hibalactone content) was evaluated by ANOVA (Table 9). Concerning the model generated, the R^2 and R^2 adj values of 0.99 and 0.98, respectively, indicated a good correlation between the experimental and predicted values of the response. Moreover, the quadratic model contribution was significant ($p < 0.0001$) and the lack-of-fit was insignificant ($p = 0.4734$), revealing that the model can adequately fit the experimental data.

ANOVA showed that all the three factors evaluated had a significant primary linear effect on the hibalactone content, as is seen in Table 9. Among the factors, *SLR* was found to have the

Table 4
ANOVA data for hibalactone linearity.

	DF	SS	MS	F	F_{tab}
Model	1	4.64894E+13	4.64894E+13	22,153.427	2.2E−16
Residual	16	2.7977E+11	1.7486E+10		
Lack-of-fit	4	2.54587E+11	6.3647E+10	30.329	3.436E−06
Pure error	12	2.5182E+10	2.0985E+09		

DF = degrees of freedom; SS = sum of squares; MS = mean squares; F = calculated F value; F_{tab} = tabulated F value.

Table 5
Data of HPLC analytical method precision at repeatability and intermediate precision levels for the hibalactone quantification in the sample.

Concentration level of the linear range of the method	Sample concentration (mg ml^{-1})	Area (µAU s)	Hibalactone content (mg ml^{-1})	Hibalactone (%)
Intra-day (day 1, analyst 1)				
	50.075	1,062,454	0.0363	0.0725
Low (37 µg ml^{-1})	50.000	1,080,791	0.0371	0.0742
	50.350	1,091,295	0.0375	0.0746
	100.130	2,107,112	0.0807	0.0806
Medium (80 µg ml^{-1})	100.055	2,108,907	0.0808	0.0808
	100.190	2,113,786	0.0810	0.0809
	150.195	2,902,183	0.1145	0.0763
High (110 µg ml^{-1})	150.250	2,740,178	0.1077	0.0716
	150.100	2,807,388	0.1105	0.0736
RSD% Intra-day (day 2, analyst 2)				4.86
	50.225	1,063,264	0.0363	0.0724
Low (37 µg ml^{-1})	50.170	1,055,136	0.0360	0.0718
	50.055	1,055,430	0.0360	0.0719
	100.300	2,075,883	0.0794	0.0792
Medium (80 µg ml^{-1})	100.315	2,066,681	0.0790	0.0788
	100.065	2,053,727	0.0785	0.0784
	150.270	2,766,609	0.1088	0.0724
High (110 µg ml^{-1})	150.360	2,687,540	0.1054	0.0701
	150.425	2,827,878	0.1114	0.0740
RSD% inter-day (intermediate precision)				4.69
RSD%				4.80

RSD% = relative standard deviation.

Table 6
Data of HPLC analytical method accuracy for the hibalactone quantification in the sample.

Concentration level of the linear range of the method	Hibalactone area in the sample (µAU s)	Hibalactone area in the sample + hibalactone standard (µAU s)	Hibalactone recovery area (µAU s)	Hibalactone content (mg ml^{-1})	Recovery (%)
	527,575	1,577,148	1,209,098	0.0425	99.72
Low (37 µg ml^{-1})	527,014	1,569,676	1,201,906	0.0422	99.00
	508,292	1,547,026	1,188,617	0.0417	97.67
	1,264,397	2,165,788	1,218,152	0.0429	100.62
Medium (80 µg ml^{-1})	1,224,099	2,112,138	1,192,710	0.0418	98.08
	1,276,744	2,166,606	1,210,327	0.0426	99.84
	1,599,658	2,557,239	1,235,807	0.0437	102.38
High (110 µg ml^{-1})	1,564,023	2,537,740	1,244,816	0.0441	103.28
	1,557,472	2,524,455	1,236,772	0.0437	102.47
Theoretical concentration of hibalactone standard (mg ml^{-1})					0.0426
Average recovery (RSD%)					100.34 (2.07)

RSD% = relative standard deviation.

Table 7
Chromatographic parameters on the robustness testing of the HPLC analytical method for hibalactone quantification in the sample.

Parameter	Average (RSD%$_{intra}$)		
	Area (µAU s)	Hibalactone (%, w/w)	RSD%$_{inter}$
Original method	2,149,913 (0.43)	0.0825 (0.47)	–
Column oven temperature (°C)			
26	2,164,591 (0.51)	0.0831 (0.57)	0.48
27	2,202,867 (1.10)	0.0848 (1.22)	1.72
Mobile phase			
Acetonitrile/methanol/water (5:70:25)	2,159,388 (0.34)	0.0829 (0.37)	0.31
Acetonitrile/methanol/water (10:60:30)	2,158,220 (0.83)	0.0829 (1.05)	0.27
Column dimension			
Zorbax Eclipse XDB-C18 (150 × 4.6 mm, 5 µm)	2,121,751 (0.30)	0.0813 (0.33)	0.93

RSD% = relative standard deviation.

