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# Preparation and thermal characterization of inclusion complex of Brazilian green propolis and hydroxypropyl-$\beta$-cyclodextrin

## Increased water solubility of the chemical constituents and antioxidant activity

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**Abstract** The propolis produced in Southeastern Brazil is known as green propolis (BGP) because of its color and the most important plant source is *Baccharis dracunculifolia*. Several authors reported biological activities such as anti-ulcer, anti-inflammator, antimutagenic, antifungal/antibacterial, antileishmanial/antiplasmodial for the BGP. For this reason, BGP has been extensively employed in food and beverages, thus helping improve health and preventing diseases. Some authors related that the biological activities of BGP are mostly due to its high levels of prenylated $\rho$-coumaric acids derivatives, mainly artepillin C. The inclusion complex between Brazilian green propolis (BGP) with hydroxypropyl-$\beta$-cyclodextrin (HP-$\beta$-CD) was prepared and its characterization was investigated by different analytical techniques (X-ray diffraction, Fourier transform infrared spectroscopy, and thermogravimetry) and suggesting that propolis was molecularly dispersed in the HP-$\beta$-CD matrix. The increasing solubility of chemical constituents was determined using quantitation methods for total flavonoids and polyphenols. Furthermore, it was developed a method for the quantitation and identification of the main compounds by high-performance liquid chromatography in order to evaluate the increasing water solubility of each constituent in aqueous BGP extract (aromadendrin, isosakuranetin, and artepillin C). The antioxidant activity was evaluated by chemical assay 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging.

**Keywords** Brazilian green propolis · Hydroxypropyl-$\beta$-cyclodextrin · Inclusion complex · Flavonoids · Polyphenols · Artepillin C

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## Introduction

Over the last few decades, interest in functional foods has been growing fast, leading to the discovery of new functional components or processes that can improve food processing, as well as products that may help to retard aging or avoid diseases. In this context, bee products have gained the attention of consumers and researchers, due to their chemical compositions and functional properties. Propolis is one of the bee products with functional properties, but it cannot be consumed as a food because it is a resinous substance [1, 2].

Propolis is a resinous material collected by bees (*Apis melilifera* L.) from exudates and buds of plants and mixed with wax and bee enzymes. In general, is composed of 50% resin and vegetable balsam, 30% wax, 10% essential and aromatic oils, 5% pollen and 5% various others substances, including organic debris [3, 4]. More than 300 compounds, among polyphenols, terpenoids, steroids, sugar, and amino acids have been detected in raw propolis. Their abundance is influenced by geographical factors and botanical origins, as well as by collection season [2, 4–7].

Propolis produced in Southeastern Brazil is known as green propolis because of its color [8]. The most important plant source of green propolis is *Baccharis dracunculifolia*

D.C. (Asteraceae) [9]. Several works report that biological activities such as antiulcer [9], anti-inflammatory [10], antimutagenic [11], antifungal/antibacterial [12–14], antileishmanial/antiplasmodial [15] activities have been reported for the BGP. For this reason, BGP has been extensively employed in food and beverages, thus helping improve health and preventing diseases [16]. Some authors related that the biological activities of BGP are mostly due to its high levels of prenylated $\rho$-coumaric acids derivatives, mainly artepillin C [17–19].

The most common propolis extraction uses ethanol as solvent. It is interesting to point out that a great number of the lipophilic compounds of propolis that has attracted much interested because of the related biological properties are ethanol soluble. However, the ethanolic extraction has some disadvantages such as strong residual flavor, adverse reactions and intolerance to alcohol in some people [1, 20]. Researches and industry are interested in producing a new type of extract with the same compounds extracted by the ethanolic method, but without disadvantages. Water alone has been tested as extraction solvent, but resulted in a product containing less extracted compounds [21]. Although there are few reports showing the characterization of these aqueous extracts of propolis it is know that its major constituents have higher antioxidant activity, higher inhibitory activity of some enzymes and a higher absorption than the ethanolic extract [22].

During the last years encapsulation methods was found to have many applications in the cosmetics and food/drug industry, as a flavor carrier and as a treatment to impart some degree of protection against evaporation, oxidation, reaction or migration of active compounds in a food, while by increasing their solubility its allows them to be used in the formulation of fortified and functional foods [4, 23, 24].

