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Original Article

Development and validation of a stability indicating method for quantification of the sesquiterpene lactone eremantholide C from *Lychnophora trichocarpha* (Brazilian arnica)

Bárbara O. Henriques[a], Dênia A. Saúde-Guimarães[a], Tamires G. Caldeira[b], Jacqueline de Souza[b,*]

[a] *Laboratório de Plantas Medicinais, Escola de Farmácia, Universidade Federal de Ouro Preto, Ouro Preto, MG, Brazil*
[b] *Laboratório de Controle de Qualidade de Medicamentos, Escola de Farmácia, Universidade Federal de Ouro Preto, Ouro Preto, MG, Brazil*

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ABSTRACT

Pharmacological activities as anti-inflammatory, anti-hyperuricemic, anti-gouty arthritis, antitumor and trypanocidal activities of the aerial parts from *Lychnophora trichocarpha* (Spreng.) Spreng. ex Sch.Bip., Asteraceae (Brazilian arnica) have already been proved. Eremantholide C is a sesquiterpene lactone and one of the active chemical constituents responsible for these activities presented by *L. trichocarpha*. Therefore, the aim of this work was to develop and validate a stability indicating HPLC method for eremantholide C. Eremantholide C stability was evaluated in *L. trichocarpha* ethanolic extract and in its isolated form. Analytical conditions employed C18 column, acetonitrile/water in gradient elution, flow of 0.8 ml/min at 30 °C. To correct for the loss of analyte during sample preparation the use of coumarin as an internal standard was necessary. The developed method provides good separation and resolution of the peaks, allowing quantification of eremantholide C, isolated or directly in the ethanolic extract, in internal standard presence. Validation results showed that this method is linear in the concentration range 2–180 μg/ml, precise, accurate and specific. Stability studies showed that *L. trichocarpha* ethanolic extract and eremantholide C remain stable for 6 months when stored at room temperature and impermeable glass bottle, therefore they can be used safely and effectively within this period. While at 40 °C there was stability loss, at 8 °C a stability increase was observed for the extract and the isolated eremantholide C. Forced degradation studies showed that eremantholide C degraded under acidic and alkaline conditions and was stable for three days under neutral and oxidative conditions, and when exposed to high temperature. Thus, with the development of a stability indicative method and the application of it in eremantholide C stability studies, the results can guide the development of new products that adequately preserve the original features of the biologically active substance with quality, safety and efficacy.

Introduction

Aerial parts of *Lychnophora* species ("Brazilian arnica", Asteraceae), a native shrub of Brazilian "cerrado", are commonly used in folk medicine, macerated in alcohol, due its anti-inflammatory, analgesic and anti-rheumatic properties (Cerqueira et al., 1987; Saúde et al., 1998).

Previous studies have demonstrated anti-inflammatory, anti-hyperuricemic, anti-gouty arthritis, antitumor and trypanocidal activities for the *Lychnophora trichocarpha* (Spreng.) Spreng. ex Sch.Bip. ethanolic extract. The sesquiterpene lactone eremantholide C (**1**) is one of the active chemical constituents present in this species responsible for the expression of these activities (Oliveira et al., 1996; Souza et al., 2012; Ferrari et al., 2013; Saúde-Guimarães et al., 2014).

Eremantholide C (**1**) showed significant reduction of carrageenan-induced paw edema in mice. The mechanism of the effect of **1** on the reduction of carrageenan-induced paw edema may be attributed to inhibition of production of TNF-α and stimulation of IL-10 production (Ferrari et al., 2013). The anti-hyperuricemic activity, in order to promote a new pharmacological basis for the treatment of gouty arthritis, was demonstrated reducing the hyperuricemia induced by potassium oxonate in Swiss mice (Souza et al., 2012). Furthermore, trypanocidal and antitumor activities also have been described for eremantholide C.

* Corresponding author.
E-mails: jacsouza@ef.ufop.br, jacsouza@usp.br (J. Souza).

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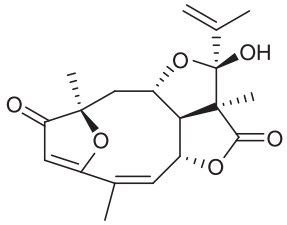
Stability studies demonstrated the ability of a biologically active substance or a drug to remain within the specifications established by keeping their physicochemical properties, potency, purity and quality over a predetermined period opposite to environmental conditions (Bilia et al., 2001; Thakur et al., 2011).

Currently, the use of standardized extract in natural drugs has become common and popular. However, during the manufacture and the extracting process of natural products, in most cases, the active components responsible for the expression of pharmacological activity are exposed to oxidation, hydrolysis, microbial attack or other environmental degradation, which represents a stability problem for the products (Thakur et al., 2011).

