Journal of CO_2 Utilization 39 (2020) 101165

Contents lists available at ScienceDirect

Journal of CO_2 Utilization

journal homepage: www.elsevier.com/locate/jcou

Influence of the supercritical CO_2 extraction in the stability of the coumarins of *Pterocaulon lorentzii* (Asteraceae)

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ARTICLE INFO

Keywords:
Pterocaulon lorentzii
Sabandinol
Supercritical fluid extraction
Epoxy-coumarins

ABSTRACT

Coumarins are important especialized metabolites that have potential application in different fields. For the purpose of obtaining fractions enriched with coumarins, extracts from the leaves and inflorescences of *Pterocaulon lorentzii* Malme (Asteraceae) were obtained by different methods and solvents, i.e. with water at 60 °C, with dichloromethane at room temperature (25 °C) and by means of supercritical CO_2 (SC-CO_2) extraction at 40 °C with gradual pressure increments (90, 120, 150, 200 and 300 bar). The coumarins content in the extracts was calculated using ultra-fast liquid chromatography analyses, which allowed the characterization of the coumarins. Sabandinol (5-(2′,3′-dihydroxy-3′-methylbutyloxy)-6,7-methylenedioxycoumarin) was the main coumarin found in the aqueous and dichlorometane extracts. The epoxy-derivative 5-(2′,3′-epoxy-3′-methylbutyloxy)-6,7-methylenedioxycoumarin was identified as the major component of all extracts obtained by SC-CO_2. The SC-CO_2 extract from the inflorescences proved to be richer in coumarins than the extract from the leaves. The results suggest that when the objective is to obtain epoxy-coumarins, the method of choice should be SC-CO_2, as this technique was effective for preventing the ring-opening of the epoxy group.

1. Introduction

Coumarins are important especialized metabolites that have been found mainly in plants from the families Apiaceae, Rutaceae and Asteraceae [1]. Although many compounds of this class have been sythezised, nature provides coumarins with interesting estructural features that can be used as they are or modified by semisynthesis. These heterocyclic molecules, both natural and synthetic, have been exhibiting numerous activities such as anti-inflammatory [2], antifungal [3], antitumoral [4]. They are also receiving increasing research attention for their potential applications as pesticides [5,6], dye for textiles [7], laser dyes and dye-sensitised solar cells [8], among others.

Pterocaulon genus (Asteraceae) has been demonstrated to be a rich source of oxygenated coumarins, most of them reported by Debenedetti and co-workers [9–11]. This genus encompasses 26 species, the majority occuring in South America. From *Pterocaulon* species, 41 coumarins have been identified being 16 dioxygenated, 22 trioxygenated, and 3 tetraoxygenated, the vast majority being oxyprenylderivatives [12].

The prenyl group (3-methyl-2-butenyloxy) commonly seen in the *Pterocaulon* coumarins, in turn, can be oxidized to yield compounds presenting the correspondent 2,3-epoxy-3-methylbutyloxy and 2,3-dihydroxy-3-methylbutyloxy derivatives. Although less frequent in these coumarins, other oxidized forms such as 2-hydroxy-3-methylbutyloxy, 2-hydroxy-3-methyl-3-butenyloxy and 3-methyl-2-butenyloxy have also been found.

Prenyl side chain has been considered as important pharmacophore feature, conferring biological activities to the molecules containing this unit [13]. The impact of this substituent on the pharmacological activity was already determined, for example regarding the cytotoxic and anti-inflammatory effects of some coumarins [14].

An ethnoveterinary study carried out in south Brazil, in 2008, indicated that among the plants used by the rural population to treat mycoses of animals, there was a species of *Pterocaulon* [15]. Subsequently, our research group has started the investigation of species from this genus aiming at coumarins isolation as well as the determination of

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https://doi.org/10.1016/j.jcou.2020.101165
Received 1 November 2019; Received in revised form 21 February 2020; Accepted 19 March 2020
Available online 07 April 2020
2212-9820/

some biological activities. Plants extracts and isolated compounds demonstrated antifungal [3,16–18], antiprotozoal [19–21] and cytotoxic [4] activities.