Cyclodextrins (CDs) are chemically and physically stable molecules and formed by the enzymatic modification of starch [25]. They have the ability to form inclusion complexes with a wide variety of organic compounds, which enter partly or entirely into the relatively hydrophobic cavity, simultaneously expelling the few high-energy water molecules from inside. Encapsulation with CDs leads to increased dissolution rate, membrane permeability and bioavailability of nutraceuticals of low solubility [26]. Moreover, CDs improve the shelf life of food products and mask or reduce undesired smell or taste [26]. Microencapsulation in $\beta$-CD has been successfully applied in complex mixtures, like olive leaf extract [27], hibiscus anthocyanins rich extract [28] and propolis balsam, but complex not characterized [24, 29].

In this study, we investigated the encapsulation and thermal characterization of chemical compounds of Brazilian green propolis extract with hydroxypropyl-$\beta$-cyclodextrin for analysis solubility in water and evaluation of antioxidant activity.

## Materials and methods

### Reagents, chemicals, and equipments

The following compounds were used as standards in HPLC analysis: caffeic acid (Fluka), $\rho$-coumaric acid (Fluka), cinnamic acid (Fluka), artepillin C (Wako), gallic acid (Synth), aromadendrin, and isosakuranetin (Fluka). Aromadendrin was isolated and identified by using authentic chromatographic standards available at the library of standard compounds of the Laboratory of Pharmacognosy, School of Pharmaceutical Sciences of Ribeirão Preto, São Paulo, Brazil, by comparing UV spectra and considering both the maximum lambda and the relative area obtained with the use of two wavelengths (A 275/320). Methanol HPLC grade was obtained from J.T. Baker and water was treated in Milli-Q water purification system.

The compounds used for antioxidant activity such as 1,1-diphenyl-2-picrylhydrazyl (DPPH) purchased in Sigma-Aldrich, and others reagents used were analytical grade.

Spectrophotometer UVmini-1240 Shimadzu, Ultrasonic bath USQ 750 Unique, Rotaevaporator 820 Fisatom, high-performance liquid chromatography (HPLC) using a Shimadzu apparatus equipped with an CBM-20A controller, a LC-20AT quaternary pump, an SPD-M 20A diode-array detector, Shimadzu LC solution software (version 1.21 SP1), and PHmeter B-476 Micronal.

Samples BGP used was obtained from Apis Flora Industrial & Comercial (Ribeirão Preto/SP, Brazil).

### Methods

#### *Preparation of inclusion complex and aqueous extract*

The sample of ethanolic extract of BGP (APP-AF®, batch 14401/10 acquired from Apis Flora Company, Ribeirão Preto/SP, Brazil) was standardized to 11% w/v dry weight, according to patent number PI 0405483-0 published in "Revista de Propriedade Industrial" n.1778, 01/02/2005). For its production, green propolis raw material was collected in Bambuí, state of Minas Gerais, Brazil during November, 2009.

The inclusion complex between BGP and HP-$\beta$-CD was prepared according to the method described by Naefady et al. [29], with some modifications. The EEP solution was concentrated to dryness under reduced pressure. This 5 g of residue was suspended in 100 mL aqueous solution con-

taining 18 g of HP-$\beta$-CD. The suspension was subjected to sonication for 4 h and was subsequently centrifuged for 10 min at 3000 rpm. Then, the supernatant was filtered through 0.45 μm PVDF filter. Finally, the extract with inclusion complex was stored for further handling. For aqueous extract (AE), the same methodology was used without addition of HP-$\beta$-CD.

## Characterization of BGP/HP-$\beta$-CD complex formation

### *X-ray diffraction*

X-rays diffraction patterns were obtained using a Kristalloflex Simens Diffractometer with a Ni filter and Cu K$\alpha$ radiation, step pass of 0.02° and a step time of 3 s, from 4° to 70° (2$\theta$ angle).

### *Fourier transform infrared spectroscopy*

The FT-IR spectra for all dried samples were obtained on a PerkinElmer Spectrum 1000 FT-IR Spectrophotometer. Pellets were prepared from mixtures of the samples and KBr (1:100 in weight mass). Scans were done at a resolution of 4 $cm^{-1}$.

### *Thermogravimetry*

The TG curves were obtained from SDT equipment from TA Instruments. The conditions used in the experiments were: nitrogen atmosphere at a flow rate of 100 mL $min^{-1}$, heating rate of 10 °C/min from 25 to 600 °C and alumina pans.