Thus, the aim of this work was to develop and validate a new stability indicating HPLC method for eremantholide C (**1**). And besides that, to evaluate the stability of the *L. trichocarpha* ethanolic extract and eremantholide C purified, analyzing the appropriateness of using the extract as phytotherapy and eremantholide C as future potential drug.



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Materials and methods

Plant material

Aerial parts of *Lychnophora trichocarpha* (Spreng.) Spreng. ex Sch.Bip., Asteraceae, were collected in July, 2007, in Ouro Preto, Minas Gerais, Brazil. Voucher specimens were deposited at the Institute of Physical and Biological Sciences, Federal University of Ouro Preto reference number 20635. The plant species were collected with the permission of Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (IBAMA – license number 009/2006).

Preparation of ethanolic extract and obtention of eremantholide C

Aerial parts of *L. trichocarpha* (245 g) were air-dried at 37 °C, ground and extracted exhaustively with ethanol 95%, by percolation. The solvent was removed under reduced pressure and temperature below 40 °C to obtain the dried crude ethanolic extract (31.2 g).

Eremantholide C (**1**) was obtained by chromatographic fractionation according to the methodology described by Saúde-Guimarães et al. (2014). Eremantholide C was identified by infrared spectrometry analysis, nuclear magnetic resonance, melting point and optical rotation whose data is shown in agreement with those published by Le Quesne et al. (1978) and Saúde et al. (1998).

Eremantholide C purity was 97.4% as determined by HPLC analysis.

Eremantholide C (**1**): 6,9-epoxi-2*H*-1,4-dioxaciclodeca [c,d] pentaleno-2,7(4a*H*)-dioxano, 2a,3,5,6,11a,11b-hexaidro-3-hidroxi-2a,6,10-trimetil-3-(1′-metiletenil)-2a*R**,3*S**,4a*R**,6*S**,10*Z*, 11a*S**,11b*S**. Colorless solid, melting point: 213.6 °C to 215.0 °C, $[\alpha]_D^{25}$ =−10.0° (*c* 0.01 g/ml, methanol). IR (KBr) υ_{max} (cm^{-1}): 3450 (OH), 2900 (CH), 1770 (C=O de γ-lactone), 1700 (C=O by conjugated ketone), 1660 (C=C), 1590 (C=C by system C=COR furanone), 1450, 1370, 1350, 1320, 1270, 1220, 1200, 1150, 1100, 1060, 1040, 1000, 960, 920, 810, 730 (C=C). ^{1}H NMR ($CDCl_3$, 400 MHz): 5.63 (s, H-2); 6.04–6.03 (m, H-5); 5.02–4.98 (m, H-6); 2.82 (dd, *J* = 4.3; 7.1 Hz, H-7); 4.09 (ddd, *J* = 2.5; 4.2; 12 Hz, H-8); 2.48 (dd, *J* = 2.5; 13.5 Hz, H-9a); 2.00 (dd, *J* = 12.0; 13.5 Hz, H-9b); 1.18 (s, H-13); 1.45 (s, H-14); 2.05 (t, *J* = 1.9 Hz, H-15); 5.31 (sl, H-2′a); 5.07 (t, *J* = 1.6 Hz); 1.91 (s, H-3′); 3.79 (s, OH). ^{13}C NMR ($CDCl_3$, 75 MHz): 205.89 (C-1); 104.54 (C-2); 187.27 (C-3); 130.00 (C-4); 134.77 (C-5); 81.46 (C-6); 62.53 (C-7); 78.37 (C-8); 43.46 (C-9); 90.24 (C-10); 59.88 (C-11); 175.72 (C-12); 21.94 (C-13); 20.48 (C-14); 20.30 (C-15); 106.09 (C-16); 142.22 (C-1′); 115.80 (C-2′); 19.00 (C-3′).

Method development

The quantification was proposed by HPLC, using Waters Alliance System 2695, consisting of a quaternary pump, degasser, automatic injector of samples and a column oven. Waters PDA 2695 detector and Empower® 2 software were carried out for data analysis. The integration parameters were 30 for peak width and 50 for threshold.

Different chromatographic conditions were evaluated. Two octadecylsilane columns with different particle size, Waters Spherisorb® (150 mm × 4.6 mm, 5 μm) (column 1) and Waters Atlantis T3® (150 mm × 4.6 mm, 3 μm) (column 2) were tested, in both cases C18 Phenomenex® pre-column was used.

Mixtures of mobile phases containing methanol HPLC grade/ultra-pure water obtained by Direct Q® system from Millipore and acetonitrile HPLC grade/ultra-pure water obtained by Direct Q® system from Millipore, using isocratic elution or gradient systems were tested. To choose the better flow rate condition, 0.5, 0.8, 1.0 and 1.2 ml/min were used, and the column temperatures evaluated were at 30 and 35 °C.

