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Short communication

Supercritical fluid extraction and high performance liquid chromatographic determination of benzopyrans and phloroglucinol derivative in *Hypericum polyanthemum*

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ABSTRACT

The aerial parts of *Hypericum polyanthemum* Klotzsch ex Reichardt (Guttiferae) were successively extracted with supercritical carbon dioxide (SC CO_2) under pressures of 90, 120, 150 and 200 bar at different temperatures (40, 50 and 60 °C), and compared with the *n*-hexane extract obtained by ultrasound-assisted extraction. The samples obtained were examined regarding extraction yield and HPLC quantification of the main secondary metabolites, the benzopyrans HP1 (6-isobutyryl-5,7-dimethoxy-2,2-dimethylbenzopyran), HP2 (7-hydroxy-6-isobutyryl-5-methoxy-2,2-dimethyl-benzopyran) and HP3 (5-hydroxy-6-isobutyryl-7-methoxy-2,2-dimethyl) and the phloroglucinol derivative, uliginosin B. SFE presented higher selectivity than the *n*-hexane maceration, and the best condition to extract the target metabolites has been determined to be at 50 °C and for the high molecular-weight compound, uliginosin B, higher pressures were required.

1. Introduction

Hypericum genus is represented in South Brazil by approximately 20 species, found in small disperse populations or restricted to a single locality. Among them, *Hypericum polyanthemum* showed in its lipophilic extract the presence of uliginosin B, a phloroglucinol derivative, and three benzopyrans: 6-isobutyryl-5,7-dimethoxy-2,2-dimethylbenzopyran (HP1), 7-hydroxy-6-isobutyryl-5-methoxy-2,2-dimethyl-benzopyran (HP2) and 5-hydroxy-6-isobutyryl-7-methoxy-2,2-dimethyl-benzopyran (HP3), isolated from aerial plant parts [1,2]. These substances have been found to be involved with some biological properties as antiviral [3], antimicrobial [4], antinociceptive [5], antitumor [6,7], acaricidal [8] and antioxidant [9], presented by the plant extracts.

Although the *H. polyanthemum* extracts and isolated compounds demonstrated different activities, the antinociceptive effect is the most relevant from the pharmacological point of view. The *n*-hexane extract presented such effect mediated by the opioid system [5] and further studies showed that the phloroglucinol derivative uliginosin B, investigated for this activity, is not mediated by the opioid system [10]. Therefore, the benzopyrans were evaluated and HP1 demonstrated to be responsible for the opioid-mediated mechanism [11].

Due to the highly restricted distribution and the therapeutic potential, protocols for *in vitro* [12] and *ex vitro* [13] propagation of *H. polyanthemum* were established and the HPLC analysis revealed that the benzopyrans (HP1, HP2 and HP3) and uliginosin B isolated from field grown plants are also accumulated in micropropagated and acclimatized plants. Nevertheless, in addition to culture conditions optimization, the extraction process is crucial for the enhancement of bioactive substances levels.

Numerous factors influence the chemical quality of medicinal plants from crop establishment to extraction of raw material [14]. Various types of extraction can be applied to the plant material resulting in significant changes in the concentrations and proportions of active components affecting safety and benefits [15].

Supercritical fluid extraction (SFE) is a technique used for separation processes, where the solvent generally used, carbon dioxide (CO_2), is not toxic, non-explosive, of low cost and easily removed from the extract. Furthermore, the supercritical fluid has properties such as high diffusivity, low viscosity and low surface tension, which confers attractive characteristics as a solvent for the extraction of components from the solid matrix [16]. The advantages of

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carbon dioxide under supercritical conditions ($P > 73$ bar; $T > 31\,^{\circ}C$) also include the selectivity [17] and the non-degradation of thermolabile compounds [18].