## Determination of total polyphenol content

Total polyphenols content in the extracts were estimated by a colourimetric assay based on procedures described by Waterman and Mole [30] with some modifications. Aliquots of each extract was separately mixed with Folin-Denis reagent and $Na_2CO_3$ (35%) and was kept in the dark at room temperature for 30 min, after which the absorbance was read at 760 nm using a UV–visible spectrophotometer. Total polyphenols contents were expressed as milligrams per milliliter of gallic acid equivalents.

## Determination of total flavonoid content

The quantitative analyses for determination of flavonoids content were obtained according to the methodology used by Costa et al. [31] and Dowd [32]. For that, UV-spectrophotometry was used at the wavelength of 425 nm. The several samples were diluted with methanol to the linear range previously determined. Then, they were put to react with aluminum chloride and, after a 30 min reaction protected of light, samples were analyzed. The flavonoid content was calculated against an analytical curve of the quercetin standard prepared under the same conditions. Total flavonoid content was expressed in terms of microgram of quercetin equivalent per milliliter of extract.

## HPLC analysis

The propolis extract was analyzed by high-performance liquid chromatography (HPLC). A Shimadzu Shim-Pack CLC-ODS (M) column (4.6 mm × 250 mm, particle diameter of 5 μm, pore diameter of 100 Å) was used. The mobile phase consisted of methanol and of a solution of water-formic acid (0.1% v/v), pH 2.7. The mixture was eluted using a linear gradient of 20–95% of methanol over a period of 77 min at a flow rate of 0.8 mL/min. Detection was set at 275 nm.

The propolis extracts were dissolved with 5 mL of methanol (HPLC grade) in 10-mL volumetric flasks, subjected to sonication for 10 min and made up to volume with Milli-Q water. All samples were filtered through a 45-μm filter before analysis.

## Scavenging of DPPH radicals assay

The stable DPPH radical was used for determination of free radical scavenging activity of the extracts [33]. Solutions of different concentrations were prepared. Aliquots for each extract/compound was dissolved in methanol and was mixed with 1 mL acetate buffer (0.1 M, pH 5.5) 1 mL of ethanol and 1 mL of solution of $DPPH^{\bullet}$ (250 μM). The mixture was shaken vigorously and kept in dark room for 30 min. The reduction of the DPPH radical was measured by decrease of absorption at 517 nm (UV–visible spectrophotometer). DPPH scavenging effect was calculated as a percentage of DPPH discolouration using equation: % scavenging effect = [(ABSDPPH − AExt)/ABSDPPH] × 100. The extract concentration providing 50% inhibition (IC50) was calculated from the graph of scavenging effect percentage against extract concentration in solution.

## Statistical analysis

All the determinations were performed in triplicate. The Student $t$ test was used to calculate statistical significance ($p < 0.05$). The level of significance was 5%. Statistical analysis of data was performed using the software Graph Pad Prism 4.

## Results and discussion

### Total polyphenols and flavonoids contents

The total polyphenols and flavonoids contents of the extracts used in this study can be seen in Table 1. For analysis of total polyphenols there was no statistical difference, both extracts have the same amount of these compounds, around 620 μg/mL of extract, showing that HP-β-CD, is not able to improve the extraction of polar compounds compared to water extraction. But, there was an increase of solubility of total flavonoids compounds in the extract containing HP-β-CD. The increment of water solubility was 2.5-fold higher.

Kalogeropoulos et al. [24] concluded that the size and chemical class of the guest molecules affect both their encapsulation efficiency and bioavailability. The more effectively encapsulated small phenolic molecules were released from the β-CD cavity with more difficulty, while the opposite was true for flavonoids, anthraquinones and terpenes. The net release of specific compounds from encapsulated PE will depend not only on their chemical properties but also on their relative abundance in the propolis extract sample.

Phenolics compounds are commonly found in both edible and non-edible plants, and they have been reported to have a multiple biological effects, including antioxidant activity [5, 34]. Contents of flavonoids and others phenolics compounds substances have been suggested to play a preventive role in the development of cancer and heart diseases [5, 34]. Some authors state that the biological activities of Brazilian propolis are mostly due to the high levels of phenolics acids [6] whilst flavonoids are considered to be responsible for the activity of European propolis extracts [35].

