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Evaluation of bioactive compounds potential and antioxidant activity of brown, green and red propolis from Brazilian northeast region

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ABSTRACT

The aim of the present study was to determine the contents of bioactive compounds present in brown, green and red species of propolis cultivated in the Brazilian northeast states of Alagoas and Sergipe. The contents of phenolic compounds, flavonoids and antioxidant activity (DPPH, $ABTS^{+}$, FRAP, ORAC) were determined. Identification and quantification of phenolic and flavonoid compounds were performed by using UHPLC-QqQ-MS/MS system. The results revealed high contents of total phenolics and flavonoids. Among the three species, the antioxidant potential had higher capacity in the red propolis. The presence of some of bioactive compounds viz. acacetin, artepellin C, eriodictyol, gallic acid, isorhamnetin, protocatechuic acid, vanillin and vanillic acid in Brazilian red propolis is reported for the first time in this work. Positive correlation between total phenolics versus the FRAP and ORAC methods was established which led to conclusion that antioxidant activity of propolis is mainly due to its phenolic compounds.

1. Introduction

Propolis is a resinous substance, dark in color and it is collected by honeybees, mainly from leaf and flower buds, stems and cracks in the bark of many species of trees. This material is transported to the hive and mixed with beeswax, producing a strongly adhesive substance, which for centuries has been used worldwide in traditional medicine (Daugsch, Moraes, Fort, & Park, 2008; Pellati, Orlandini, Pinetti, & Benvenuti, 2011).

In South America, Brazil is well known for its green propolis, produced by *Apis mellifera*, which collect resins mainly from a native shrub *Baccharis dracunculifolia* (López, Schmidt, Eberlin, & Sawaya, 2014). However, due to the large Brazilian biodiversity, there are 13 types of propolis, which include less common brown and red propolis and these are classified based on the place of production where these are found (Sawaya et al., 2004). Since propolis is a natural product characterizing for several biological and pharmacological properties, it has attracted interest of researchers in the last decades. The therapeutic properties of propolis are well known in popular medicine, due its antiseptic, antitumoral (Franchi et al., 2012; Frozza et al., 2017), antiinflammatory (Bufalo et al., 2013; Franchi et al., 2012), immunomodulatory (Bufalo et al., 2013), antioxidant (Franchi et al., 2012), antibacterial, antimicrobial activities (Bufalo et al., 2013; Franchi et al., 2012; Sforcin & Bankova, 2011; Szliszka et al., 2013; Viuda-Martos, Ruiz-Navajas, Fernandez-Lopez, & Perez-Alvarez, 2008).

The chemical composition of propolis varies according to the geographic region, climate, environmental conditions and collecting season (López et al., 2014; Sawaya, Barbosa da Silva Cunha, & Marcucci, 2011). Although phenolic compounds are the most abundant in propolis, yet > 300 compounds have been identified in different species, such as phenolic acids, flavonoids including flavones, flavanones, flavonols and chalcones, terpenes, aromatic aldehydes, alcohols, fatty acids, stilbenes, steroids, amino acids, lignans and sugars (Akyol et al., 2013; da Silva Frozza et al., 2013; Righi et al., 2011).

Raw propolis cannot be used as feedstock and it must be purified. This process should remove the waxy material and preserve the fraction of polyphenols, which are considered to contribute most to the curative effects than the other constituents of propolis. The most popular technique for the production of propolis extracts is ethanol extraction due to the fact that active substances of propolis are more easily soluble in etanol (Pietta, Gardana, & Pietta, 2002). According to other authors, extraction using the hydroalcoholic solvent (ethanol) have been described as the most suitable means for the extraction of biologically active phenolic components from propolis materials (Cottica et al., 2011; Frozza et al., 2017; López et al., 2014; Pellati et al., 2011). Sun, Wu, Wang, and Zhang (2015) stated in his study that ethanol/water solvents have a significant effect on the phenolic composition and antioxidant properties of the propolis extracts.