Various natural extracts containing bioactive compounds can be obtained using supercritical extraction procedures [19–22], which afford a simple, inexpensive, fast, effective and virtually solvent-free sample pretreatment technique. Majority of analytical SFE are performed with CO_2 for the several practical reason cited above. Due to its low polarity, it shows a great selectivity for lipophilic compounds [23,24], which suggests applicability for benzopyrans and phloroglucinol derivatives extraction. Nevertheless, pressure and temperature changes through extraction process modify the CO_2 density and alter the supercritical fluid selectivity as well as the solubility of the substance under extraction by this solvent, affecting the yield and presence of the compounds in the resulting extracts. Therefore, the extraction of specific components from medicinal plants requires research concerning the conditions [21], which give a high yield of total extract and high content of desired pharmacological active compounds.

Intending to establish an efficient benzopyrans and uliginosin B extraction method, the objective of this study was to determine the influence of SFE using CO_2 as fluid on the yield of total extract of aerial parts of *H. polyanthemum*, under successively increased pressures and different temperatures. Additionally, bioactive compounds contents resulted from the different treatments were quantified by HPLC means and compared to those obtained from the *n*-hexane extract of the plant.

2. Experimental

2.1. Plant material

Plants of *H. polyanthemum* Klotzsch ex Reichardt were harvested during its flowering stage, in Guaritas, Caçapava do Sul, Rio Grande do Sul state, Brazil, in October, 2008. Voucher specimens were deposited in the Herbarium of the Universidade Federal do Rio Grande do Sul (ICN) (*H. polyanthemum*, Bordignon et al. 1405). Plant material was carefully dried and powdered to obtain the *n*-hexane extract and to submit to the SFE with CO_2.

2.2. Preparation of the extracts

2.2.1. n-Hexane extract

Powdered aerial parts (0.05 g DW) were extracted 15 times during 20 min with 5 mL of *n*-hexane in an ultrasonic bath (Ultrasonic, São Paulo, Brazil) until colorless liquid extract was obtained. Each triplicate sample was evaporated to dryness under reduced pressure.

2.2.2. Supercritical extraction

Supercritical extractions were carried out on pilot-scale automated equipment (Fig. 1) previously described [25]. The extraction vessel is supplied with a heating jacket and an automated temperature controller. Heating tapes were used throughout the apparatus to maintain constant temperature in the extraction section. To ensure constant and steady solvent delivery the pump head was cooled by a circulating fluid, which passes through a chiller. Flow rates and accumulated gas volumes passing through the apparatus were measured using a flowmeter assay, 1–300 g min^{-1} (Thar 06618-2, USA). Ke (USA) micrometering valves (VC1) were used for flow control throughout the apparatus. Heating tapes with automated temperature controller were also used around this valve to prevent both freezing of the solvents and solid solute precipitation following depressurization. Pressure in the extractor was monitored with a digital transducer system, Novus 8800021600, acquired from Novus Produtos Eletrônicos (Brazil) with a precision of ±1.0 bar. The temperature controller (TC) was connected to thermocouples (PT-100), with an accuracy of 0.5 K. Powdered aerial plant material (180 g DW) was used and the extractions were conducted at different temperatures (40, 50 and 60 °C) while the pressures ranged from 90 to 200 bar. SC CO_2 extraction was successively performed at 90, 120, 150 and 200 bar, collecting the samples of each pressure after 80, 40, 40 and 55 min, respectively. In each extraction pressure the end of the process was confirmed when no variation in mass extract was observed after 30 min (3 × 10 min). The supercritical carbon dioxide flowed at rate of 6.67×10^{-4} kg s^{-1} through the extraction vessel.

2.3. HPLC analysis

Extracts obtained by *n*-hexane ultrasound-assisted maceration and supercritical fluid technique were treated with acetone in order to remove waxes and insoluble impurities, evaporated to dryness and dissolved in HPLC grade methanol, filtered (0.22 μm pore size, Merck) and analyzed by high performance liquid chromatography.

2.3.1. Benzopyrans determination

HP1, HP2 and HP3 analysis were performed according to the method previously described [12] using a Waters 600 pump and a Waters 2487 dual λ absorbance detector set to 220 and 270 nm. The separation was carried out with an isocratic solvent system (60% CH_3CN, 40% H_2O) through a Waters Nova-Pack C18 column (4 μm, 3.9 mm × 150 mm) adapted to a guard column Waters Nova-Pack C18 60A (3.9 mm × 20 mm) and flow rate of 1 mL min^{-1}.