Considering the relevant activities already found and those that may be determined in the near future, we are striving to develop alternative methods for obtaining these compounds. In this way, Medeiros-Neves et al. [22] demonstrated that some coumarins from *P. balansae* can be obtained using hot water and Torres et al. [23] showed that the same plant submitted to $SC-CO_2$ extraction afforded the same coumarins but in very different amounts. When *P. balansae* coumarins were extracted with water, there was a very small amount of coumarins containing the epoxy-3-methylbutyloxy unit, with predominance of the hydrolysis products having the 2,3-dihydroxy-3-methylbutyloxy substituent [22]. On the other hand, the same plant material, when subjected to supercritical fluid extraction, provided high concentrations of epoxy derivatives and minimal amounts of hydrolysis products [23]. These results suggest that the most aggressive conditions used in aqueous extraction would be responsible for the hydrolysis of the epoxide ring. This ring has high instability and the milder conditions of supercritical extraction would protect this grouping.

Besides the advantages of using mild condition, the use of $SC-CO_2$, a non-toxic solvent that is completely removed from the extract after extraction, is of great interest, especially in processes for obtaining compounds that can be used to develop pharmaceutical products. Therefore, the use of supercritical carbon dioxide as a solvent is indicated, as it avoids the use of organic solvents that pollute the environment, the product and the residue, which would be disposed of in nature [24]. Non-toxicity, non-flammability and easy availability [25] are characteristics that give to the supercritical carbon dioxide the condition of a desirable solvent for the development of extractive processes. Bearing in mind the growing demand of society for clean and environmentally friendly technologies, the development of processes that use green solvents instead of chlorinated solvents is highlighted [26] even though the supercritical processing requires large investments due to the use of vessels, valves and connections compatible with high pressure conditions. Amid advantages and disadvantages, another positive aspect for the use of supercritical CO_2 as a solvent is its ability to modify its solvation power with small changes in temperature and pressure values. With this broad and easy ability to adjust selectivity for compounds of interest, supercritical fluid extraction is a technology that has not yet been exhausted in terms of research.

Continuing the studies of species of *Pterocaulon*, in this work we investigated the coumarins of *P. lorentzii*. As far as we know, until now only three studies were carried out with this plant. All of them report the presence of sabandinol as the main component [4,27,28] of the chloroform or dichloromethane extracts.

Therefore, the purpose of this study was to apply the $SC-CO_2$ extraction method to obtain coumarins from the leaves and inflorescences of *P. lorentzii*. The chemical profile of the $SC-CO_2$ extracts was compared with that of the extracts obtained by maceration in chlorinated solvent, as well as with the aqueous extracts.

2. Material and methods

2.1. Chemical and materials

Acetonitrile and formic acid were acquired from Tedia (HPLC grade, USA). Ultrapure water was obtained from a Milli-Q® Plus apparatus by Millipore (Billerica, USA). Reagent grade acetone, dichloromethane, ethyl acetate and *n*-hexane (Anidrol, Brazil), ethanol and methanol (Nuclear, Brazil) were regularly used in extraction and isolation procedures. DMSO (dimethyl sulfoxide) deuterated was acquired from Sigma-Aldrich (Brazil).

2.2. Plant material

Aerial flowering parts of *Pterocaulon lorentzii* were collected in Imbituba, SC, Brazil, in March 2018. Plant collection was authorized by Ministério do Meio Ambiente, Conselho de Gestão do Patrimônio Genético - SisGen (A608FA6) and voucher specimen was deposited in the herbarium of the Universidade Federal do Rio Grande do Sul. The plant material was dried at room temperature (25 °C), protected from direct light and comminuted in a cutting mill. The ground material produced a set of fibers in the form of tangled agglomerates. The average thickness of these fibers was determined by a caliper, resulting in 0.061 mm for the inflorescences and 0.148 mm for the leaves. The plant material moisture was determined in a thermobalance (BEL Engineering - Model i-Thermo 163 L). The values obtained were 5.27 % and 2.08 % for inflorescences and leaves, respectively. All experiments were conducted using the same batch of plant material.

2.3. Extracts preparation

2.3.1. Dichloromethane extract

The dichloromethane extracts were obtained using dried leaves (200 g) or inflorescences (226 g) under static maceration at room temperature (25 °C) until exhaustion, which was determined when no mass variation was observed in the extracts. The process was concluded renewing the solvent every 24 h for 5 days. The extracts were filtered and concentrated in vacuum at 40 °C. The residues were solubilized with acetone to remove the insoluble waxes.

