Extraction of saponins from *Pfaffia glomerata* (Spreng) Pedersen roots by sequential extraction in a fixed bed using $scCO_2$, ethanol and water as solvents

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ABSTRACT

Saponins are surfactants that reduce the surface tension of aqueous solutions and also stand out for their pharmacological actions. This study aimed to extract saponins from *P. glomerata* roots by a sequential process in fixed bed using $scCO_2$, ethanol and water as solvents. Extractions were performed in a particle bed, at 50 ^oC e 300 bar, and in four sequential steps. The efficiency of extraction and fractionation process was evaluated based on: 1) the extraction yield, 2) presence of saponins, which were monitored by thin layer chromatography (TLC) and 3) the potential of extracts to reduce the surface tension of aqueous solutions. The extraction yields for the four steps were 0.16, 0.55, 1.00 and 6.90 %, respectively, showing predominant presence of more polar compounds in these species. Analyses by TLC indicated that process was selective in accordance with the polarity of the solvent used, providing fractions enriched in different compounds. The extract obtained by mixing carbon dioxide and ethanol (SCEE) showed the highest surface tension reduction: 72.4 $mN.m^{-1}$ (deionized water) to 25.0 $mN.m^{-1}$. This result suggests this potential is associated with the presence of less polar saponins. Furthermore, it was found that SCEE presented critical micelle concentration of 2.0 $g.L^{-1}$. Finally, the supercritical carbon dioxide showed to be selective for saponins with the highest capacity to reduce the surface tension of water when ethanol was used as cosolvent.

INTRODUCTION

Pfaffia glomerata (Spreng) Pedersen species is a native plant of Brazil widely used in Brazil for years due to its medicinal properties. They are popularly known as Brazilian ginseng, and saponins are cited as one of its active constituents [1]. Saponins are usually produced by plants against pathogens agents and herbivores. Apart from their role in plant defense, these compounds are of great interest because they are active components of drugs with valuable pharmacological properties. Among the biological actions attributed to saponins, immunostimulatory properties, as well as anticancer, antimicrobial, antifungal, anti-inflammatory and antiviral activities are reported [2]. Structurally, saponins are high molecular weight glycosides having a fundamental nucleus, known as aglycone (triterpene or steroid) connected to different types and numbers of sugar units. Therefore, they represent a diverse group of amphiphilic compounds, responsible for surface and interfacial activities of these compounds [3]. Although studies on the surface-active properties of saponins of plant origin were mostly performed with *Quillaja* saponins [4,5], the soyasaponins [6], Asian ginseng (*Panax ginseng*) [7], juá and sisal saponins [8] have also been investigated.

The supercritical technology has been highlighted for obtaining high purity extracts, besides being a green technology. Among the solvents commonly used as supercritical fluid,

carbon dioxide stands out, an inert and non-toxic compound that is applied to foods and pharmaceuticals, producing clean extracts and residues. However, the extraction using pure supercritical carbon dioxide ($scCO_2$) is restricted to apolar and/or low polarity substances. Facing this situation, one alternative to use $scCO_2$ for separation of more polar compounds such as saponins is to use water or ethanol as cosolvent to increase $scCO_2$ polarity. Moreover, extraction techniques involving several sequential steps with $scCO_2$ and combinations of $scCO_2$, ethanol, and water have been very successful for extracting and fractionating anthocyanins and phenolics [9-12], as a prior step to the conventional extraction with water and ethanol to give extracts enriched in compounds of interest [13].

The aims of this study were to extract saponins from *P. glomerata* roots by sequential extraction process in fixed bed, using $scCO_2$, ethanol, and water as solvents, and to characterize the extracts for their ability to reduce the surface tension.