For the analyses, sample injection volume was 10 μl, containing internal standard solution with concentration of 100 μg/ml and using Photo Diode Array (PDA) as detector. First, a scan was performed between 200 and 400 nm to extract fingerprint. Eremantholide C (**1**) presented maximum absorption at 265 nm. Because of that, the analytes were evaluated at 265 nm using PDA.

Method validation

Method validation was performed according to ICH recommendations (ICH, 1995). The analytical standard of coumarin was used with ≥99% content, provided by Fênix distributor of excipients. Internal standard was applied in a constant concentration of 100 μg/ml, prepared using a stock solution containing 1000 μg/ml in methanol and then diluted in the working solution also in methanol.

Preparation of standard solutions

The extract samples were prepared by dissolution in methanol HPLC grade to obtain final concentration of 2000 μg/ml. Eremantholide C and coumarin (IS) standard solutions were prepared in methanol HPLC grade with final concentration of 200 and 1000 μg/ml, respectively.

Linearity

Linearity was evaluated by three analytical curves using eight different concentrations for the eremantholide C: 2, 4, 5, 20, 60, 100, 140 and 180 μg/ml.

Precision and accuracy

Repeatability and intermediate precision were investigated by analyzing three replicates of eremantholide C (**1**) standard solution at 5, 60 and 140 μg/ml. The results were expressed in terms of percentage Relative Standard Deviation (% RSD). The accuracy, in turn, was evaluated using the same concentrations mentioned above, however, the results were expressed in terms of percentage relative to Theoretical Value (% TV).

Analytes recovery

Recovery was examined by addition of eremantholide C standard solution at 2, 5, 10 and 15 μg/ml in 1000 μg/ml of extract solution. The results were calculated by eremantholide C concentration obtained after extraction procedure in the extract without standard addition compared to the extract after standard addition.

Specificity

Specificity was evaluated by standard addition method. The extract was analyzed at 1000 μg/ml without standard addition, and after adding 2, 5, 10 and 15 μg/ml of eremantholide C standard solution and IS at 100 μg/ml. In addition, confirmation of the specificity method was performed by analysis of peak purity.

Limits of detection and quantification

Limits of detection (LOD) and quantification (LOQ) were calculated from the slope of the linearity mean curve and the Standard Deviation (SD) between the interception of axis Y of three linearity curves.

Stability studies

Lychnophora trichocarpha ethanolic extract and eremantholide C stability were evaluated during the storage time, at different temperatures and regarding the photostability. The parameters used to evaluate the samples at the end of the stability study were eremantholide C content and the extract fingerprint.

Stability studies of long-term, accelerated and low temperature

Lychnophora trichocarpha ethanolic extract and eremantholide C were stored at different sealed amber bottles and subjected to stability studies of long-term, accelerated (40 °C) and low temperature (8 °C). These studies were carried out for six months and extract samples and isolated substance were collected at the end of months: 1, 2, 3, 4.5 and 6. The proposition of the temperature to be used in each of the stability studies and the times to perform the analysis of the samples were based on the recommendation of the Anvisa guide to stability studies of medicines (Anvisa, 2005). During the planning of the stability study, the conditions were proposed and the number of samples was calculated to perform each study. The aliquots were placed in glass vials separately and at each given time a sample was withdrawn and analyzed. Due to the characteristic variability of natural substances, the aliquots may already vary from one another. In the experiment the variation was less than 3%. Thus decay of the content in values equal to or lower than this variation cannot be directly attributed to the instability of the extract. Fingerprint extract and quantification of eremantholide C content was performed in order to evaluate the decrease of the biologically active substance as well the possible appearance of secondary peaks, indicating the presence of degradation products. The comparison was made by quantifying the eremantholide C in purified sample and extract, confronting with solution freshly prepared.

Box 1
Conditions used in the forced degradation studies of eremantholide C.

Degradation	Experimental conditions
Acid	1 ml of the eremantholide C solution at 1000 μg/ml + 1 ml of the HCl 1 M solution. The resulting solution was stored at room temperature and protected from light for 3 days. Was added 1 ml of NaOH 1 M solution and the volume were completed to 5 ml with methanol to give a eremantholide C final concentration at 200 μg/ml.
Alkaline	1 ml of the eremantholide C solution at 1000 μg/ml + 1 ml of the NaOH 1 M solution. The resulting solution was stored at room temperature and protected from light for 3 days. Was added 1 ml of HCl 1 M solution and the volume were completed to 5 ml with methanol to give a eremantholide C final concentration at 200 μg/ml.
Neutral	1 ml of the eremantholide C solution at 1000 μg/ml + 1 ml of the ultra-pure water. The volume were completed to 5 ml with methanol to give a eremantholide C final concentration at 200 μg/ml. The resulting solution was stored at room temperature and protected from light for 3 days.
Oxidative	1 ml of the eremantholide C solution at 1000 μg/ml + 1 ml of the H_2O_2 10%. The volume were completed to 5 ml with methanol and the resulting solution was stored at room temperature and protected from light for 3 days.