2.3.2. Supercritical fluid extraction

Extraction with supercritical carbon dioxide was performed in a 0.5 L high-pressure extraction vessel (internal diameter of 5.4 cm), in which the previously dried raw material, 30 g DW (dry weight) of inflorescences and 40 g DW of leaves, was inserted. This extraction vessel is part of a pilot unit, described in detail by Scopel et al. (2013) [29], which has pressure, temperature and flow controllers. Pressure values were monitored with a Novus 8800021600 digital transducer system, whose accuracy was ± 1.0 bar. Temperature controllers (PT-100) are positioned on the equipment to measure the temperature of the carbon dioxide inlet and inside the extraction vessel. The control software of this unit was developed using the Elipse SCADA platform developed by the company Elipse Software Ltda. [30].

The vegetable matter introduced into the extraction vessel constitutes an extraction bed through which the solvent percolates extracting compounds that are solubilized. The solvent used was supercritical carbon dioxide with a degree of purity equal to 99.9 % (Air Products) which flowed with a flow rate of 1 $kg\,h^{-1}$ measured by a flowmeter test (Sitraus FC Massflo 2100 - Siemens) with 0.1 % accuracy. The flow of the solvent is carried out by a pump (Thar P - 200, USA). In the extraction experiments there was no recycling of carbon dioxide.

Using the extraction protocol employed by Cargnin et al. [30] and Torres et al. [23], the same plant material was subjected to extraction at temperature of 40 °C and under successively increased pressures (90, 120, 150, 200 and 300 bar), collecting the samples of each pressure after 20, 20, 20, 20, 20 min for leaves and 20, 30, 20, 20, 20 for inflorescences. The system was maintained in the same pressure condition until the achievement of a constant extract weight, that is, when no mass variation in mass extract was observed after three consecutive measurements carried out by a digital scale [30]. The extracts were solubilized with acetone to remove waxes. The acetone soluble fractions were evaporated to dryness.

2.3.3. Aqueous extract

The aqueous extracts were obtained using a biomass weight to solvent volume ratio of 2 % [31]. The extracts were prepared by dynamic maceration, using 1.0 g of dry leaves or inflorescences immersed

in 50 mL of distilled water. The maceration took place in a multipoint magnetic stirrer (Dist-DI920) with a water bath (60 °C) for 4 h. The extracts were filtered in Whatman® filter paper and stores at room temperature (25 °C).

2.4. UFLC analysis

The ultra fast liquid chromatography (UFLC) analyses were carried out using a system developed and validated by our research group [31]. The system used was Shimadzu Prominence UFLC System (Shimadzu, Japan) equipped with a diode array detector (SPD-M20A). The output signal was monitored and processed using Shimadzu LC-solution Multi-PDA software (Kyoto, Japan). Chromatographic separation was performed on a Shim-pack XR ODS column 100 × 2.0 mm i.d.; particle size, 2.2 µm guarded by an in-line pre-column C_{18} SecurityGuard™ ULTRA (Phenomenex, USA).

The mobile phase consisted of (A) formic acid 0.1 % (v/v) and (B) acetonitrile. The flow rate was set in 0.55 mL/ min up to 8 min, the wavelength was adjusted to 327 nm, injection volume 5 µL, and the analysis was carried out at 55 °C [31].

The samples of the aqueous, dichloromethane and $SC-CO_2$ extracts were prepared in triplicate and analyzed by UFLC. The dichloromethane and supercritical fluid extracts were analyzed in the concentration of 200 µg/mL, diluted in ACN/H_2O (1:1). In counterpart, the aqueous extracts were diluted (1:10) in ACN/H_2O (1:1).

2.5. Isolation of sabandinol

The acetone-soluble fraction (6 g) of *P. lorentzii* was subjected to vacuum liquid chromatography on silica gel using a gradient elution of *n*-hexane:ethyl acetate (100:0 − 0:100) to afford 6 fractions (Fr. 1–6). Fr. 4–6, eluted with *n*-hexane/ethyl acetate mixtures 75:25 and 50:50, showed by thin layer chromatography the presence of a main compound. These fractions were combined and subjected to column chromatography using the same mobile phase cited above to obtain 10 subfractions. Fractions 6–8 were joined up affording a precipitated which was recrystallized in methanol. The structure of the coumarin isolated from *P. lorentzii* was determined by analyzing the NMR spectra (^{1}H-NMR, COSY, ^{13}C-NMR, HSQC) (Supplementary material).