MATERIALS AND METHODS

Raw Material and Characterization

P. glomerata roots (voucher at Chemical, Biological and Agricultural Pluridisciplinary Research Center - CPQBA Herbarium N^o 567) have been cultivated in the experimental field at CPQBA - UNICAMP, Campinas, Brazil, at the geographical coordinates 22^o 48' South and 47^o 07' West, and collected in December 2009. After collection, the roots were perforated (Trapp), dried in an air circulation oven at 40 ° C for four days and milled (Ametek, Model 114584) in dry ice. To better particle size distribution, the samples were milled again (MA 340, Marconi, Brazil), and stored in glass flasks with lids in freezer at -26 ºC (Brastemp Flex, Brazil).

Extraction Method

The extractions were performed at 50 °C and 300 bar in four sequencial steps in a fixed bed extractor in the experimental unit (Laboratory of extraction, applied thermodynamics and equilibrium - ExTrAE, UNICAMP, Brazil) as shown in Figure 1. It was used CO_2 (99.5 % w/w) (White Martins Gases Industriais, Brazil), absolute ethyl alcohol (Êxodo Científica, AE09700RA, Brazil), and ultra pure Milli-Q water (Millipore direct-Q3 UV, Millipore Corporation, EUA) as solvents.

The extractor (7) was packaged with the raw material mixed with 5 mm diameter glass beads. The thermostatic bath was set at the desired temperature, and the pressure was reached by pumping up (3) CO_2 into the extractor. The extraction was initiated by draining CO_2 at a flow rate of 1.0 $L.min^{-1}$ (1.65 $g.min^{-1}$) through the bed (extractor) and collecting the extract in the collection flask (8). Specifically for the extractions using $scCO_2$, this was led to a gas flow meter (9) to control the CO_2 flow and to a volume totalizer (10) to measure the carbon dioxide used in the experiment. The residue 1 from the first extraction step remained inside the extractor, and the second step was initiated by pumping (3) ethanol concomitantly CO_2 (Figure 2). At the end of this process, the system was depressurized. Then, the third step was performed from the residue 2, in which pure ethanol was used as a solvent and finally the residue 3 was extracted by a hydroalcoholic solution. The material remaining in the extract after the completion of the four extraction steps was denominated final residue.

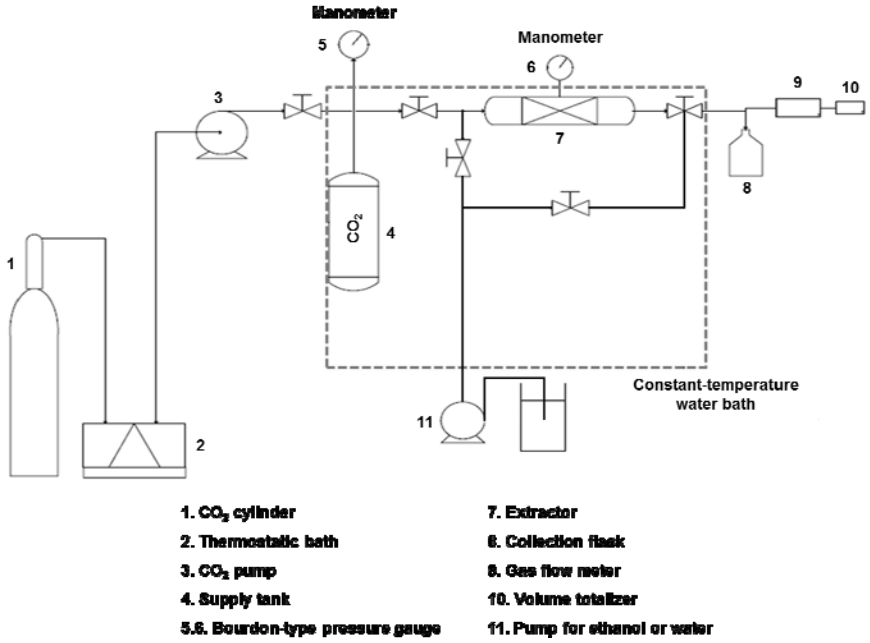

Figure 1: Experimental extraction unit.