Table 8
Box–Behnken design arrangement for UAE parameters and the response of the hibalactone content.

Run	SLR (g ml⁻¹)	ES (%)	ET (°C)	Hibalactone (mg ml⁻¹)
1	1:10	50	55	0.0906
2	1:10	70	55	0.1022
3	1:5	50	55	0.1731
4	1:5	70	55	0.2100
5	1:7.5	50	45	0.1053
6	1:7.5	70	45	0.1228
7	1:7.5	50	65	0.1338
8	1:7.5	70	65	0.1480
9	1:10	60	45	0.0859
10	1:5	60	45	0.1788
11	1:10	60	65	0.1057
12	1:5	60	65	0.2124
13	1:7.5	60	55	0.1293
14	1:7.5	60	55	0.1391
15	1:7.5	60	55	0.1379
16	1:7.5	60	55	0.1355
17	1:7.5	60	55	0.1374

Table 9
Analysis of variance (ANOVA) for the quadratic polynomial regression model factor.

Source	Sum of squares	Degrees of freedom	Mean squares	F value	p
Linear					
SLR	0.019007	1	0.019007	1247.267	<0.0001
ES	0.000803	1	0.000803	52.69839	0.0002
ET	0.00143	1	0.00143	93.80788	<0.0001
Quadratic					
SLR^2	0.000732	1	0.000732	48.04676	0.0002
ES^2	0.000107	1	0.000107	7.013122	0.0330
ET^2	0.0000464	1	0.0000464	3.047984	0.1243
Interaction					
SLR × ES	0.000159	1	0.000159	10.44798	0.0144
SLR × ET	0.0000478	1	0.0000478	3.139741	0.1197
ES × ET	0.00000262	1	0.00000262	0.172102	0.6907
Model	0.022296	9	0.002477	162.5661	<0.0001
Lack-of-fit	0.0000461	3	0.0000154	1.01614	0.4734
Pure error	0.0000605	4	0.0000151		
Sum of squares total	0.022403	16			
R^2	0.9909				
Adjusted R^2	0.9855				

higher influence in hibalactone extraction ($p < 0.001$), followed by ET ($p < 0.001$) and ES ($p = 0.0002$) (Fig. 3). The SLR and the ES also displayed quadratic behaviors on the response. Moreover, the interactive effect of these both factors (SLR × ES) was the only significant ($p = 0.0144$). The fitted equation obtained for the hibalactone content response, with correlation coefficient of $r = 0.9909$ and adjusted $R^2 = 0.9855$, is shown in Eq. (5):

$$\text{Hib. } (\mu g\ ml^{-1}) = 0.13 + 0.01ES + 0.049SLR + 0.013ET + 0.00063ES \times SLR - 0.0052ES^2 + 0.013SLR^2. \quad (5)$$
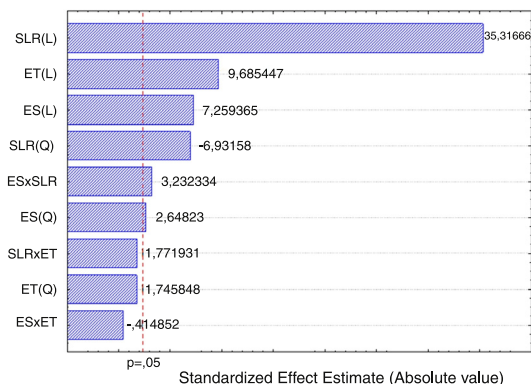

Fig. 3. Pareto's chart of the main effects with interactions and their significance on the hibalactone content.

Fig. 4 shows the surface response plot for hibalactone content as a function of SLR and ES. The plot clearly shows that higher levels of SLR (1:5 g ml⁻¹) can promote increased levels of hibalactone, which is also associated with higher levels of ES (70%, v/v).

Increasing the SLR ratio it was demonstrated to be highly significant for the hibalactone extraction. The use of high quantities of solvent can lead to high operating cost and energy consumption, being not considered cost-effective (Chemat et al., 2012). Guo et al. (2015) also found that the SLR was the extraction factor most significant in the extraction of three lignans structurally related with hibalactone, namely, (−)-fargesin, sesamin, and L-asarinin.