Photostability study

Lychnophora trichocarpha ethanolic extract and eremantholide C samples were stored in different sealed bottles that were subdivided into two groups: colorless bottles, which were exposed to light radiation, and amber bottles which were protected from light radiation. Then, the samples were placed in a dark chamber containing a fluorescent lamp for ten days.

At the end of the exposure period, fingerprint extract and quantification of eremantholide C content of samples subjected to the study were analyzed. Content of samples was compared with content of samples not exposed to light radiation.

Forced degradation studies of eremantholide C

Eremantholide C solutions at 1000 μg/ml were submitted to acid, alkaline, neutral and oxidative degradation according to the protocol described in Box 1. After exposure, the samples were evaluated in comparison to eremantholide C that was used as a control solution at 200 μg/ml.

Furthermore, a sealed amber bottle containing eremantholide C was subjected to 90 °C for three days. After this period, the biologically active substance was dissolved in methanol resulting in a solution at 200 μg/ml, which was taken for quantification. The result obtained was compared with one control solution of eremantholide C, freshly prepared.

Results and discussion

Method development

The best conditions for the analytical method were chromatographic column 2, mobile phase acetonitrile and water in elution gradient, mobile phase flow rate 0.8 ml/min and column temperature 30 °C.

The mobile phase gradient considered the most suitable started with 10% acetonitrile in 5 min, 30% acetonitrile in 10 min, 50% of