As result of the four-step sequential extraction, four extracts were obtained, as follows: SCE (supercritical extract), SCEE (supercritical + ethanol extract), EE (ethanolic extract), and HE (hydroalcoholic extract). The extracts were concentrated in a rotary evaporator (Rotary evaporator Marconi, MA-120, Brazil), and dried in an oven (Marconi, MA030/12, Brazil) under vacuum (Vacuum Pump Marconi, MA057 / 1, Brazil) at 50 °C to constant weight. The extraction yields (percentage) were calculated as the ratio of the total mass of the extract to the initial mass of the raw material used to assemble the extractor. Kinetic extraction curves (mass of extract x extraction time) were constructed by collecting samples at set time intervals.

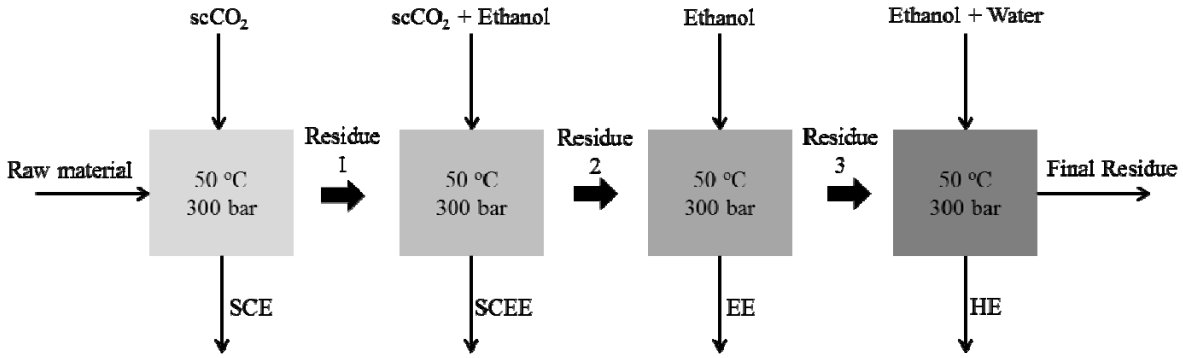

Figure 2: Flowchart of four-step sequential extraction.

Thin Layer Chromatography (TLC)

The chromatographic profiles of the extracts were analyzed by thin layer chromatography (TLC). Plates precoated with Silica gel G 60 F254 were used as stationary phase (1.05554.0001, Merck, Germany), and a mixture of chloroform (Synth, Brazil): methanol (Synth, Brazil): 0.5% trifluoroacetic acid (Vetec, Brazil) (60:40:5 v/v/v) was used

as mobile phase. An anisaldehyde solution (universal stain) was used to reveal the compounds. β-ecdysone (20-hydroxyecdysone, Sigma-Aldrich H5142, 93% purity) and extracts obtained by conventional Soxhlet extraction using methanol as solvent were used as reference.

Surface Tension (TS)

The sample extracts were solubilized in deionized water to a concentration of 20 $g.L^{-1}$. The reduction of surface tension in the saponin-containing extracts was compared with the surface tension of a standard synthetic surfactant, sodium dodecyl sulfate (SDS). Aqueous solutions of sodium dodecyl sulfate (Synth, Brazil) with a concentration ranging from 0.01 to 50 $g.L^{-1}$ were prepared. The surface tension measurements were determined at 24 °C in a tensiometer (Digital Tensiometer K10ST, Kruss, Germany) using a Pt-Ir ring. The critical micelle concentration (CMC) was determined by the intersection between the straight lines formed in the surface tension graph as a function of the logarithm of SDS concentration.

Statistical analysis

All analyses were performed in triplicate, and the results were submitted to analysis of variance to compare means with one-way ANOVA and post hoc Tukey's test ($p < 0.05$), using the statistical software Minitab version 16 (Minitab, State College, PA, USA).