3. Results and discussion

Coumarins are compounds that usually are soluble in organic solvent, mostly in those of moderate polarity such as chloroform or dichloromethane. Some of them can also be solubilized in hot water but modification in the structures, in some cases, can occur [22].

Considering the nature of coumarins, it can be estimated that supercritical CO_2, due to its moderate polarity in the experimental contitions used in the present study, may be the ideal solvent to extract them. Specifically addressing *Pterocaulon*, our research group has already verified the efficiency of this method in the extraction of coumarins from *P. balansae* [23].

To corroborate the above statement, the main purpose of the present study was to determine by UFLC the chemical profile of the extracts obtained from the leaves and inflorescences of *P. lorentzii* using three different extraction techniques. The compounds were determined in the samples by comparing the retention times and ultraviolet profiles with authentic samples previously identified by Medeiros-Neves et al. [22]. The chemical structure of sabandinol and their epoxy-derivative are shown in Fig. 1.

Although sabandinol was easily characterized in our samples and was previously reported for this plant as the main coumarin, the compound was isolated from the dichloromethane extract of the leaves and had its structure elucidated by spectroscopic methods (Figures S1, S2, S3 and S4).

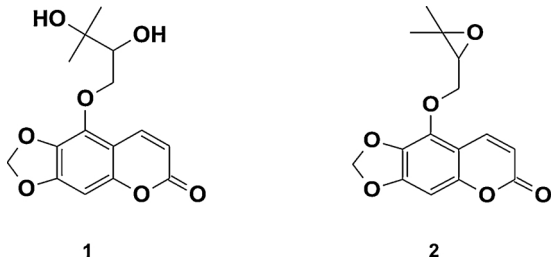

Fig. 1. Chemical structure of the main coumarins present in *P. lorentzii* extracts. **1.** 5-(2′,3′-dihydroxy-3′-methylbutyloxy)-6,7-methylenedioxycoumarin (dihydroxy-derivative also called sabandinol); **2.** 5-(2′,3′-epoxy-3′-methylbutyloxy)-6,7-methylenedioxycoumarin (epoxy-derivative).

3.1. Dichloromethane extracts

The amount of coumarins in each g of extract was determined and shown in the Table 1. The percentual of about 6 % of the dichloromethane extract obtained from the leaves comprised sabandinol (**1**), as the main compound, and 0.7 % of the epoxy-derivative (**2**) (Fig. 2; Table 1). This extract also showed the presence of traces of other coumarins characterized as 7-(2′,3′′-dihydroxy-3′-methylbutyloxy)-6-methoxycoumarin (retention time 2.33 min) and its precursor 7-(2′,3′-epoxy-3′-methyl-3′-butyloxy)-6-methoxycoumarin (retention time 4.55 min) (Figure S5). These two coumarins had previously been characterized by thin-layer chromatography and high-performance liquid chromatography in the chloroformic extract of this plant [27].

For the inflorescences, the percentual of about 8 % of the dichloromethane extract was obtained. Sabandinol (**1**) was the main compound determined in the dichloromethane extract of the inflorescences (5 %). As it was observed in the extract from the leaves, the epoxy-derivative (**2**) was present in smaller amount (2 %) than the dihydroxy-derivative (Table 1; Fig. 3).

Apart from the coumarins, other two compounds were detected in this extract. The compounds, with retention time of 4.64 and 6.06 min, were not identified but the UV-spectra are somewhat similar to that of luteolin, a flavone previously found in *Pterocaulon* species (profile shown in the supplementary material, Figure S6).

An experiment aiming to recover luteolin from *Arrabidaea chica* (Bignoniaceae) demonstrated that this flavonoid was not obtained using pure supercritical carbon dioxide [32]. According to the authors, the presence of four hydroxyl groups in the molecule turns luteolin insoluble in carbon dioxide. This could explain the presence of compounds with the ultraviolet profile of a flavonoid in the dichloromethane extract and their absence in the supercritical extracts. The isolation of several flavonoids from dichloromethane extract of *P. alopecuroides* and *P. purpuracens* has been reported [33–36]. To extract flavonoids with supercritical carbon dioxide, generally ethanol is used as modifier.