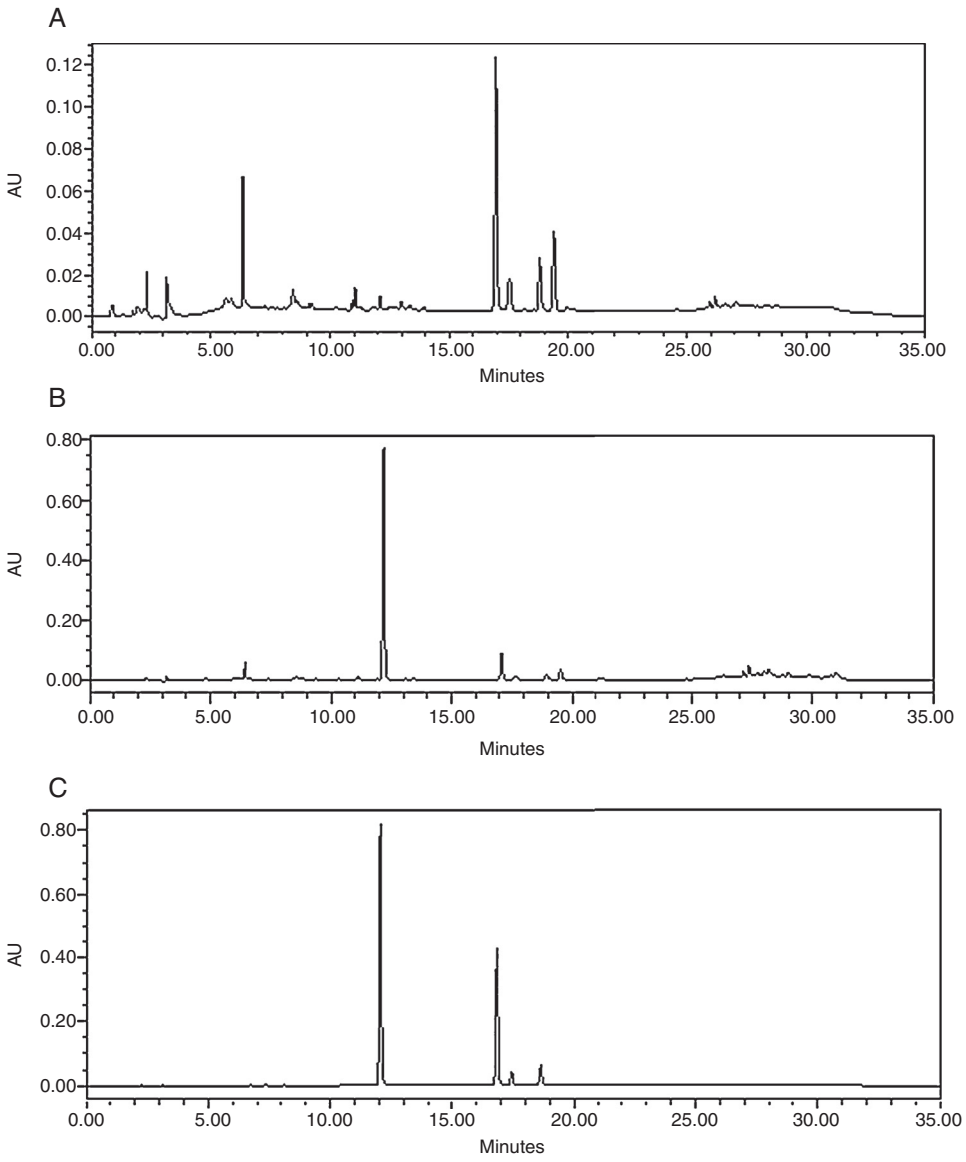

Fig. 1. Chromatograms obtained using the developed method. *Lychnophora trichocarpha* ethanolic extract 500 μg/ml (A); *Lychnophora trichocarpha* ethanolic extract 500 μg/ml + coumarin 100 μg/ml (B); and eremantholide C 180 μg/ml + coumarin 100 μg/ml (C).

each one; in 15 min, 60% acetonitrile in 20 min, 65% acetonitrile; in 23 min, 90% acetonitrile and from 30 to 35 min, 10% acetonitrile. The retention times obtained with this method were 12.3 min for coumarin and 17.1 min for eremantholide C, and the chromatograms obtained are shown in Fig. 1.

The chromatographic column 2 showed better performance in separation and resolution of the peaks. Both columns used have the same filling, with octadecylsilane, but the presence of the stationary phase with smaller particle provides better separation of analytes. Atlantis® HPLC columns are produced by controlling the reaction of a trifunctional C18 silane with specially pretreated silica particles followed by novel endcaping technology, they produced a new sorbent, Atlantis T3®, with an intermediate primary-ligand density (1.6 moles/m^2) approximately half that of the most modern high-ligand-density C18 phases. It has provided good performance, versatility, and retention for polar compounds, while also affording balanced retention for complex mixtures as *L. trichocarpha* ethanolic extract. Latest refinements reflect improving retention, selectivity, peak shape, and stability via substrate synthesis, particle morphology manipulation, and surface modification (Gilar et al., 2005; Mac Donalds et al., 2007). This also allowed obtaining suitable chromatographs profiles, with better resolution (coumarin to eremantholide C (**1**) resolution was 18 and eremantholide C to impurity peak resolution was 2.6) and separation and lower total analysis time, 35 min. It is important to note that these analytical conditions are adequate for the quantification of isolated eremantholide C and also contained in the plant extract, attesting an adequate resolution between the signal of the analyte of interest and the adjacent signals.

Santos et al. (2003) obtained a method with analysis time of 40 min, but employing a mixture of furanoheliangolides standards isolated from the vegetal matrix. According to the master resolution equation, the parameters of Rs = Resolution; n = number of dishes (efficiency); α = separation factor (k_2/k_1); k = retention factor were calculated aiming that changes in k that cause changes in α and n thus improving the Rs of the analytes of interest (Cass and Cassiano, 2015). The number of theoretical plates obtained in the established analytical condition, calculated from the eremantholide C analysis, was 396,906 N/meter. This was a great advantage over the conditions previously described in literature, which used

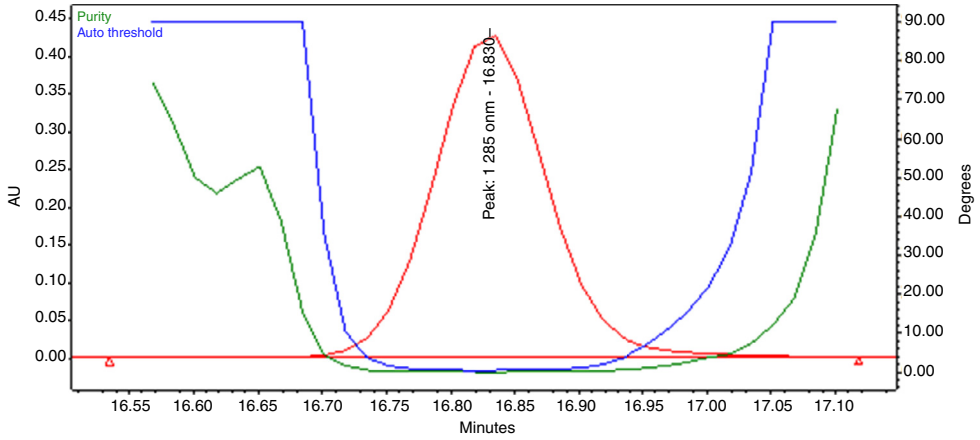

Fig. 2. Purity peak obtained for eremantholide C at 180 μg/ml.

longer chromatographic analyses with 60 min (Gobbo-Neto and Lopes, 2008).