2.3.2. Uliginosin B determination

Uliginosin B yield was determined using the same equipment under isocratic solvent condition (95% CH_3CN, 5% H_2O, 0.01% TFA), flow rate of 1 mL min^{-1} and detection at 220 nm. For quantification, excellent linearity (found in the concentration range between 2 and 800 μg mL^{-1}, with high reproducibility and accuracy) of the calibration curve was achieved ($r^2 > 0.999$). For intra-assay determination, three replicates of eight concentrations of the analyte were used. The detection limit of the compound was 5.38 μg mL^{-1} and for quantitative analysis, peak areas were used to calculate the amount of uliginosin B present in the extracts and compared to standard isolated from the aerial parts of *in natura H. polyanthemum*. The identification was made on the basis of the ultraviolet absorption spectra and retention time (21.76 min).

2.4. Statistical analysis

Means difference among each extraction condition was tested for significance by ANOVA using a probability value of $P < 0.05$. Tukey's test was used to indicate mean separation among these conditions (SPSS Software, version 10).

3. Results and discussion

The performance goals of SFE with CO_2 are to reduce the use of hazardous chemicals, the extraction time and enrich the bioactive compounds contents [19–22]. This fact promotes a non-toxic condition of the process and leads to extracts without residues. The advantages presented justify the SFE technology application to obtain extracts from plants with regard to *n*-hexane extraction. To examine the effect of temperature and pressure on extraction yield and benzopyrans and uliginosin B contents of the resulting *H. polyanthemum* extracts, SC CO_2 extraction was successively performed at 90, 120, 150 and 200 bar and at 40, 50 or 60 °C.

The *H. polyanthemum* lipophilic extracts, besides the dominating benzopyrans and phloroglucinol derivative, mainly contain alka-

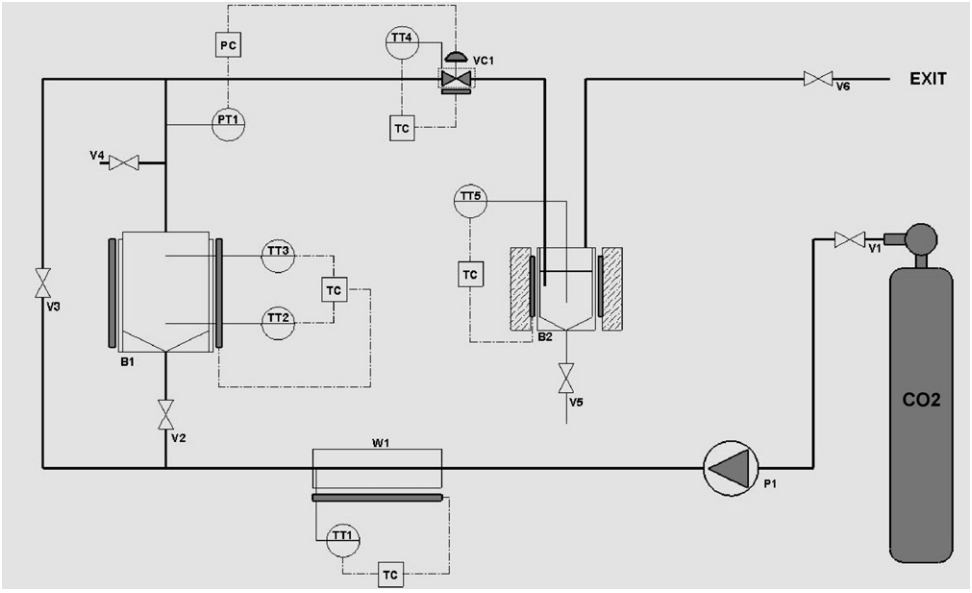

Fig. 1. Schematic diagram of the experimental apparatus: P1, high pressure pump; W1, preheater; B1, extraction vessel; B2, separation vessel; VC1, micrometering; TC, temperature controllers, V1–V6, sphere valves.

