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Propolis extracts obtained by low pressure methods and supercritical fluid extraction

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ABSTRACT

Propolis is a natural product used for centuries by human kind, due to several evidenced biological activities: antioxidant, antimicrobial, anti-inflammatory, antitumor and anti-HIV. Extracts from propolis, used in food, pharmaceutical and cosmetic industries, present quality and composition related to the extraction method applied. Natural compounds with biological activity can be obtained by conventional techniques, such as Soxhlet and Maceration, or by alternative methods such as supercritical fluid extraction (SFE). Thus, the aim of this work was to compare propolis extraction yields obtained by different procedures, for instance, SFE in one stage, with CO_2 and CO_2 plus co-solvent, and SFE in two stages, as well as Soxhlet and Maceration as low pressure extraction methods using ethanol, ethyl acetate, chloroform, n-hexane, water and mixtures of water/ethanol. The operational conditions for SFE in one stage with pure CO_2 were: 30, 40 and 50 °C and from 100 to 250 bar. The SFE with co-solvent was performed at 150 bar and 40 °C and ethanol concentrations of 2, 5 and 7% (w/w). The highest yield was obtained by chloroform Soxhlet extraction (73 ± 2%, w/w) whereas for SFE the maximum yield was 24.8 ± 0.9%, using 5% ethanol as co-solvent. For SFE in two stages, 100 and 150 bar were used in the first stage while 250 and 300 bar were applied in the second stage, at 40 °C. The yields were 8.4 ± 0.7 (150 bar) and 5.1 ± 0.7 (250 bar), for stages 1 and 2, respectively. The chemical composition of the propolis material was determined by HPLC analysis. The experimental data were correlated using four models based on differential mass balance equations: (1) the Sovová's model; (2) the logistic model (3) the diffusion model and (4) the simple single plate model (SSP). The logistic model provided the best adjustment for propolis SFE curves.

1. Introduction

Propolis is a complex resinous mixture produced by bees through the mixture of different plant exudates, beeswax and salivary secretions [1–3].

Because of its popularity in folk medicine, propolis has become the subject of intense pharmacological and chemical studies for the last 30 years. Numerous studies have proven its versatile activities: antioxidant [4–6]; antimicrobial [2,7–8]; anti-inflammatory [9–10]; anticancer [11–12] and anti-HIV [13]. These biological activities are attributed to compounds such as phenolic acids, flavonoids, terpenes and sesquiterpenes [2,14].

The chemical composition of propolis is complex and variable, being related to the vegetation of the region visited by bees [15]. In general, it consists of 50–60% resins and balsams, 30–40% wax, 5–10% essential oils, 5% pollen granum, and microelements [3].

Although low variability in composition is observed for propolis from temperate regions like Europe, where the main bioactive compounds are flavonoids [2,15], this behavior is not detected for Brazilian propolis. Therefore, because of the composition variability, Marcucci in 2006, classified Brazilian propolis (BRP) according to chemical markers [16] such as 3-prenyl-4-hidroxycinnamic acid (PHCA), 2,2-dimetyl-6-carboxietenyl-2H-1-benzopirane (DCBEN), 6-propenoic-2-2-dimetyl-8-prenyl-2H-1-benzopirane acid (DPB) and 3,5-diprenyl-4-hydroxicinnamic acid (DHCA). The DHCA, also known as Artepillin C, was initially isolated in 1994 and has been the focus of several researches due to antimicrobial potential, anticancer and anti-inflammatory properties [10,12], and antioxidant activities [17]. Therefore, the presence of Artepillin C, labels the propolis from Brazil as "*the Brazilian Própolis*", placing Brazil as Japan main supplier, with 80% of the demand [18].

Propolis extract has applications in several products such as nutraceuticals, cosmetics, dental hygiene goods, creams and food supplements [14]. The most used product is the ethanolic extract, but nowadays, more people has allergy to ethanol, restricting its use. Propolis extracts are obtained by conventional techniques such as Soxhlet extraction and solvent Maceration, or by alternative methods like supercritical fluid extraction (SFE). According to Reverchon and De Marco [19], SFE presents desirable characteristics: high flexibility by adjusting solvent power and process

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selectivity; high product quality due to low use of polluting organic solvents; and reduced costs from solvent elimination. Carbon dioxide is the most common supercritical solvent and behaves as a lipophilic solvent (non-polar). Then, the use of co-solvents improves the SFE performance, as shown by Campos et al. [20]. A variation for SFE is the separation in sequence steps, where the process temperature and pressure are varied. In this strategy, different families of compounds are obtained from one raw material. For instance, moderate solvent density at the first step extracts high soluble components (essential oils), while high density solvent at the second step extracts heavy substances such as antioxidant compounds [19]. Paviani et al. [21] studied the SFE of dried ethanolic extract of Brazilian propolis, investigating the fractionation of components of interest present in propolis extract and the results indicated higher selectivity at low solvent density.

Therefore, different extraction methods for Brazilian propolis (Soxhlet and Maceration with various solvents, SFE with CO_2 and with co-solvent and SFE in two steps) were compared in this work in terms of yield and composition. Also, the extraction curves obtained by SFE-CO_2 were adjusted using four mass transfer models: Sovová's model [22], logistic model by Martínez et al. [23], diffusion model by Crank presented by Reverchon [24] and SSP model by Gaspar et al. [25].

2. Materials and methods

2.1. Raw material characterization

Pulverized crude propolis, supplied by *Breyer Ltda.* an apiary company (PR, Brazil), was stored at −18 °C in a domestic freezer. The propolis sample consisted of a material representative from the South of Brazil. The pulverized material was classified in a sieve separator and the fraction of mesh −16/+65, was selected to perform all the extractions. The raw material moisture content was determined according to AOAC [26]. The wax and flavonoid contents of propolis sample were determined by Natural Labor laboratory, according to Bankova and Marcucci [27].