This extract also showed the presence of another coumarin (retention time 6.46) that could be the prenyl derivative (5-(3-methyl-2-butenyloxy)-6,7-methylenedioxycoumarin), previously characterized in very small amount in the plant [27]

Regarding the yields, the dichloromethane extracts were 6.13 % and 3.59 %, for leaves and inflorescences, respectively. The yield of coumarins (mg/g plant material) in the dichloromethane extract of the leaves was 3.3 mg of sabandinol and 0.4 mg of the epoxy-derivative/g of plant material. The extract of the inflorescence, in turn, showed 1.73 mg of sabandinol/g of and 0.87 mg of the epoxy-derivative/g of plant material.

3.2. Supercritical fluid extracts

The supercritical fluid extracts were obtained at constant temperature and gradual pressure increments. The largest amounts of

Table 1
Coumarins content (mg/g of extract) determined in the different extracts obtained from leaves and inflorescences of *P. lorentzii*.

Methods	Static maceration (mg/g of extract)	Supercritical fluid extract (SC-CO_2) (mg/g of extract)				
	Dichloromethane	90 bar	120 bar	150 bar	200 bar	300 bar
LEAVES						
Dihydroxy-derivative (1)	58.70 ± 8.94	4.25 ± 0.37	1.31 ± 0.16	1.76 ± 0.21	2.06 ± 0.47	3.33 ± 0.17
Epoxy-derivative (2)	7.05 ± 0.02	34.68 ± 3.10	25.12 ± 1.92	14.15 ± 2.05	10.87 ± 3.41	10.96 ± 0.51
Total coumarins	**65.75**	**38.93**	**27.04**	**15.91**	**12.93**	**14.24**
Total coumarins of SC-CO_2				**109.05**		
INFLORESCENCES						
Dihydroxy-derivative (1)	51.98 ± 2.04	2.72 ± 0.28	12.58 ± 1.05	16.91 ± 1.49	28.19 ± 3.94	23.11 ± 0.17
Epoxy-derivative (2)	26.07 ± 0.33	53.60 ± 5.40	87.00 ± 5.92	102.78 ± 5.41	172.74 ± 3.14	110.40 ± 7.17
Total coumarins	**78.05**	**56.32**	**99.58**	**119.69**	**200.93**	**133.51**
Total coumarins of SC-CO_2				**610.03**		