In relation to the mobile phase, it was constituted by acetonitrile, which presents a disadvantage of higher cost in comparison to methanol but it showed better resolution and separation between peaks of eremantholide C, coumarin and interfering substances. Tail factor calculated by dividing the peak width at 5% of height; by twice the value of the anterior portion of the peak, relative to the width at 5% of the height for the signal relative to eremantholide C was equal to 1. Indicating that the experimental conditions allowed to minimize the tail of the chromatographic signal (Snyder et al., 1997). Thus, the gradient elution was defined, with low flow (0.8 ml/min) and temperature of 30 °C. The flow of the mobile phase was altered by monitoring the system pressure. Thus the pressure becomes a dependent variable and the flow was selected that provided better resolution, with pressure appropriate to the analysis.

Coumarin was chosen as internal standard due to citation in other studies in the literature (Savournin et al., 2001; Krieger et al., 2013). The presence of lactone ring α,β-unsaturated 6-membered in the chemical structure of coumarin and the 5-membered in sesquiterpene lactones provides a common chromophore group, allowing maximum absorption at the same wavelengths.

PDA detector was used due to its ability of evaluate different wavelengths simultaneously. This is important in stability studies for monitoring new peaks that can be related to degradation products. The PDA detector enables verify chromatographic purity of a peak and develop a method capable of detecting eremantholide C without interference from other substances. Thus, this detector provides more parameters for monitoring the stability of the extract.

This method, compared to reports in the literature (Saúde, 1994; Santos et al., 2003; Gobbo-Neto and Lopes, 2008), enables to reduce the analysis time, solvent consumption and total cost. Furthermore, it allows the quantification of eremantholide C in presence of IS, isolated and directly in *L. trichocarpa* ethanolic extract, which is a very complex matrix. Thus, this method can be employed in studies of stability of the extracts, quantification of eremantholide C, taking fingerprints of the extract and identification of potential degradation products.

Method validation

Validation results to quantification method of eremantholide C were linear in the range of 2–180 μg/ml, with respectively limits of detection and quantification equal to 0.76 and 2.54 μg/ml. The method was precise since it showed a value of % RSD of less than 5%, and accurate with % TV values between 98.0 and 102.0% (Green, 1996).

Table 1
Validation results to quantification method of eremantholide C in presence of IS, showing the correlation coefficient (*R*) obtained to linearity, deviations and the Relative Standard Deviation (% RSD) precision, accuracy, recovery and specificity.

		Eremantholide C
Linearity	*R* (mean curve) ± DPR	0.998 ± 0.06
Repeatability (% RSD)	5 μg/ml	0.27
	60 μg/ml	0.70
	140 μg/ml	0.39
Intermediary precision (% RSD)	5 μg/ml	2.04
	60 μg/ml	1.21
	140 μg/ml	1.87
Accuracy (% content)	5 μg/ml	101.02
	60 μg/ml	100.09
	140 μg/ml	99.81
Recovery (%) Added amount	2 μg/ml	103.91
	5 μg/ml	98.97
	10 μg/ml	102.02
	15 μg/ml	98.18
Specificity (% RSD) Added amount	2 μg/ml	0.20
	5 μg/ml	3.85
	10 μg/ml	4.84
	15 μg/ml	4.28

Adequate recovery of eremantholide C was evidenced by the concentration values obtained with respect to the amount added, all between 95.0 and 105.0%. The results are shown in Table 1.

The method specificity was confirmed by the standard addition method, and the deviations obtained were less than 5% for eremantholide C (**1**). Fig. 2 shows the purity peak obtained for eremantholide C at 180 μg/ml. The purity angle (0.126) in green was lower than the purity threshold (0.789), in blue, indicating the specificity of the method.

Stability studies

Lychnophora trichocarpha ethanolic extract and eremantholide C were exposed for six months at room temperature, 40 °C and 8 °C. In Fig. 3 the overlapping of the chromatograms relative to the fingerprint of the extract performed at the beginning of the stability study and after six months allowed the decay of the analyte signal of interest with retention time of approximately 17 min and increase relative to other chromatographic signals that can be products formed in the approximate time of 6.5 and 20.5 min. Other chromatographic signals appeared, however, with less intensity