Hibalactone is soluble in organic solvents and slightly soluble in water (Yamashita and Matsui, 1960) and thus solutions with higher ES (70%, v/v) have superior efficiency of its extraction. The use of hydroethanolic solutions have been described as the optimal extraction solvent for extract lignans in RSM studies, besides its low toxicity compared to other organic solvents (Zhang et al., 2007; Cheng et al., 2016).
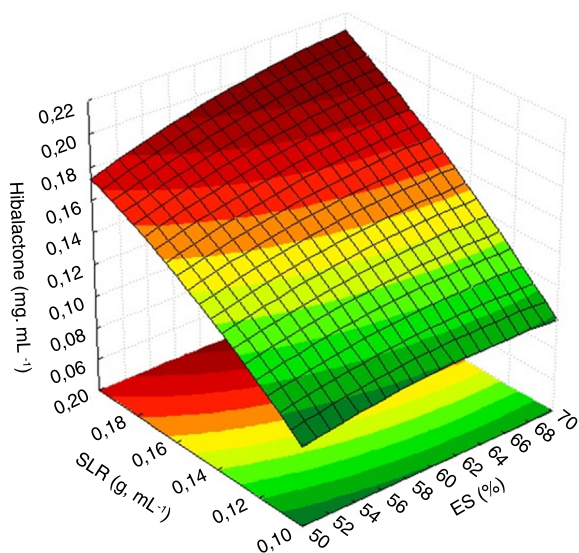
Fig. 4. Surface plot of hibalactone content from UAE experiments as a function of *SLR* and *ES*.

With respect to *ET*, this effect showed significance on hibalactone extraction at higher levels (65 °C). In UAE, higher temperatures may increase the cavitation effects, in addition to enhancing the desorption and solubility of compounds from the cells, thus favoring the extraction process (Capelo-Martínez, 2009). However, there is no data regarding thermal stability of hibalactone, which studies may contribute to a better knowledge of the influence of this factor in the hibalactone extraction.

Verification of the RSM model

The experimental results showed good agreement with the predicted data, as can be seen in Fig. 5. Thus, the statistical models generated were able to explain the influence of the main factors affecting the hibalactone extraction efficiency from *H. umbellata* subterraneous parts.

The best conditions for UAE of hibalactone (**1**) from *H. umbellata* subterraneous parts within the investigated ranges and obtained from the RSM optimization function were: *SLR* of 1:5 g ml^{-1}, *ES* of 70% (v/v), and *ET* of 65 °C. The value predicted for hibalactone content, under these conditions, was 210 μg ml^{-1}. The independent experiments conducted in triplicate resulted in an average of hibalactone content of 207.005 μg ml^{-1}, which corresponds to 98.57% of
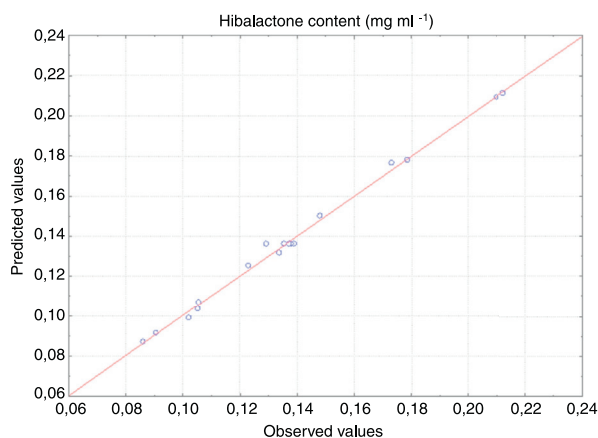
the predicted value, demonstrating that the model was suitable to predict the data. Moreover, the optimum conditions obtained from the RSM may be useful to scale up the ultrasonic extraction process of hibalactone from *H. umbellata*.

Conclusion

The HPLC-PDA analytical method developed for hibalactone quantification was validated and demonstrated to be selective, linear, precise, accurate and robust, being useful for the analysis of this lignan in *H. umbellata* subterraneous parts extracts. The RSM has been successfully employed in determining the optimum conditions for the UAE of hibalactone, and the following conditions were established: solid-to-liquid ratio of 1:5 g ml^{-1}, ethanolic strength of 70% (v/v) and extraction temperature of 65 °C. Thus, the results can provide support of the quality control and standardization of raw materials from the specie.

Authors' contributions

MGO contributed in running the laboratory work and drafted the paper. PHGA contributed in running the laboratory work. TLSO contributed in collecting plant sample and running the laboratory work. LSS and FSC contributed in the identification of the isolated compound. SFA contributed to chromatographic analysis. LLB contributed to statistical analysis. PAS and VBS contributed to critical reading of the manuscript. JRP contributed in plant identification and herbarium confection, supervised the laboratory work and contributed to critical reading of the manuscript. All the authors have read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgment

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bjp.2018.10.003.

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Fig. 5. Correlation between experimental values and predicted values for the hibalactone content.

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