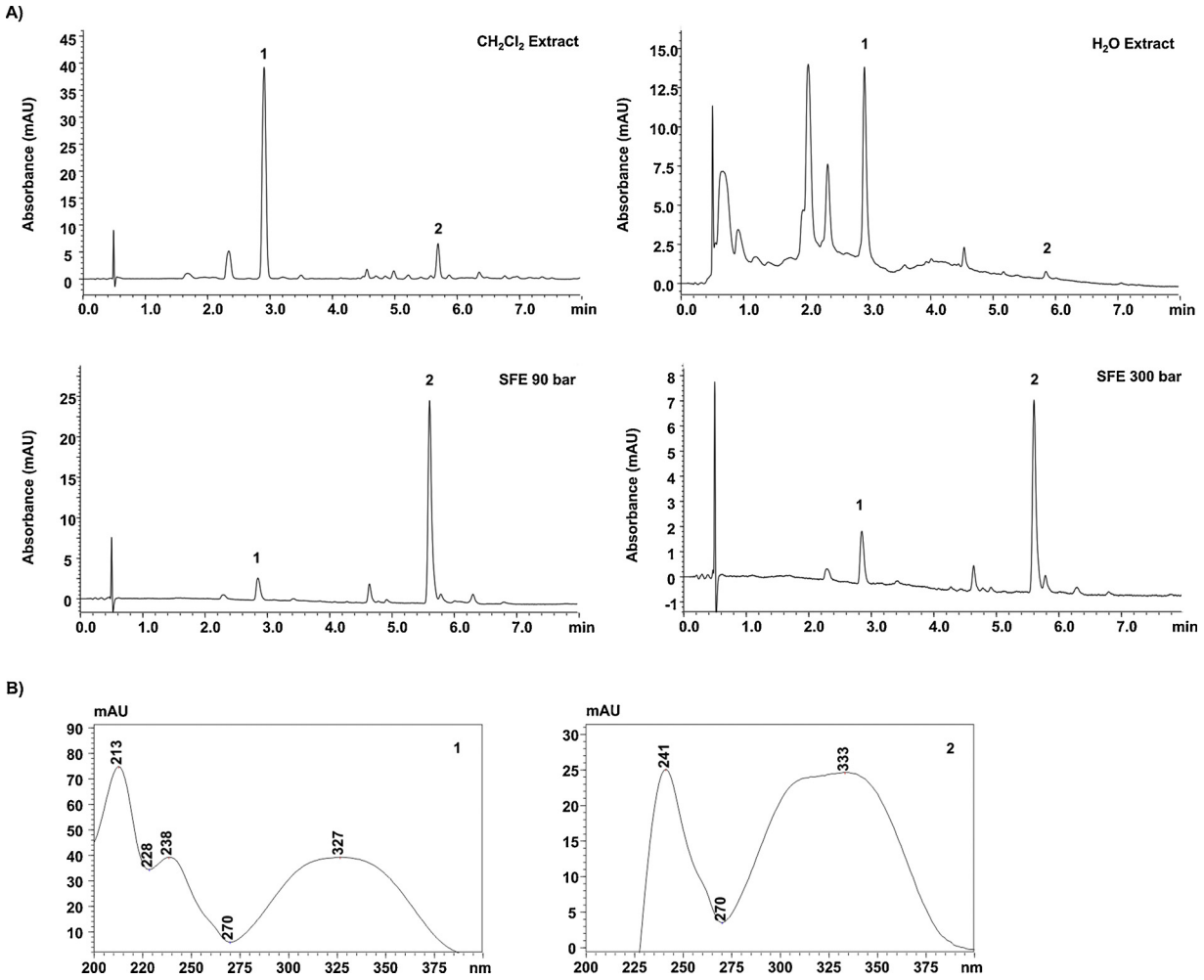

Fig. 2. 2A) Chromatographic profiles of the different extracts of *P. lorentzii* leaves. **2B)** UV-spectra of dihydroxy-derivative (1) and epoxy-derivative (2). **SFE:** supercritical fluid extraction.

coumarins in leaves (around 60 %) were obtained at the beginning of the process, with pressures of 90 and 120 bar.

In contrast to the results obtained in the experiments (dichloromethane extract and aqueous extract), SC − CO_2 afforded the epoxy-derivative (**2**) as the main compound, being sabandinol (**1**) obtained in minute amount (Fig. 2). Small amount of a compound characterized as the 7-(2′,3′-epoxy-3′-methyl-3′-butyloxy)-6-methoxycoumarin (retention time 4.55 min) was found in all the extracts obtained by SC − CO_2.

In another study carried out by our research group with the aerial parts (leaves and branches) of other *Pterocaulon* species (*P. balansae*), the SFE with carbon dioxide afforded ca. 3 % [23], lower than the yield obtained in the dichloromethane extract (ca. 11 %) [4]. However, it was observed that the selectivity in SC − CO_2 has greatly increased. In the present study, the supercritical CO_2 also allowed selective extraction of the coumarins from *P. lorentzii*.

As the aim of the study was to verify the stability of the coumarins and the selectivity of the SC − CO_2 for these compounds, the yield of the fractions obtained by the SFE was not estimated. To evaluate the selectivity, the coumarins content in the different fractions was determined. For that, the samples were solubilized in acetone to remove the waxes and equal aliquots, in triplicate, were collected for analyses