nes, fatty acids and wax esters. In fact, the occurrence of alkanes in *Hypericum* species is noteworthy [23] and these compounds were found in all the investigated native species [26]. To obtain purified extracts, each fraction was treated with acetone, producing an insoluble fatty residue, containing epicuticular waxes, which was eliminated through a paper filtration. This is a well-established procedure which is efficient to eliminate these undesirable components [27]. After this procedure, the HPLC analysis of the fractions showed the presence of the three benzopyrans (Fig. 2) and of uliginosin B (Fig. 3) isolated from *in natura H. polyanthemum* [1,2].

The total extraction contents obtained with successive increase of pressure and different temperatures were 30.01 mg/g (40 °C), 22.15 mg/g (50 °C), 17.39 mg/g (60 °C), while the *n*-hexane extraction afforded an amount of 23.40 mg/g (Table 1). Although the yield achieved by the *n*-hexane ultrasound-assisted extraction was equivalent or superior those obtained with SFE at 50 and 60 °C, respectively, these fractions were richest than the *n*-hexane extract regarding the metabolite concentrations. For example, the best supercritical conditions for the extraction of the two most important bioactive compounds afforded concentrations more than

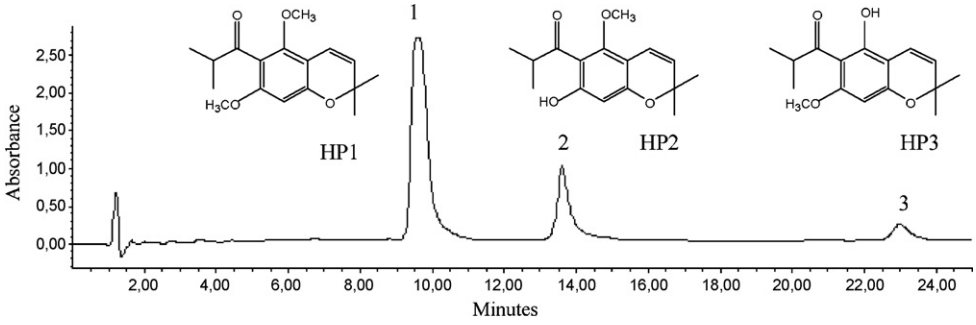

Fig. 2. HPLC profile of benzopyrans (1) HP1, (2) HP2 and (3) HP3 from the CO_2 SFE fraction obtained at 60 °C temperature and 120 bar pressure. Conditions used: Waters Nova-Pack C18 column (4 μm, 3.9 mm × 150 mm) adapted to a guard column Waters Nova-Pack C18 60A (3.9 mm × 20 mm); isocratic solvent system (60% CH_3CN; 40% H_2O); flow rate of 1 mL min^{-1}; injection volume of 20 μL; detection performed at 270 nm at room temperature.

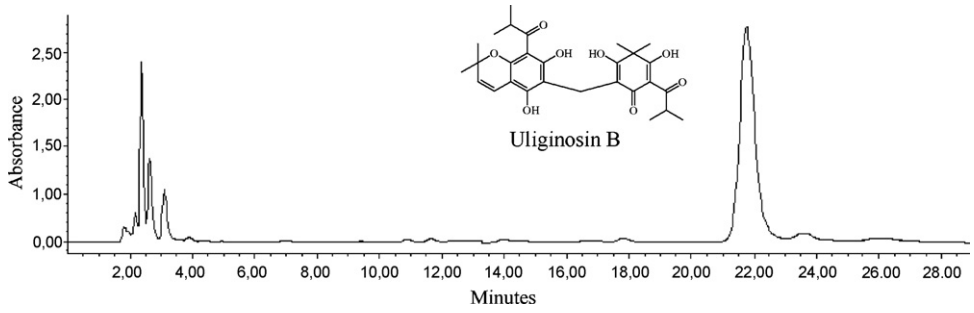

Fig. 3. HPLC profile of uliginosin B from the CO_2 SFE fraction obtained at 50 °C temperature and 150 bar pressure. Conditions used: Waters Nova-Pack C18 column (4 μm, 3.9 mm × 150 mm) adapted to a guard column Waters Nova-Pack C18 60A (3.9 mm × 20 mm); isocratic solvent system (95% CH_3CN; 5% H_2O; 0.01% TFA); flow rate of 1 mL min^{-1}; injection volume of 20 μL; detection performed at 220 nm at room temperature.

