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Standardizing the analysis of phenolic profile in propolis

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ABSTRACT

The analysis of propolis is controversial, hampering the comparison of its biological properties and estimation of its commercial value. This work evaluates the effectiveness of combining maceration and ultrasonication extraction techniques on the yield, total phenol content (Folin-Ciocalteau) and the specific phenolic compounds (HPLC-UV), on propolis from different origins. The extraction method was not significant in any case; therefore ultrasonication is recommended (time-saving) but only when a double extraction is performed. Propolis yield varies significantly between samples, as it includes impurities, consequently the results should be expressed considering the yield (as balsam) instead of raw propolis. Of the 13 quantified phenolic compounds, CAPE and pinocembrin (803 and 701 mg/g balsam) stand out. The phenolic profile of a propolis must be fixed using both total phenol content (with a consensus reference compound) and the specific phenolic compounds, since the latter provides information about compounds that can play a significant antioxidant role.

1. Introduction

Bees (*Apis mellifera*) use propolis as a sealant to protect their hives against invaders, heat, humidity and wind. They produce this product by collecting resinous substances from the exudates of certain plants that are modified by mixing with enzymes, pollen and wax; therefore, it is composed of resins (50%), waxes (30%), essential oils (10%), pollen (5%) and other organic substances (5%) (Cheng, Qin, Guo, Hu, & Wu, 2013; Gómez-Caravaca, Gómez-Romero, Arráez-Román, Segura-Carretero, & Fernández-Gutiérrez, 2006; Pellati, Prencipe, & Benvenuti, 2013; Pietta, Gardana, & Pietta, 2002). Propolis has traditionally been used for its antioxidant and medicinal properties (antimicrobial, antiviral, antiinflammatory, antitumoral, hepatoprotective and immunomodulary activies) (Osés, Pascual-Maté, Fernández-Muiño, López-Díaz, & Sancho, 2016). Among the > 300 compounds identified in this product, phenolic compounds should be highlighted, as they are mainly responsible for its pharmacological and biological activity (Alm-Eldeen, Basyony, Elfiky, & Ghalwash, 2017; de Francisco et al., 2018; Díaz-Carballo, Malak, Bardenheuer, Freistuehler, & Reusch, 2008; Freires, de Alencar, & Rosalen, 2016; Pellati et al., 2013; Sampietro, Vattuone, & Vattuone, 2016; Sforcin, 2016; Soltani et al., 2017; Yang et al., 2015). The presence in these bioactive compounds in propolis is strongly influenced by the vegetation and climate in the region where the bees are kept (Bankova, 2005; Cheng et al., 2013; Soltani et al., 2017).

Different solvents may be used for extraction of the active components of propolis. Among them, an ethanol/water mixture (70/30) is the most commonly used as it is non-toxic and very efficient at extraction, specially of polyphenols and flavonoids, commonly used as quality criterion in this substance (Alm-Eldeen et al., 2017; Cunha et al., 2004; Popova et al., 2004; Popova et al., 2007; Sampietro et al., 2016; Trusheva, Trunkova, & Bankova, 2007).

The final extraction of the bioactive compounds depends on the type and quantity of solvent, temperature and time, and even the procedure used to interact with the crude propolis (Sawaya, da Silva Cunha, & Marcucci, 2011). Maceration is the traditional extraction procedure, although in recent years sonication and microwaves have also been recommended due to their efficiency, time saving and selectivity (Sforcin, 2016; Trusheva et al., 2007).

Spectrophotometry, especially the Folin-Ciocalteu method, is the most widely used for the routine determination of total content of phenols and certain groups of flavonoids in propolis (Cottica et al., 2015; González, Guzmán, Rudyk, Romano, & Molina, 2003; Kumazawa, Hamasaka, & Nakayama, 2004; Popova et al., 2004). However, other spectrophotometry methodologies have also been used: (DPPH) 1,1-diphenyl-2-picrylhydrazyl (Cottica et al., 2015; Laskar, Sk, Roy, & Begum, 2010); (DNP) 2,4-dinitrophenylhydrazine (Popova et al., 2007) and (ABTS) 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acids) (Gülçin, Bursal, Şehitoğlu, Bilsel, & Gören, 2010; Sun, Wu, Wang, & Zhang, 2015). There is a significant discrepancy in the results reported in the bibliography about total phenolic content. This is mainly due to the difference in the reference compounds chosen for the construction of the calibration curves necessary to express the quantitative result (Cicco, Lanorte, Paraggio, Viggiano, & Lattanzio, 2009).

Chromatographic methods, especially HPLC, are used for the

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separation and quantification of the specific constituent compounds of the phenolic profile, although they are not recommended as routine procedures due to their high cost (Castro et al., 2014; Popova et al., 2004).

The discrepancy found in everything related to the analysis of propolis (method of extraction or quantification, and criteria to express the results) by researchers and laboratories decisively influences the disparity of results (Andrade, Marina, Santos de Oliveira, Nunes, & Narendra, 2017; de Francisco et al., 2018). Consequently, it is difficult to compare the biological properties of different "types" of propolis. For this reason, it is necessary to standardize an analytical procedure to determine valid common criteria, and therefore accurately classify propolis according to its composition and commercial value.

For the aforementioned reasons, the objective of this work was to evaluate the effectiveness of the most used bioactive compound extraction techniques (maceration and ultrasonication) applying different extraction combinations (double maceration, double ultrasonication and maceration followed by ultrasonication) on the yield (with respect to crude propolis), on the total phenol content and on the quantification of specific compounds of the phenolic profile of propolis.