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The compounds daidzein, biochanin A (López et al., 2014; Silva et al., 2015), pinocembrin and quercetin (Daugsch et al., 2008; de Mendonça et al., 2015) are biomarkers of Brazilian red propolis. Besides these compounds, the red propolis is known to possess chemical substances which were not found in other varieties of propolis, such as vestitol and neovestitol, *C*-glycoside, liquiritigenin, isoliquiritigenin, formononetin, and medicarpin (Bueno-Silva et al., 2013; da Silva Frozza et al., 2013; López et al., 2014; Piccinelli et al., 2011). However, the principal constituents of Brazilian green propolis are caffeic acid, *p*-coumaric acid, ferulic acid, naringenin, kaempferol, isorhamnetin, sakurametin, pinocembrin, kaempferide and artepellin C (Szliszka et al., 2013).

In Brazil, green and red propolis species are the most studied (Frozza et al., 2017; Hatano et al., 2012; Silva et al., 2015), although there are some reports in literature on other species, like brown propolis (Bittencourt et al., 2015). However, there are no reports on the presence and comparative data on phenolic compounds and antioxidant activity among the various varieties of Brazilian propolis. Thus the investigation on the chemical composition of different species and their comparative evaluation becomes important.

In recent years, the use of Ultra-high Performance Liquid Chromatographic system coupled with Tandem Mass Spectrometry (UHPLC-QqQ-MS/MS) is becoming the most commonly employed method for determination of phenolic compounds, due to the system's high sensitivity, selectivity and high-throughput capability. Thus the objective of this study was to use UHPLC-QqQ-MS/MS system to identify and quantify phenolic compounds and to evaluate their antioxidant activity in brown, red and green propolis species grown in the states of Alagoas and Sergipe, pertaining to the Northeast region in Brazil.

2. Materials and methods

2.1. Analytical standards and reagents

All the organic solvents employed were of HPLC grade. Water used for the mobile phase was purified through a Milli-Q system (Millipore, São Paulo, Brazil; Direct-Q® 3UV). The solvents acetonitrile and formic acid used were of HPLC grade 98% of purity obtained from Sigma Aldrich and Fluka Analytica (St Louis, MO, USA). Apigenin ($C_{15}H_{10}O_5$), acacetin ($C_{16}H_{12}O_5$), artepellin C ($C_{19}H_{24}O_3$), biochanin A ($C_{16}H_{12}O_5$), cinnamic acid ($C_9H_8O_2$), α-cyano-4-hydroxycinnamic acid ($C_{10}H_7O_3N$), caffeic acid ($C_9H_8O_4$), ferulic acid ($C_{10}H_{10}O_4$), caffeic acid phenyl ester (CAPE) ($C_{17}H_{16}O_4$), (+)-catechin ($C_{15}H_{14}O_6$), chrysin ($C_{15}H_{10}O_4$), epicatechin ($C_{15}H_{14}O_6$), eriodictyol ($C_{15}H_{12}O_6$), ethyl gallate ($C_9H_{10}O_5$), gallic acid ($C_7H_6O_5$), isorhamnetin ($C_{16}H_{12}O_7$), kaempferide ($C_{16}H_{12}O_6$), kaempferol ($C_{15}H_{10}O_6$), luteolin ($C_{15}H_{10}O_6$), narigenin ($C_{15}H_{12}O_5$), *p*-coumaric acid ($C_9H_8O_3$), protocatechuic acid ($C_7H_6O_4$), pinocembrin ($C_{15}H_{12}O_4$), quercetin-3-glucoside ($C_{21}H_{20}O_{12}$), chrologenic acid ($C_{16}H_{18}O_9$), rutin ($C_{27}H_{30}O_{16}$), vanillin ($C_8H_8O_3$) and vanillic acid ($C_8H_8O_4$) were purchased from Sigma-Aldrich (Saint Louis, MO, USA). The reagents and standards: etanol; aluminum chloride; sodium carbonate, potassium phosphate buffer, sodium citrate, ferrous sulfate; Folin-Ciocalteu phenol reagent; fluorescein; 6-hydroxy-2,5,7,8-tetramethylchromo-2-carboxylic acid (Trolox); 2,2-diphenyl-1-picrylhydrazyl radical (DPPH·); 2,2′-azino-bis (3-ethylbenzthiazoline) 6-sulfonic acid ($ABTS^+$); 2,2-Azobis (2-Amidino-Propane) dichloride (AAPH); and FRAP reagent were obtained from Sigma Aldrich and Fluka Analytica (St Louis, MO, USA).