Table 1
Summary of the extraction yields and benzopyrans and uliginosin B contents in the SFE extract of *H. polyanthemum* at different pressures and temperatures, and comparison with the *n*-hexane extract. Different letters[a,b,c] among the same pressures indicate significant differences among the different temperatures ($P<0.05$).

Extraction conditions			HP1		HP2		HP3		Uliginosin B	
T (°C)	P (bar)	Extraction yield (g/100 g)	mg/100 g plant	mg/g in the extract (% ± S.D.)	mg/100 g plant	mg/g in the extract (% ± S.D.)	mg/100 g plant	mg/g in the extract (% ± S.D.)	mg/100 g plant	mg/g in the extract (% ± S.D.)
40	90	1.315	239	182.6 ± 6.7[b]	62	47.5 ± 1.6[b]	70	53.3 ± 2.0[c]	184	140.9 ± 8.3
	120	0.265	13	51.0 ± 12.3[c]	3.6	13.7 ± 3.2[c]	4	14.8 ± 3.6[b]	16	60.6 ± 6.7[b]
	150	1.091	81	74.3 ± 10.8[c]	21	19.5 ± 2.8[c]	24	21.7 ± 3.2[b]	29	26.9 ± 2.9[c]
	200	0.330	35	107.2 ± 10.9[a]	13	38.4 ± 16.8[a]	15	28.9 ± 2.6[a]	23	69.9 ± 13.5[b]
50	90	0.338	92	272 ± 1.1[a]	68	187.6 ± 6.0[a]	38	111.4 ± 10.4[b]	nd	nd
	120	0.621	170	274.3 ± 27.6[b]	142	213.2 ± 17.6[a]	97	155.7 ± 38.6[a]	75	120.3 ± 6.0[a]
	150	0.393	55	141.3 ± 13.2[b]	46	117.5 ± 5.8[b]	40	102.6 ± 9.8[a]	138	352.5 ± 14.8[a]
	200	0.863	17	19.9 ± 0.1[b]	16	18.1 ± 1.1[b]	15	17 ± 1.6[b]	139	161.6 ± 3.0[b]
60	90	0.077	20	264.7 ± 8.6[a]	14	200.8 ± 13.6[a]	10	133.9 ± 6.8[a]	nd	nd
	120	0.531	198	373.2 ± 7.7[a]	110	202.4 ± 11.4[a]	105	167.6 ± 18.9[a]	nd	nd
	150	0.376	75	198.2 ± 10.7[a]	40	158.1 ± 12.2[a]	36	97 ± 5.1[a]	68	180.6 ± 19.0[b]
	200	0.755	14	18.1 ± 2.9[b]	11	14.9 ± 1.2[b]	12	15.8 ± 1.2[b]	158	209.9 ± 19.3[a]
n-Hexane		2.340	275	117.5 ± 1.8	148	63.3 ± 0.5	194	82.7 ± 22.7	163	69.7 ± 3.4

nd: not detected.

3-fold higher for HP1 (60 °C, 120 bar) and 5-fold higher for uliginosin B (50 °C, 150 bar) when compared to the *n*-hexane extraction (Table 1). Furthermore, it is important to highlight that, except for the HP3 content, the superior extract yield obtained with the organic solvent was not enough to provide higher quantity of the analyzed metabolites. This fact suggests that the SFE was more selective than the conventional technique to obtain the target metabolites, probably due to the differences of the extractor polarity mean and the affinity to the solute, providing a rentable source of bioactive metabolites.