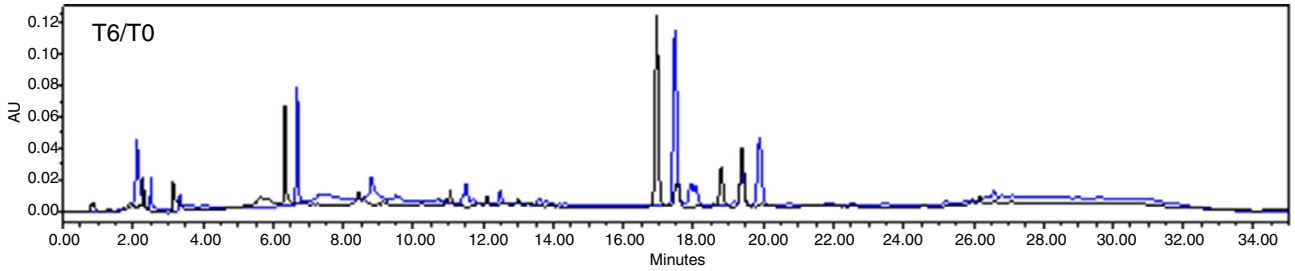

Fig. 3. Overlap of the fingerprints of *Lychnophora trichocarpa* ethanolic extract obtained from the samples in a long-term stability study with a storage time of 6 h (in blue) in relation to time zero (in black) at the concentration of 500 μg/ml.

and in time that corresponds to retention time different from that observed for eremantholide C.

The maximum decrease of eremantholide C content in the extract was 10% and the results obtained are described in Table 2.

Although in Fig. 4 the fingerprint for the accelerated stability study performed with the extract samples showed decay of the chromatographic signal relative to the analyte of interest, this did not exceed 10% of the initial concentration. Peaks of degradation products that interfere with quantification of eremantholide C or are present at high intensity were also not observed.

For eremantholide C, a purified substance, the maximum allowed decrease was 5%. The results are listed in Table 3.

The drug shelf-life is the time that the concentration of the biologically active substance presented a maximum decrease until 5% when the stability studies are used. However, in the case of medicinal plants and derivatives, where constituents with known therapeutic activity are still unknown, the variation in content during the proposed shelf life should not exceed ± 10% of the initial assay value (Bilia et al., 2001).

According to studies performed, *L. trichocarpa* extract and eremantholide C remain stable for six months when stored at room temperature and impermeable glass bottle, and therefore can be used safely and effectively within this period.

Accelerated stability study assists in estimating the shelf-life, since the degradation of substances occurs with greater speed at higher temperatures favoring the chemical reactions.

Under 40 °C, the eremantholide C concentration in ethanolic extract decrease 12.94%, while in its isolated form it decreases 9.51%. Therefore, stability was observed at 4.5 months for the extract and 3 months for eremantholide C in isolated form.

The decrease in the eremantholide C concentration was higher in the extract than in its isolated form. One possible explanation for this is that at high temperatures, chemical reactions between various substances that make up the extract are favored due to lost connections and the formation of new intermolecular interactions (Ev, 2001).

The stability study at low temperatures, most of the time, reduces the degradation of biologically active substances. This was confirmed by subjecting the ethanolic extract and eremantholide C at 8 °C, yielding decrease of 7.55% and 2.52% of eremantholide C concentration in the extract and its isolated form, respectively. Therefore, in these conditions, the stability time or the safety and effective use time of the extract or isolated substance could be higher.

Regarding the photostability studies, the mean content of eremantholide C in the extract in the protected samples from light was 28.91 μg/mg, while the mean content of the samples exposed to light was 27.88 μg/mg. Thus, the exposure of the extract to light produced a decrease of the 3.55% in the eremantholide C concentration.

For isolated eremantholide C, the mean content of protected samples from light was 72.59 μg/mg, while the mean content of the samples exposed to light was 69.30 μg/mg, yielding decrease of 4.53% in eremantholide C concentration.

Photolysis is a major cause of bioactive substances degradation. The results obtained in this type of study can guide the development of formulation, most appropriate packaging choice and even the need for special conditions to manipulate this product (Ev, 2001). It is not the case for ethanolic extract and isolated eremantholide C, since that the decrease is presented within the established limits.

Forced degradation studies of eremantholide C

Eremantholide C concentration decrease was calculated from the comparison between sample submitted to degradation conditions and the control.