2. Materials and methods

2.1. Raw samples

Crude propolis from different countries were used in this study to consider a possible range of variability in the phenolic profile. Specifically, 3 samples from Rumania (Suceava County), 2 from Spain (Gestalgar and Montroy municipalities, in Valencian region) and 1 from Honduras (municipality of Siguatepeque, department of Comayagua) were analyzed. The samples were collected at the end of summer and beginning of autumn. Each sample from Romania and Spain was harvested from a specific professional apiary, composed of no < 150 hives. In the case of Honduras the sample came from wild hives collected by the Lencas communities. The samples were stored at −18 °C until analysis.

2.2. Reagents and solutions

The standards used: caffeic acid, rutin, p-coumaric acid, ferulic acid, m-coumaric acid, quercetin, trans-cinnamic acid, apigenin, genistein, kaempferol, chrysin, pinocembrin, caffeic acid phenylethyl ester (CAPE), and acetonitrile were purchased from Sigma–Aldrich (St. Louis, MO, USA). Gallic acid as well as Folin-Ciocalteau reagent were acquired in Scharlab (Barcelona, Spain). All reagents and standards used were HPLC grade, and purified water from a Milli Q system was used throughout the experiments.

2.3. Extraction procedure

Each crude sample (10–15 g) while still frozen, was ground to homogenize it before extraction. Three different extraction methods were carried out: double maceration (MM), double ultrasonication (UU) and maceration followed by ultrasonication (MU). Each extraction was executed in triplicate.

2.3.1. Maceration-Maceration (MM)

One gram of pulverized sample was weighed and dissolved in 30 mL of 70% ethanolic solution (70:30 ethanol:water). Then, this solution was stirred constantly for 24 h in a dark room. After that, a 5 min centrifugation (5000 rpm at 5 °C) was carried out and the supernatant was separated from the residue by filtration (Whatman 3) (1st extraction). This process was repeated on the residue, to obtain a second supernatant (2nd extraction). Both supernatants (1st + 2nd extraction) where collected in a volumetric flask and topped up to 100 mL using the same 70% ethanol solvent.

2.3.2. Ultrasonication-Ultrasonication (UU)

Again 1 g of pulverized sample was weighed and dissolved in 30 mL of 70% ethanolic solution (70:30 ethanol:water). Then, rather than the solution being stirred constantly for 24 h in a dark room, the extraction process was carried out in an ultrasonic bath at 25 °C for 30 min. Then, centrifugation and filtration was carried out as for MM.

2.3.3. Maceration–Ultrasonication (MU)

The first extraction was carried out in the same manner as described for maceration, and then submitted to a second extraction as described for ultrasonication.

In the UU and MU extraction methods the same number of extracts were considered as described in the MM method: 1st extraction, 2nd extraction and 1st + 2nd extraction.

The extracts were preserved at −18 °C until the quantification of the "propolis yield" (balsam content), and the determination of the total polyphenols (by spectrophotometry) and specific compounds (by HPLC).

2.4. Establishing a standardized quantitative criterion

In order to standardize the expression of phenol compounds present in a propolis extract, the yield of the propolis samples (eliminating the impurities) was calculated. The yield was expressed as balsam content (soluble ethanolic fraction) and determined according to Popova et al., 2007. To this end, an aliquot (2.0 mL) of each ethanolic extract was evaporated in a vacuum oven to constant weight. The percentage of yield was calculated following the Eq. (1):

$$Yield = \left(\frac{\text{weight of the dry ethanolic extract}}{\text{weight of crude propolis}}\right) * 100 \quad (1)$$

2.5. Spectrophotometric determination of total phenolic content

The method used to determine the total phenolic content of the propolis extract was based on a slight modification of the procedure described by Cicco et al. (2009). 100 µL of each extract of propolis (1th, 2nd and 1st + 2nd) plus 1900 µL distilled water were placed in a glass tube and then the solution was oxidized by adding 100 µL of Folin-Ciocalteau reagent. After exactly 2 min, 800 µL of 5% sodium carbonate (*w/v*) was added. This solution was maintained in a water bath at 40 °C for 20 min, and then the tube was rapidly cooled with crushed ice to stop the reaction. The generated blue colour was measured using a spectrophotometer at 760 nm. As the result of the total content of phenolic compounds is clearly dependent on the reference substance used in the calibration curve, three different standards were essayed in this study: rutin, gallic acid and a mixture of pinocembrin/galangin (2:1) (Popova et al., 2004; Woisky & Salatino, 1998). In order to prepare the stock standard solutions, 25 mg of rutin, gallic acid or a pinocembrin/galangin mix (2:1) were dissolved to a final volume of 25 mL methanol in each case and stored at −20 °C. The calibration curves were carried out at the beginning of each working day from six working standard solutions, which were prepared by appropriate dilution of each stock standard solution with 70% ethanol. Concentration ranges were: 50–600 µg/mL for rutin, and 50–300 µg/mL for both gallic acid and pinocembrin/galangin (2:1). The ethanolic solution was used as a blank.

2.6. Chromatographic determination of the phenolic profile

Individual stock solutions of each standard were prepared in methanol at 10 mg/mL, and stored at −20 °C. The working standard mixture solutions were made by diluting the appropriate amount of each stock standard solution to obtain 5 calibration levels (final concentrations of 5, 10, 20, 200 and 1000 µg/mL).