The contents of HP1, HP2, HP3 and uliginosin B obtained in the supercritical fluid extraction process showed a different concentration trend with pressure increase as well as temperature variation (Table 1). Higher total yields of HP1 (368 mg/100 g plant) were obtained at 40 °C, being 65% of this amount obtained at 90 bar. Nevertheless, calculating on the basis of the percentage of extract, the HP1 richest fraction (ca. 373.2 mg/g) was verified at 60 °C and 120 bar. Higher total yields of HP2 (272 mg/100 g plant) were obtained at 50 °C, being half of this amount obtained at 120 bar. Coincidently, this condition afforded a fraction with high concentration of the compound (213.2 mg/g).

A similar profile was observed at 60 °C/120 bar (202.4 mg/g), even though the total extraction of HP2 at this temperature was inferior to the amount achieved at 50 °C.

The same behavior reported for HP2 was observed in the extraction of HP3 (Table 1).

For the uliginosin B extraction, the pressure of 90 bar and temperature of 40 °C afforded high amounts of the metabolite (184 mg/100 g plant). Nevertheless, considering the total amount recovered at the 50 °C experiment it can be seen that the increase of temperature and pressure had a determinant role in extraction yields (Table 1). It is interesting to point out that the temperature of 50 °C was also found to be the optimum condition for the extraction of phloroglucinols from *Hypericum perforatum* using supercritical carbon dioxide [28].

The results obtained in this work indicated that when higher temperatures are used (i.e. 50 and 60 °C) higher pressures are necessary to obtain uliginosin B, due to its higher molecular-weight. As demonstrated in Table 1, at 60 °C this compound was detected only at the pressure of 150 bar. Therefore, the extraction of uliginosin B at higher temperatures strongly depends on the CO_2 density. These results are in accordance with the reports of studies with other medicinal plants where higher molecular-weight compounds are extracted in larger quantities at the end of the extraction process [24].

Taking into account the total amount of the benzopyrans and phloroglucinol derivative in the experiments performed at successive increased pressures and different temperatures, it was observed that the increase of temperature leads to a decrease in the mass amount of extract (g/100 g) and an increase of HP1, HP2, HP3 and uliginosin B concentration. The total concentration of these metabolites represents 27.80% of the extract obtained at 40 °C, while at 50 and 60 °C, it corresponds to 51.82% and 50.1% of the extract obtained, respectively. These data are in accordance to a study performed with *H. perforatum* where an increased amount of extract was followed by a decrease of hyperforin concentration, as a consequence of the increased yield of other compounds [23]. Nevertheless, in the work of Glisic et al. [21] this effect was not observed and the authors reported that the absolute extracted amount of hyperforin and adhyperforin was almost constant, not depending of the total yield of extract, i.e., on density of SC CO_2, determined by the temperature.

The results obtained in the present work cannot be compared in details with those achieved with other *Hypericum* species, considering that the Brazilian species do not present hyperforin or adhyperforin. The literature of SC CO_2 extraction focuses on *H.*

perforatum, which accumulates the above mentioned compounds [21] and no report regarding plants of the genus without hyperforin is available. The dimeric phloroglucinol derivatives found in Brazilian species differ from hyperforin and adhyperforin, which possess a bicyclononane skeleton substituted with several isoprene chains. The dimeric compounds frequently present a benzopyran nucleus formed by the cyclization of the prenyl unit with an adjacent hydroxyl group. Together with these dimeric compounds, some species, such as *H. polyanthemum*, also present the benzopyran nucleus found as monomers [1,2]. Therefore, such difference in the composition of the lipophilic fraction may account for specific extraction conditions of the plant studied in this work.

4. Conclusions

The experimental results for the bioactive metabolites yields obtained in the extraction of *H. polyanthemum* demonstrated that the compounds were easily extracted by SC CO_2 with successively extractions at 90, 120, 150 and 200 bar in the three studied temperatures, being the best condition to extract the benzopyrans and uliginosin B determined to be at 50 °C. Further optimization of the extraction protocol is under investigation to obtain these pharmacological active compounds.

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