RESULTS

Three sequential extractions in fixed bed were performed for *P. glomerata* roots. In two of them, samples were taken at short intervals to build the overall extraction curves shown in Figure 3. The third extraction was carried out so that only a sample of each step was removed to be characterized for thin layer chromatography (TLC) and surface tension. Supercritical extraction (Step 1) was performed in 3 hours. In this step, the mass of the supercritical extracts (SCE) was small, making measurements more difficult, which may explain the irregularities observed in this step for the supercritical extracts (Figure 3A). SCEE extracts (Figure 3B) were collected for 5 hours. The curve of mass of extract versus extraction time showed that the extraction rate decreased in the last hours of the process, since approximately 75% of the SCEE extract was extracted during the first 3 hours. The last two steps (Figure 3C and 3D) were carried out for 6 hours each, and 68 and 72% of the EE and HE extracts, respectively, were obtained until the first 3 hours.

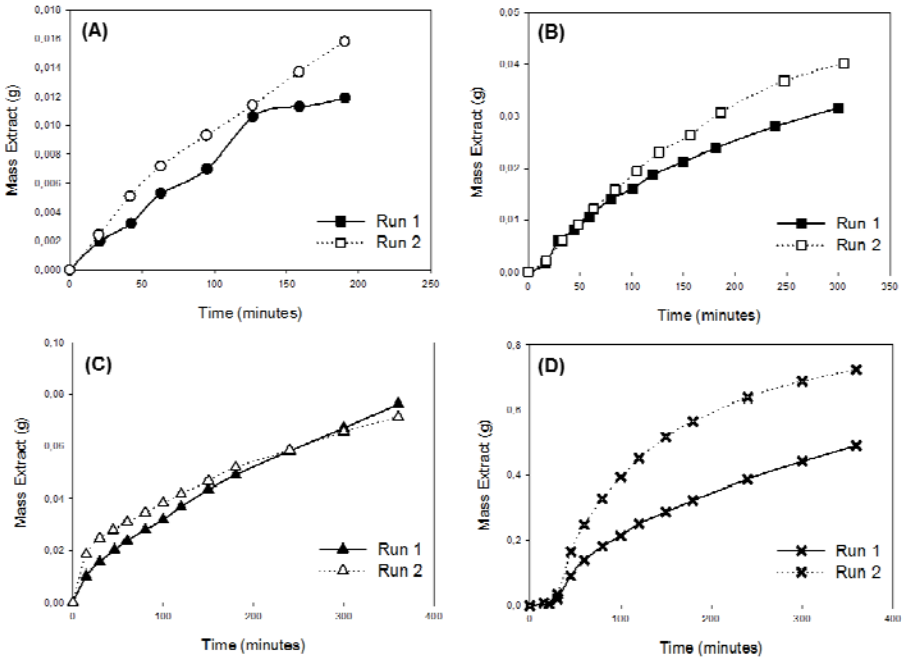

Figure 3: Overall extraction curves of *P.glomerata* in fixed bed at 300 bar and 50 °C: SCE (A), SCEE (B), EE (C) and HE (D).

Table 1: Extraction yields at 50 °C and 300 bar, and surface tension values at 24 °C for aqueous extracts at 20 $g.L^{-1}$.

Extract	Yield (%)	Surface Tension at 20 °C ($mN.m^{-1}$)
SCE	0.16 ± 0.07^{b}	-
SCEE	0.55 ± 0.09^{b}	25.0 ± 0.2^{e}
EE	1.00 ± 0.09^{b}	29.5 ± 0.9^{d}
HE	6.9 ± 3.3^{a}	41.8 ± 1.2^{b}
Global	8.6 ± 3.5^{a}	-
SDS	-	$36,3 \pm 1,3^{*c}$
Deionized Water	-	$72,4 \pm 0,4^{a}$

SCE: supercritical extract; SCEE: supercritical + ethanol extract; EE: ethanolic extract; HE: hydroalcoholic extract; Global: SCE + SCEE + EE + HE;
* mean values of surface tension of aqueous solutions of sodium dodecyl sulfate (SDS) above the CMC.