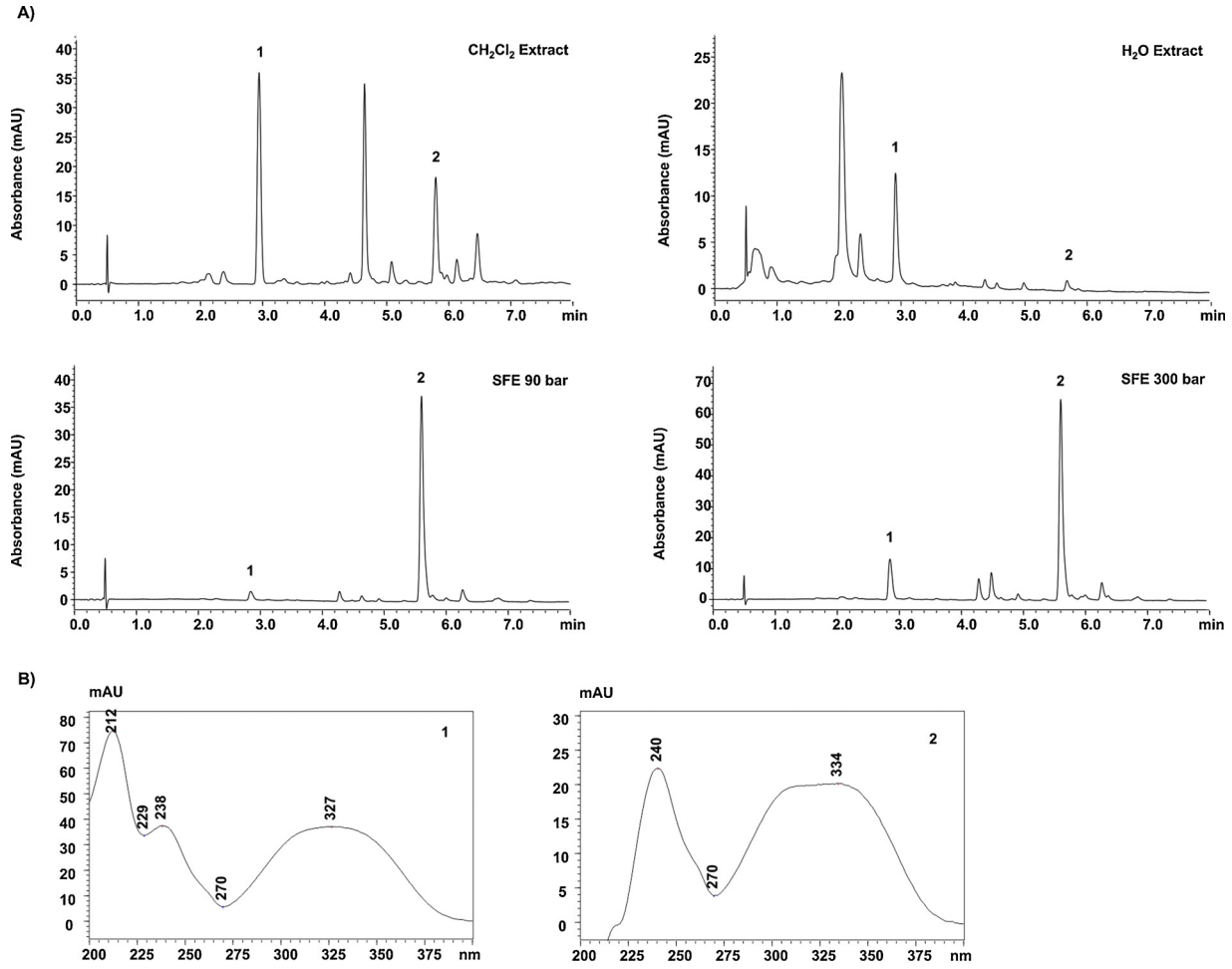

Fig. 3. 3A) Chromatographic profiles of the different extracts of *P. lorentzii* inflorescences. **3B)** UV-spectra of dihydroxy-derivative (1) and epoxy-derivative (2). **SFE:** supercritical fluid extraction.

at the UFLC.

Unlike the results observed in leaves extracts, in the $SC-CO_2$ extracts of the inflorescences the coumarins content increases with increasing pressure probably due to the intensification of the density and some characteristic of the raw material that hinders the access of CO_2 in lower pressures (Fig. 3). In the case of leaves, the highest amounts of coumarins were obtained at the lowest pressures, while in the inflorescences the highest quantities were extracted at 200 bar. These results suggest that the compounds probably are more available on the leaves surface and that on inflorescences they are more internalized. Thus, increased pressure allows carbon dioxide access to more internal regions of the plant material, resulting in higher coumarins content.