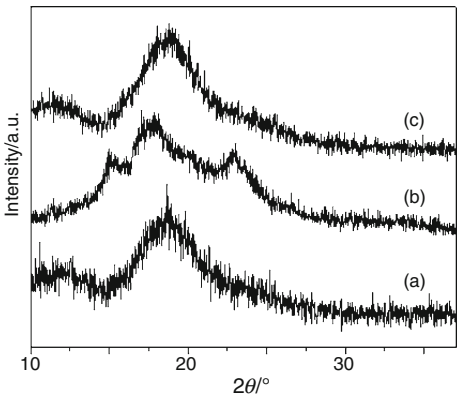


**Fig. 1** XDR analyses of: (*a*) HP-β-CD, (*b*) propolis, and (*c*) HP-β-CD/propolis

### Characterization of BGP/HP-β-CD complex formation

The powder X-ray diffraction patterns of HP-β-CD, Brazilian green propolis water extract and HP-β-CD/propolis are shown in Fig. 1. The powder diffraction pattern of HP-β-CD, Fig. 1a, displayed an amorphous structure, and a broad peak around 18.8° was identified [36]. Dried propolis extract, Fig. 1b, shows an amorphous structure also, and three broad peaks in 15°, 17°, and 23° could be marked. The diffraction pattern of HP-β-CD/propolis, Fig. 1c is similar of the HP-β-CD diffractogram, and characteristics peaks of propolis extracts was not observed in HP-β-CD/propolis diffractogram, suggesting that propolis was molecularly dispersed in the HP-β-CD matrix [36].

The IR spectra of HP-β-CD, dried propolis extract and HP-β-CD/propolis are shown in Fig. 2. Since there are seven repeating units in the HP-β-CD molecule, the spectra of HP-β-CD/propolis largely dominated by the vibrational bands of the cyclodextrin molecule [37]. There are no

**Table 1** Quantitation of chemical constituents of extracts and total polyphenol and flavonoids contents

| | Aqueous extract | Inclusion complex solution | Increase of solubility |
|---|---|---|---|
| Total polyphenol (μg GAE[a]/mL[b]) | 650 ± 26 | 620 ± 24 | – |
| Total flavonoids (μg QCE[c]/mL[b]) | 116 ± 3 | 290 ± 7 | 2.5× |
| Caffeic acid[d] | 52.4 ± 2.798 | 41.5 ± 0.248 | 0.8× |
| ρ-Coumaric acid[d] | 236.5 ± 0.246 | 194.0 ± 0.180 | 0.8× |
| Cinnamic acid[d] | 11.0 ± 0.465 | 14.6 ± 0.216 | 1.3× |
| Aromadendrin[d] | 26.7 ± 1.404 | 70.9 ± 0.282 | 2.6× |
| Isosakuranetin[d] | 23.8 ± 0.475 | 180.6 ± 4.487 | 7.5× |
| Artepillin C[d] | 0.0 ± 0.000 | 169.7 ± 1.434 | 17.0× |

Values are mean ± SD obtained from analyses in triplicate ($n = 3$)

[a] GAE = gallic acid equivalent

[b] ext = propolis extract

[c] QCE = quercetin equivalent

[d] μg/mL of extract

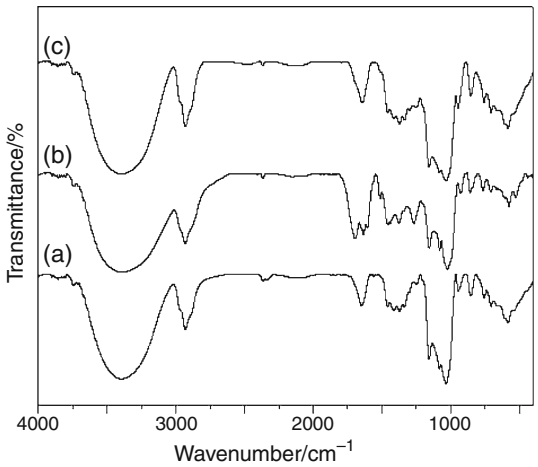


**Fig. 2** FTIR analyses of: (*a*) HP-*β*-CD, (*b*) propolis, and (*c*) *β*-CD/propolis

significant modifications detected in the hydroxyl stretching region [38]. The possible changes of the contribution of the OH groups of the guest compound were masked by the enormous amount of *β*-cyclodextrin hydroxyls; most of them were not perturbed by the presence of propolis. In the region 3600–3100 cm$^{-1}$, absorptions that characterize an intense and broad band corresponding to O–H stretching in the glucose units of the CD and the presence of water were observed [39]. The broad band localized around 3450 cm$^{-1}$ observed in *β*-CD, propolis and HP-*β*-CD/propolis, suggests the occurrence of intermolecular interactions between propolis and CD. In fact, it was considered that propolis had been completely dispersed in HP-*β*-CD and they should have some interaction, such as the combination of hydrogen bonds or van der Waals forces [37–39].