Particle diameter and porosity of the fixed bed, formed by grinded propolis and used in SFE, were determined. The mean particle diameter was obtained by particle size distribution method using the following equations [28]:

$$\bar{D}_s = \sqrt{\frac{\sum_1^n \Delta\ell_i/\bar{D}_i}{\sum_1^n \Delta\ell_i/\bar{D}_i^3}} \quad (1)$$

$$\Delta\ell_i = \frac{m_i}{M} \quad (2)$$

where $\bar{D}_s$ is the superficial mean particle diameter (cm), m_i is the sample mass retained in sieve i (g), M is the sample total mass (g), $\bar{D}_i$ is the mean diameter of sieve i (cm) and n is the total fractions number.

Gas pycnometer analyses (Ultrapycnometer 1000, Quantachrome), with helium displacement, were used to evaluate the solid phase density, the real density (dr). The bed porosity (ε) was calculated considering apparent density (da) by: $\varepsilon = 1 - (da/dr)$.

2.2. Low pressure extractions (LPE)

The Maceration (Mac) was performed according to Cunha et al. [1] using absolute ethanol (EtOH) (Nuclear, CAQ Ind. & Com. LTDA.) and ethanol water solutions at 50% (v/v) (EtH_2O, 50%) and 70% (v/v) (EtH_2O, 70%). Briefly, 10 g of pulverized propolis were placed in contact with 40 mL of solvent for seven days at room temperature. The separation of propolis sample and extract was done by filtration at room temperature.

The Soxhlet extraction (Sox) was performed according to Cunha et al. [1]. Pulverized propolis (5 g) placed inside a paper timber was submitted to 6 h Soxhlet extraction at a maximum temperature of 60 °C, using 150 mL of solvent. Different solvents were used: n-hexane (Hex), chloroform ($CHCl_3$), ethyl acetate (EtAc), ethanol (EtOH) (Nuclear, CAQ Ind. & Com. LTDA.) and distilled water (H_2O); with polarities of 0.0, 4.1, 4.4, 5.2 and 9.0 [29], respectively.

The extracts were maintained at −18 °C overnight and then filtered at 0 °C to remove waxes. The resulting extracts were evaporated at reduced pressure to obtain the dry extract and the yield results were calculated based on the initial amount of propolis (w/w). All extractions were performed in duplicate.

2.3. Supercritical fluid extraction (SFE)

The supercritical assays were carried out using the dynamic method to obtain extraction curves and the global yield (X_0). The SFE with CO_2 was performed by two procedures: one step (OS-SFE) and two sequential steps (TSS-SFE). The OS-SFE consists of using one condition of pressure, temperature and solvent flow rate for each assay. The same procedure was used for SFE with co-solvent. For the TSS-SFE, two levels of pressure, at constant temperature and solvent flow rate, were applied for each assay. The objective of TSS-SFE is to obtain two fractions of extract (in each assay) with different chemical and biological features. The first step was performed at moderate pressure (100 or 150 bar), where soluble compounds such as wax and essential oils should be extracted. The second step was performed at higher pressure levels (250 or 300 bar) in order to extract phenolic acids and flavonoids, with important biological activities, for instance, antioxidant and antimicrobial properties.

The high-pressure unit used for the SFE with CO_2 and solvent mixtures (CO_2 plus co-solvent) was modified from the unit described by Zetzl et al. [30]. In the present work, a co-solvent pump (Constametric, 3200, EUA), was connected to the extraction line in order to supply the modifier (co-solvent) to the extraction vessel. This pump works with flow rate from 0.01 to 9.99 mL min^{-1}. Ethanol (EtOH) was used as co-solvent in concentrations of 2, 5 and 7% (w/w). The CO_2 was 99.9% pure delivered at pressure up to 6 MPa (White Martins, Brazil). The extraction methodology, described by Michielin et al. [31], consisted of placing 20.00 ± 0.03 g of pulverized propolis inside the extractor cell to form the fixed bed. The temperature and the pressure were adjusted and the extraction proceeded by collecting the solute in amber flasks, after 5 h extraction, weighted in analytical balance (OHAUS—AS200S, NJ, USA). The yield assays to obtain the X_0 values were performed in duplicate and divided in three groups:

(1) OS-SFE with CO_2: this group followed a complete factorial design with two factors (temperature and pressure) at three levels (30, 40, 50 °C and 100, 150, 200 bar), and the solvent flow rate (Q) at 3.0 and 5.0 g CO_2 min^{-1}. The extraction length (time) was defined by the use of 900 g CO_2 as solvent, i.e., for Q = 3.0 g CO_2 min^{-1}, the extraction time was 5 h, and for Q = 5.0 g CO_2 min^{-1}, the extraction time was 3 h.
(2) OS-SFE with CO_2 and co-solvent at concentrations of 2, 5 and 7% (w/w): these assays were performed at 40 °C, 150 bar, 5.0 g min^{-1} and 3 h extraction. The extracts were then evaporated at reduced pressure (Fisatom 802, Brazil) and weighted in analytical balance to obtain X_0.
(3) TSS-SFE: in sequential extraction two different conditions were applied. The first step was performed at 100 bar and at 150 bar at fixed 40 °C and 5.0 g CO_2 min^{-1}, for 2 h extraction. For the second step, the pressure levels were 250 and 300 bar, at constant 40 °C for a period of 3 h extraction.