In the present experiments, the total resistance to mass transfer can be understood as the sum of the internal resistance of the solute when transported through the solid phase (plant material) with the external resistance of the solute when transported through the flowing solvent (supercritical fluid). So, the fact that lower mass rates were obtained from the leaves, even though the pressure increased, can be associated with the internal resistance being dominant. Therefore, higher pressures cause lower diffusion coefficients [37]. Consequently, smaller amounts of coumarin mass were obtained. On the other hand, in the case of inflorescences higher mass rates are observed. Assuming that the controlling resistance is in the solvent phase, increasing the pressure will increase the solvent density of the solvent and consequently, the coumarins solubility [38].

Comparing the coumarins content obtained from the inflorescences using organic solvent and supercritical carbon dioxide, it was found that the amounts of these compounds were much higher in the supercritical extraction. The lower coumarins content observed in dichloromethane extract is possibly due to the parallel extraction of several other mass-adding compounds, this method being less selective for coumarins extraction.

3.3. Aqueous extract

The aqueous extracts obtained from the leaves and inflorescences presented sabandinol (**1**) as the main coumarin and only a minute amount of the epoxy-derivative (**2**) (Figs. 2 and 3). The yield of sabandinol was ca. 2 mg/g plant material both for leaves and inflorescences. On the other hand, the yields of the epoxy-derivative were 0.06 and 0.17 mg in one gram of the leaves and inflorescences, respectively (Table S1). The extract from the leaves also shows the presence of two compounds (retention time 2.03 and 2.34 min), characterized as phenolic acids by the ultraviolet profiles (Figure S5). The profile of the extract from the inflorescences is very similar to that obtained from the leaves, with the presence of sabandinol (**1**) as the main coumarin, and phenolic acids (Figure S6).

4. Conclusions

This study is part of our ongoing research aiming at obtaining coumarins from different plant. The genus *Pterocaulon* is an abundant source of these compounds, some of them endowed with relevant biological activities. As supercritical extraction had never been used before

by other research groups for species of *Pterocaulon,* we consider that it is relevant to study other species of the genus besides *P. balansae*, previously analyzed by us. Thus, in this study, we investigated the coumarins of *P. lorentzii* obtained by different extraction processes. Besides the leaves, the inflorescences of the plant were also analyzed. No species of *Pterocaulon* has been previously studied for the chemical composition of inflorescences.

Studies carried out with *Pterocaulon* species have been conducted with aqueous extracts or with those obtained with chlorinated solvents. In both extracts, it has been found predominance of coumarins having the 2,3-dihydroxy-3-methylbutyloxy substituent instead of the epoxy-derivatives.

The aqueous and chlorinated extracts afforded sabandinol, a dihydroxy-derivative. On the other hand, in the SFE, the $SC-CO_2$ proved to be effective in protecting the epoxy group of coumarins and permitted the obtainment of an epoxy-derivative never reported before for this species.

Taking into account the biological activities, it would be very important to evaluate extracts obtained under the mild conditions provided by the SFE with $SC-CO_2$ to determine whether the epoxy-derivative is active or perhaps more effective than sabandinol, obtained with the other solvents. Which substituent would contribute to greater biological activity has not yet been defined but the researcher should keep in mind that depending on the solvent / method used, relevant variations in the component proportions will be observed. Consequently, different results would be achieved in biological assays and thus, the importance of the different substituents could be established.

CRediT authorship contribution statement

Bruna Medeiros-Neves: Conceptualization, Methodology, Software, Formal analysis, Investigation, Data curation, Writing - original draft, Writing - review & editing, Visualization. **Kriptsan Abdon Poletto Diel:** Conceptualization, Methodology, Software, Formal analysis, Investigation, Data curation, Writing - original draft, Writing - review & editing, Visualization. **Vera L. Eifler-Lima:** Writing - original draft, Writing - review & editing. **Helder Ferreira Teixeira:** Resources, Writing - original draft, Writing - review & editing. **Eduardo Cassel:** Resources, Writing - original draft, Writing - review & editing. **Rubem Mário Figueiró Vargas:** Resources, Writing - original draft, Writing - review & editing. **Gilsane Lino von Poser:** Resources, Investigation, Writing - original draft, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This study was carried out with the financial support of CNPq (406282/2016-1) and FAPERGS (17/2551-0001186-4) (Brazil). B.M.N. and K.A.P.D. would like to thank CAPES and CNPq, respectively, for the scholarship. E.C., R.M.F.V., H.T. and G.V.P. are recipients of CNPq research fellowship.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi: https://doi.org/10.1016/j.jcou.2020.101165.

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