Figure 3 shows the thermal behavior of the all samples studied. *β*-CD, Fig. 3a shows thermoanalytical profile can be divided into three parts. The first one involves water loss (6.4%) from ambient temperature up to 100 °C. Between 100 and 280 °C the TG curve is flat and no mass loss is detected, and the thermal decomposition (around 80%) occurs after 280 °C, with maxima decomposition temperature at 348 °C, confirmed by DTG curve [40].

The TG/DTG curve for green propolis water extract, Fig. 3b, presents a loss weight (7%) from ambient temperature up to 120 °C assigned to evaporation of volatile compounds. The second loss mass (72%) occurs in the temperature range 140–600 °C, with maxima decomposition temperature at 301 °C, confirmed by DTG curve. This loss is assigned to the decomposition of the mixture of organic compounds presents in the propolis extract.

The TG/DTG thermal profile of HP-*β*-CD/propolis Fig. 3c is close to thermal behavior of pure HP-*β*-CD. The first event (9%) from ambient temperature up to 250 °C is

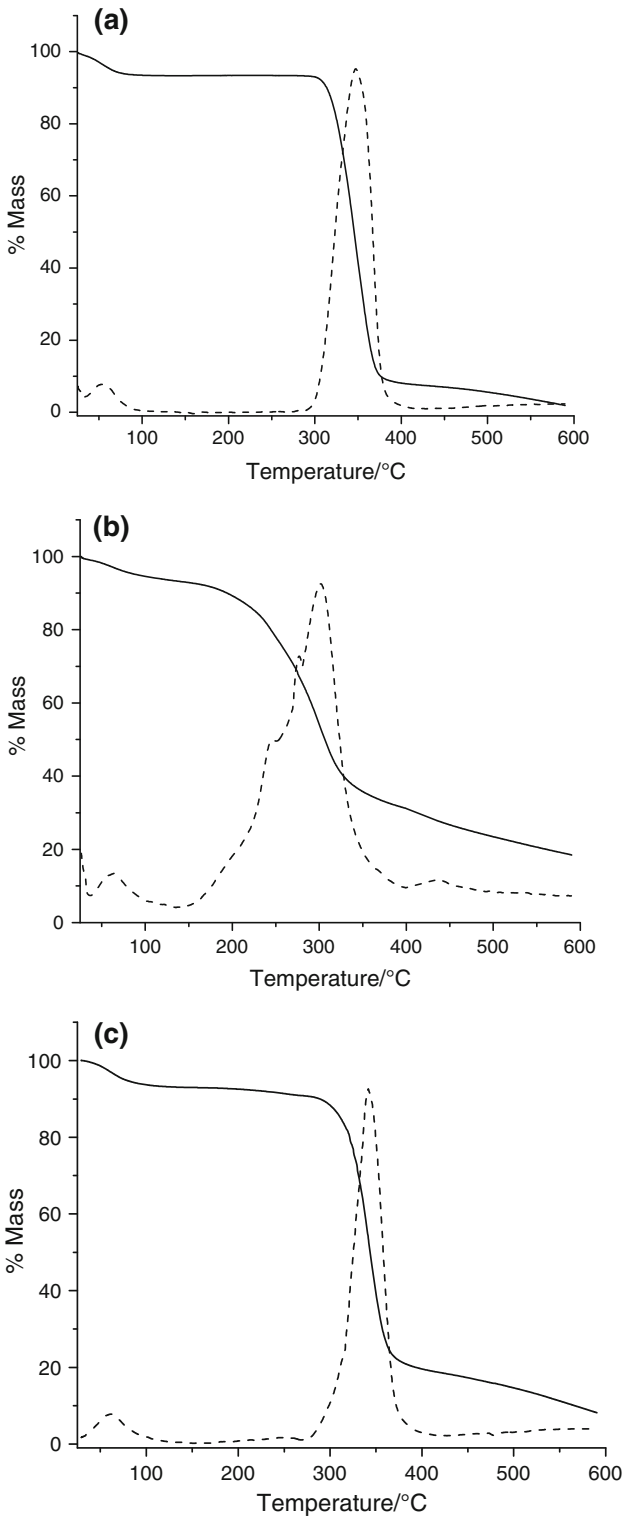


**Fig. 3** TG/DTG curves of: **a** HP-*β*-CD, **b** propolis, and **c** *β*-CD/propolis

associated with evaporation of water loss and volatile compounds. The thermal decomposition (around 80%) occurs after 280 °C, with maxima decomposition