Eremantholide C under acid and alkaline degradation conditions was completely degraded, while under oxidative conditions

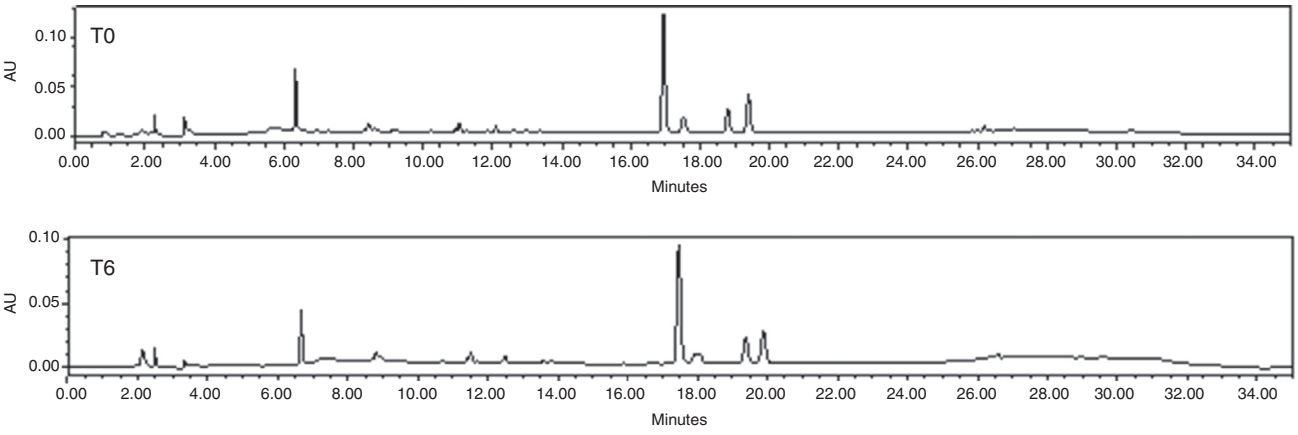

Fig. 4. Fingerprints of the *Lychnophora trichocarpa* ethanolic extract obtained from the 500 μg/ml accelerated stability study samples using validated method conditions.

Table 2
Quantities of eremantholide C in *Lychnophora trichocarpa* ethanolic extract in the long-term, accelerated and low temperature stability after different times of storage and deviations related to concentrations of freshly prepared sample.

Time (months)	Long-term		Accelerated		Low temperature	
	Eremantholide C (μg/mg)	Decrease[a] (%)	Eremantholide C (μg/mg)	Decrease[a] (%)	Eremantholide C (μg/mg)	Decrease[a] (%)
0	31.64	–	31.64	–	31.64	–
1	30.81	2.63	31.72	0	31.50	0.44
2	31.70	0	30.87	2.42	30.79	2.68
3	29.52	7.69	29.37	7.19	30.11	4.82
4.5	28.89	8.70	28.59	9.62	29.65	6.28
6	28.66	9.43	27.55	12.94	29.25	7.55

[a] The percentage of degradation was calculated by determining the amount of Erec present in the sample quantified at a given time subtracted from the amount of Erec present in the sample quantified at time 0.

Table 3
Quantities of isolated eremantholide C in the long-term, accelerated and low temperature stability after different times of storage and deviations related to concentrations of freshly prepared sample.

Time (months)	Long-term		Accelerated		Low temperature	
	Eremantholide C (μg/100 μg)	Decrease[a] (%)	Eremantholide C (μg/100 μg)	Decrease[a] (%)	Eremantholide C (μg/100 μg)	Decrease[a] (%)$_0$
0	94.72	–	94.72	–	94.72	–
1	92.45	2.39	93.62	1.15	96.42	0
2	91.28	3.63	95.91	0	94.89	0
3	92.56	2.28	90.63	4.37	92.47	2.27
4.5	90.34	4.62	88.00	7.09	91.64	3.24
6	91.67	3.22	85.71	9.51	92.33	2.52

[a] The percentage of degradation was calculated by determining the amount of Erec present in the sample quantified at a given time subtracted from the amount of Erec present in the sample quantified at time 0.

decrease was 0.35%, since that the eremantholide C concentration after forced degradation study was 81.43 μg/ml and 81.72 μg/ml for the control. When subjected to 90 °C it was observed a decrease of 7.45% and under neutral conditions, there is no degradation.

Forced degradation tests of the isolated compounds aim to define conditions which impact significantly on the stability, guiding the appropriate development of formulations that protect from the degradation conditions.

The results demonstrate the instability of eremantholide C only in acidic and basic media persisting after neutralization of the sample, suggesting that the degradation reactions suffered are irreversible. The eremantholide C degradation in alkaline media was expected, since this condition causes opening of the lactone ring.

Through the knowledge of these products behavior due different conditions, one can choose the most appropriate components to prepare formulations trying to slow down the degradation process. Eremantholide C instability in acid and alkaline media indicates that the formulation which will incorporate the extract or the isolated substance should have a pH near neutrality. One can also infer that there are no restrictions regarding the handling processes resulting in the incorporation of air in the product or requiring the use of heat.

With this information, products that adequately preserve the original features of the biologically active substance with quality, safety and efficacy can be developed.

Authors' contributions

TGC carried out the data analyses and wrote the manuscript. BHO carried out the laboratory work, the data analyses and wrote the manuscript. DASG and JS designed the study, 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.

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