The extraction yields of each step are shown in Table 1. The lower yields were obtained during the first three extraction steps (SCE, SCEE, and EE). It was observed that the higher the polarity of the solvent or solvent mixture, the higher the extraction yields were, and the greatest yield was observed in the extraction step carried out with ethanol/water (70:30 v/v) as solvents. However, the lowest values of surface tension of the extracts (Table 2) evidenced the presence of saponins mainly in the SCEE and EE extracts. These data are extremely relevant, since the surface tension analysis is a quick and inexpensive method to show that the last extraction step (hydroalcoholic) is not very important to the extraction of saponins

The chemical profile of the extracts in study was monitored by the TLC analysis (Figure 4). The crude extracts obtained by Soxhlet extraction with methanol were used as reference. (sample 6). It has five groups of compounds represented by their retention factors (R_f) which is desired to separate. Based on the retention factor of the compounds and the chromatographic conditions that were previously studied at CPQBA-UNICAMP (FAPESP-process 2006/06059-3, unpublished data), it is known that the less polar compound (R_f^1) is the steroid β-ecdysone, and the others are saponins of different polarities. The compounds in the hatched area highlighted in Figure 4 are not of interest in this study, once the saponins are more polar than the β-ecdysone due to the sugar units linked to its structure.

It was observed that the solvents used were partially selective obtaining extracts enriched in different compounds or groups of compounds. The sample 1 (SCE) showed apolar compounds with no interest in this study. The SCEE extract (sample 2) showed predominantly β-ecdysone and the less polar saponins represented by R_f^2 and R_f^3. The ethanolic extract (sample 3) had a mixture of β-ecdysone (R_f^1) with more polar and less polar saponins, thus it was not possible to state about the presence of a major compound in this extract. Finally, the hydroalcoholic extract (sample 4) differed from the EE extract, mostly for presenting the compound R_f^5.

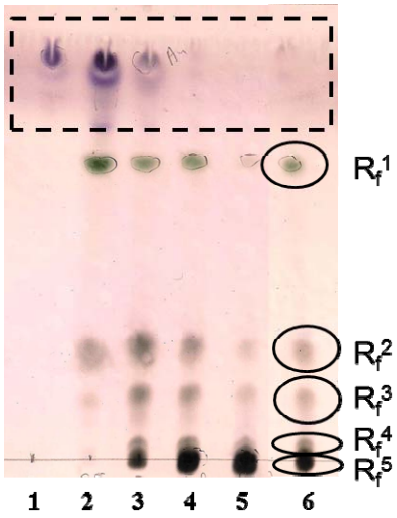

Figure 4: TLC analysis of *P. glomerata* extracts 1: SCE; 2: SCEE; 3: EE; 4: HE; 5: final reside; 6: methanolic extract (reference sample). Solvent phase: chloroform: methanol: trifluoroacetic acid 0,5% (60:40:5 v/v/v). Detection: p-anisaldehyde solution.

The surface tension analyses were performed on the extracts whose chromatographic profile showed the presence of saponins (natural surfactants). Therefore, the extracts analyzed were SCEE, EE and HE. The results are shown in Table 1.

In dilute solutions, the surfactant molecules are dispersed, whereas in more concentrated solutions, they are organized in molecular aggregates called micelles. The concentration value where the formation of micelles begins is known as critical micelle concentration (CMC). Near the surface, the polar groups are oriented towards the aqueous solution, while the apolar groups are located in the air-water interface, thus minimizing the contact with water. Therefore, the cohesive force between the solvent molecules decreases,

reducing the surface tension. After the CMC, unlike the monomers, the micelles are dispersed in the solution with no more effect on the surface tension [14, 15].