temperature at 342 °C, confirmed by DTG curve. The slightly decreasing on thermal degradation of pure $\beta$-CD (348 °C) in comparison with the $\beta$-CD/propolis (342 °C) and the similarity of the TG curves of these compounds strongly suggest green propolis water extract was molecularly dispersed in the HP-$\beta$-CD matrix, as discussed in XRD analyses [36, 40].

## Quantification of chemical constituents and solubility studies

The inclusion complex HP-$\beta$-CD/BGP aqueous solution obtained were clear and bright yellow. The visual aspect showed that the HP-$\beta$-CD made BGP components more soluble being an evidence of complexation. The Fig. 4 shows the fingerprint of extracts obtained and demonstrated that the two extracts have similar chemical profiles considering the polar compounds. Therefore, when the chromatogram of the extract with HP-$\beta$-CD is analyzed separately, it can be seen more peaks of less polar compounds. In other words, HP-$\beta$-CD can extract more insoluble compounds of propolis that water only. The increment of propolis solubility due the formation of inclusion complexes was estimated by the increase of certain constituents quantified in water with the presence o HP-$\beta$-CD. The constituents that can be dissolved with or without HP-$\beta$-CD were determined by HPLC–DAD analysis and the results can be seen in Table 1 and Fig. 5. The water solubility of caffeic acid, $\rho$-coumaric acid and cinnamic acid demonstrated no significant difference, however, some compounds like aromadendrin, isosakuranetin, and artepillin C (compounds normally water insoluble) had their water solubility increased ranged from 0.8 to 17-fold. The complexation with HP-$\beta$-CD resulted in solubility of less polars compounds (artepillin C and isosakuranetin) of propolis as observed by Kalogeropoulos [24].

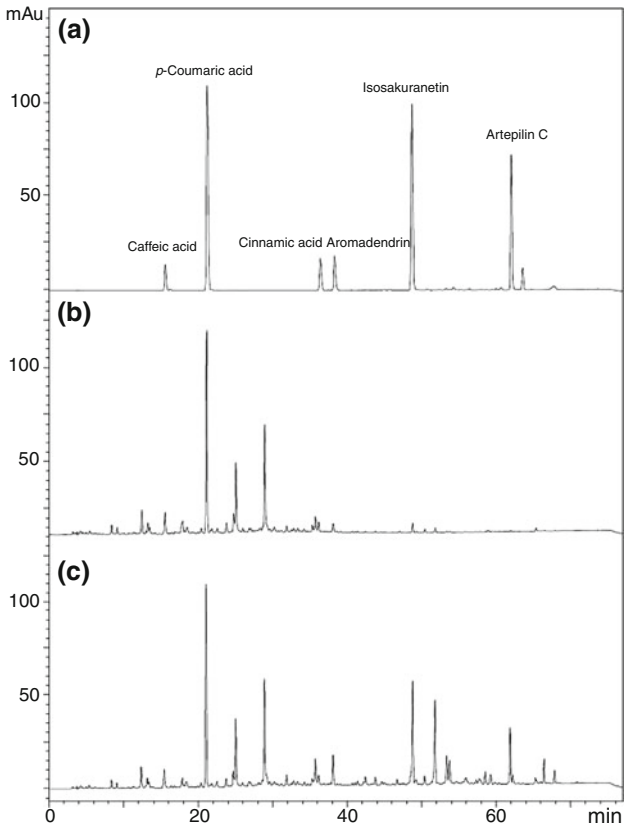


**Fig. 4** Representation of the chromatographic profile of the chemical markers of BGP (**a**), aqueous extract of BGP solution (**b**), and aqueous solution of complex inclusion of propolis/HP-$\beta$-CD (**c**) (mAU = milli Absorbance unit, min = minutes)

## DPPH radical scavenging activity

Because the free radical scavenging activity of antioxidants is due to their hydrogen-donating ability, we used a method based on the reduction of DPPH, a stable free radical, to evaluate the antioxidant activity of various propolis extracts [5].