2.2. Propolis samples

Brown, red and green raw propolis samples were collected from apiaries located at Marechal Deodoro, Alagoas state, Brazil, at coordinates 9° 45′34.454″ S, 35° 50′24.986″ W in January 2016 while the raw red propolis was collected from apiary, located in Brejo Grande, Sergipe, Brazil, at coordinates 10° 25′28″ S, 36° 27′44″ W, in July 2016. The samples of brown, green and red propolis were collected for a period of 7 days in the afternoon time at about 16 h. Three batches of propolis sample weighing about 50 g each were collected to carry out the analysis. Raw propolis were ground using a mill (IKA, Brazil, A11 basic) and the powder was later stored in glass containers which were maintained at 0 °C.

2.3. Preparation of propolis extracts

Raw propolis sample (2 g) was extracted with 15 mL of ethanol:-water (70:30, v/v) in an ultrasound bath apparatus (3L Alpha Plus) at 35 °C for 60 min. The extracts were centrifuged at 400*g* for 10 min, using a centrifuge (Eppendorf, 5810R). The supernatants were collected, dried at 35 °C in a drying oven, and resuspended in 2 mL of ethanol:water (70:30, v/v). Finally, samples were filtered through 0.2 μm cellulose filters (Millipore, Bedford, MA, USA) and injected in the UHPLC-QqQ-MS/MS system.

2.4. Determination of total phenolics content

The total content of phenolic compounds was determined by using Folin-Ciocalteu phenol reagent (Shetty, Curtis, Levin, Witkowsky, & Ang, 1995). One milliliter of the ethanolic extract was transferred to test tubes and 1 mL of 95% ethanol solution, 5 mL of distilled water and 0.5 mL of Folin-Ciocalteu reagent (1N) were added, followed by homogenization in vórtex. Later, 1 mL of sodium carbonate solution 5% (w/v) was added. The test tubes were kept in dark for 60 min, and then homogenized in vórtex. The absorbance was measured at a wavelength of 725 nm against a blank consisting of 95% ethanol solution, using a spectrophotometer (Molecular Devices, Sunnyvale, CA, USA; SpectraMax M2). For the quantification of these extracts, a calibration curve was constructed from the analysis of different concentrations of gallic acid varying from 0.0008–0.1 mg/mL, and its data on absorbances based on calibration equation: $y = 10.571x + 0.0111$ ($r^2 = 0.9958$). Results were expressed in terms of milligrams of gallic acid equivalent (GAE) per g of fresh sample weight.

2.5. Determination of total flavonoids contents

The total flavonoids content was determined according to the method proposed by Meda, Lamien, Romito, Millogo, and Nacoulma (2005). Five hundred microliters of extract were transferred to test tube and 0.5 mL of a 20 $mg{\cdot}mL^{-1}$ methanolic solution of aluminum chloride ($ALCL_3$) was added. Samples were homogenized on a vortex and left in the dark for 30 min. The spectrophotometer (Molecular Devices, Sunnyvale, CA, USA; SpectraMax M2) was set at the wavelength of 415 nm and absorbance reading measured. The calibration curve was constructed from different concentrations of quercetin varying from 0.0008–0.1 mg/mL, and its data on absorbances based on calibration equation: $y = 21.874x - 0.0047$ ($r^2 = 0.9998$). The results were expressed in terms of milligrams of quercetin per g of propolis.

2.6. Determination of antioxidant activity

2.6.1. DPPH assay

The antioxidant capacity was determined by the DPPH radical (2,2-diphenyl-1-picrylhydrazyl) scavenging method recommended by Kwon, Vattem, and Shetty (2006) with slight modifications. A 250 μL aliquot of extract was mixed with 1.25 mL of DPPH. After 5 min, the absorbance was read at 517 nm using a spectrophotometer (Molecular Devices, Sunnyvale, CA, USA; SpectraMax M2). The readings were compared with the controls, containing 95% ethanol instead of extract. The percentage inhibition was calculated by Eq. (1). Different concentrations of Trolox varying from 0 to 0.0014 mmol Trolox/mL were used to construct the calibration curve based on calibration equation: $y = -421.1x + 0.7193$ ($r^2 = 0.9958$), and expressed the results in terms of μmol Trolox per g of propolis.