The extracts showed significant differences ($p \leq 0.05$) in their ability to reduce the surface tension. The supercritical + ethanol extract (SCEE) presented the lowest surface tension (25.0 $mN.m^{-1}$), followed by the ethanolic extract (29.5 $mN.m^{-1}$) and hydroalcoholic extract (41.8 $mN.m^{-1}$). It was also observed that the SCEE and EE extracts reduced the surface tension to values less than the minimum reached by sodium dodecyl sulfate (36 $mN.m^{-1}$), and until values comparable to surfactin. Surfactin is a biosurfactant produced by Bacillus subtilis, known to have exceptional surface activity, capable of reducing the surface tension of water (20 °C) from 72 to 27 $mN.m^{-1}$ [16].

Comparing this result with the chromatogram shown in Figure 4, it is observed that the compounds with greater ability to reduce the surface tension are less polar saponins represented by R_f^2 and R_f^3. According to Decroos et al. [6], the number of sugar chains attached to the aglycone affects the CMC of saponins, once monodesmosidic saponins (with a sugar chain linked to the aglycone) isolated from soybeans presented greater ability to reduce the surface tension when compared to bidesmosidic saponin (with two sugar chains).

From this result, the SCEE extract were analyzed at concentrations ranging from 0.05 to 20 $g.L^{-1}$, and the surface tension values are shown in Figure 5. The SCEE extract presented CMC values of 2.0 $g.L^{-1}$. Stanimirova et al. [4] examined extracts of Quillaja saponaria from Chile and found that the CMC values at 25 °C were around 0.25 $g.L^{-1}$, and the lowest surface tension values were around 40 $mN.m^{-1}$. Soyasaponins studied by Decroos et al. [6] presented CMC < 1 $g.L^{-1}$, but all the surface tension values were greater than 40 $mN.m^{-1}$. Ribeiro et al. [8] studied saponin-rich plant extracts and found that Jua and Sisal extracts presented decreased surface tension values varying from 35 to 40 $mN.m^{-1}$. Asian ginseng extracts containing ginsenosides presented CMC values of 0.339 $g.L^{-1}$, and the lowest surface tension was found near 40 $mN.m^{-1}$ [7]. Despite SCEE extract of *P. glomerata* resented CMC values higher than those reported in the literature, these reduced the surface tension to values significantly lower.

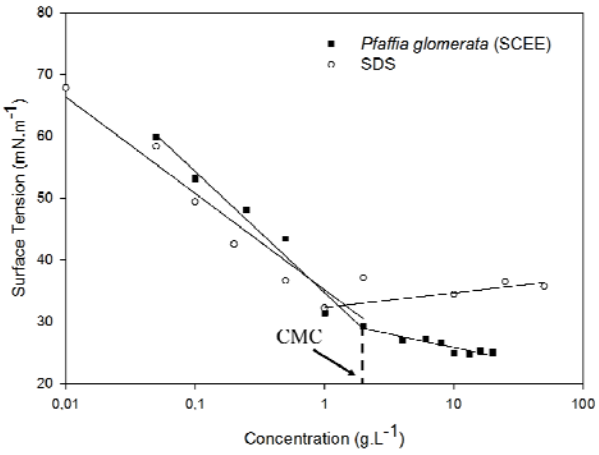

Figure 5: CMC values for SDS (dodecyl sulfate sodium) and SCEE extract of *P. glomerata*.

CONCLUSION

The sequential extraction in fixed bed using green solvents proved to be a good alternative to organic solvents to extract saponins from *Pfaffia glomerata* roots. The process was selective according to the polarity of the solvent providing fractions enriched in different groups of compounds (saponins). The extract obtained by mixing carbon dioxide and ethanol (SCEE) exhibited excellent capacity to reduce the surface tension suggesting that this potential is associated with the presence of less polar saponins in the extract. Furthermore, the results indicate the selectivity of supercritical carbon dioxide for saponins with the highest capacity to reduce the surface tension of water when used with ethanol as cosolvent.

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