The DPPH solution has a violet color and the radical scavenging activity of antioxidants compounds can be measured spectrophotometrically at 517 nm by the loss of absorbance as the pale yellow non-radical form (DPPH-H) is produced [41]. The hydrogen atom or electron donation abilities of some compounds are measured by bleaching a purple-colored ethanol solution of the stable DPPH radical. When a hydrogen atom or electron is transferred to the odd electron in DPPH, the absorbance decrease proportionally to the increase of the no-radical forms of DPPH [42].

Phenolics acids are plant metabolites widely spread throughout the plant kingdom. The recent focus of interest on phenolic acids stems is due their protective role potential, through ingestion of fruits and vegetables,

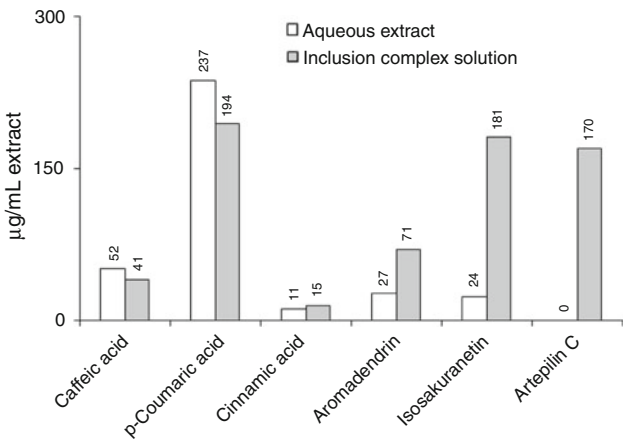


**Fig. 5** Aqueous solubility of BGP constituents with absence or presence of HP-$\beta$-CD

**Table 2** Concentration required for 50% of DPPH scavenging activity of BGP extracts

| Extract | DPHH$^{\bullet}$ scavenging[a] | |
|---|---|---|
| | $IC_{50}$ | $r^2$ |
| Aqueous extract | 0.27 ± 0.01 | 0.9838 |
| Inclusion complex solution | 0.76 ± 0.07 | 0.9696 |

Values are mean ± SD obtained from analyses in triplicate ($n = 3$)

[a] The values were expressed as mg/mL concentration. Lower $IC_{50}$ values indicate higher antioxidant activity. HP-$\beta$-CD do not have

against oxidative damage diseases (coronary heart diseases, stroke, and cancers) [43]. It was reported that the main compounds found in propolis including phenolics acids such as caffeic, ferulic, $\rho$-coumaric, and cinnamic acids. Various pharmacological activities of propolis are attributed to phenolics such as the flavonoids it contains [44].

In this work, the free radical scavenging activities of extracts were evaluated for their ability to quench the synthetic DPPH radical. The concentration of these providing 50% inhibition (IC50) was calculated from the graph plotted inhibition percentage against extract concentration and the IC50 values were very low indicating a high antioxidant activity. Table 2 demonstrates the IC50 values for both extracts.

The aqueous extract of BGP possesses a higher scavenging activity of DPPH radical than the solution containing the inclusion complex. This fact can be explained, since, this extract contains higher amounts of phenolics compounds than the solution containing the inclusion complex, this class of compounds is the most responsible for this biological activity as reported. The increased solubility of some components in the inclusion complex solution did not affect its DPPH radical scavenging, because, these components does not have significant activity.

## Conclusions

Due to the presence of some effective phenolic compounds in propolis it is known that it can be used as a mild antioxidant and also it may contribute to the human health. The characterization of BGP/HP-$\beta$-CD complex suggests that propolis was molecularly dispersed in the HP-$\beta$-CD matrix, providing an increasing of solubility of propolis compounds in water, but not for polar compounds such as caffeic and $\rho$-coumaric acids. Moreover, the HP-$\beta$-CD complexes improved the solubility of some water insoluble or less soluble components, such as, cinnamic acid, aromadendrin, isosakuranetin, and artepillin C. This fact can resolve the disadvantages of using the ethanol extract such as strong residual flavor, adverse reactions and intolerance to alcohol in some people. So, it can be concluded that the main objective of this work, which was to obtain a BGP extract soluble in water, maintaining the biological potential activities, was successfully achieved.

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