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Isolation and Quantification of the Main Prenylated Compounds from Brazilian Green Propolis with Antioxidant Properties

Isolamento e Quantificação dos Principais Compostos Prenilados da Própolis Verde Brasileira com Propriedades Antioxidantes

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Brazilian green propolis is produced by *Apis mellifera* L. in the Southeast region of the country. It is known for its wide spectrum of biological activities, such as antimicrobial, cytotoxic, and antioxidant. Brazilian green propolis is composed of prenylated cinnamic acids collected from *Baccharis dracunculifolia* DC, with Artepillin C serving as a biomarker for identification and quality control. This study aimed to identify and quantify the main prenylated substances found in hexane and ethanol extracts of Brazilian green propolis. Total phenolic and flavonoid contents were determined by colorimetric methods, and DPPH free radical scavenging was used to measure the antioxidative activities. Fractionation of crude hexane extract by flash chromatography led to a sub-fraction rich in prenylated substances, characterized by ^{1}H and ^{13}C NMR. Analytic HPLC-PDA was used to quantify prenylated substances in crude propolis extracts and the results were used to compare the antioxidant properties of each substance to crude extracts.

Keywords: Phytochemicals; artepillin C; antioxidant activity; HPLC

1. Introduction

Propolis is the product of a complex combination of plant-based substances collected by honeybees from a variety of botanical sources, such as with beeswax, volatile substances (such as essential oils), and pollen, and which are modified by enzymes found in bee saliva.[1] In addition to repairing and smoothing internal walls and controlling the hive's temperature, propolis also protects the colony against diseases, prevents the intruders from decomposing, and keeps the colony clean.[2]

Propolis' chemical composition depends on its location, collection time, and the availability of local plants. Seasonal variations in propolis chemicals pose the most significant challenge for standardizing and identifying its active compounds among the more than 300 different metabolites already identified.[3-5] Raw propolis is primarily composed of resinous compounds (50%) derived from plants found in the local flora (leaves, twigs, flowers, and exudates) and wax produced by honeybees (30%), as well as essential and aromatic oils (10%), pollen (5%) and other inorganic substances such as minerals (5%).[6]

Brazilian flora is distinguished by its diversity of plant species. This enables the production of different types of propolis with distinct chemical compositions and a wide spectrum of biological properties.[2,6-7] Brazilian green propolis (BGP) is produced by honeybees (*Apis mellifera* L.) from young apical tissues of *Baccharis dracunculifolia* DC. (Asteraceae), a perennial shrub known as "alecrim-do-campo" and widely distributed in the country's Southeast region.[2,8,9] BGP is also described as the most important type of propolis found in Brazil, known for its medicinal and cosmetic properties, as shown in studies conducted in Japan and China since the 1990s.[10] According to recent data from the BGP market, more than 140 metric tons of propolis are exported each year, generating millions of dollars in revenue.[9] BGP's chemical and ecological interactions with its botanical source have been extensively described in the literature, while chromatographic and UV-Vis techniques have revealed chemical similarities between them.[9,11-14]

Sesquiterpenes make up the majority of the volatile fraction in BGP from different locations in the Southeast region, and their seasonal variations are mainly quantitative.[15-17] Propolis has sensory and biological properties closely related to volatile substances and essential oils, contributing to its antimicrobial properties.[16] BGP's biological properties are primarily determined by its hydroalcoholic fraction, which contains prenylated cinnamic acids found in its main botanical source, B. *dracunculifolia* DC (Figure 1). The major compound of BGP is Artepillin C (**1**), a

prenyl-containing cinnamic acid, which has been established as a biomarker for the quality control of green propolis.[18]

Artepillin C (**1**) is also the most active component of BGP, and several *in vitro* and *in vivo* studies have indicated that the prenylated substance is associated with the modulation of several biological properties including antimicrobial,[19-22] antioxidant,[22-24] anti-inflammatory,[25-29] and antitumor agents.[14,30-34] Drupanin (**2**) is a mono-prenylated compound found in hydroalcoholic extracts of BGP and is both an antioxidant[25,35] and a cytotoxic agent.[30,32,36] Drupanal (**3**) was recently isolated from ethanol extract of BGP and the aldehyde exhibited activity as PPARα agonist, while both **1** and Capillartemisin A (**4**) exhibited activity as PPARγ agonist. This indicates a close relationship between the structural pattern and the biological target of prenylated cinnamic acids..[37] Baccharin (**5**) is found in high concentrations in non-polar extracts of BGP and is not as active as **1** or **2** in antioxidant and antimicrobial assays, but has exhibited activity in several human cancer cell lines.[14,34,38] The exposure of **1** to high temperatures can lead to its degradation products, resulting in heterocyclic derivatives between the prenyl side chain and the hydroxyl group, like the chroman Culifolin (**6**), which have exhibited both leishmanicidal and cytotoxic activities.[39,40]

Various civilizations have used propolis as a folk medicine to treat wounds and ulcers since ancient times.[41] In recent years, however, since the discovery of its main chemicals and their biological activities in the early 20th century, there has been an increasing demand for methods that are more efficient and effective for isolating and identifying its active compounds.[42] Furthermore, there are few effective synthetic routes available to obtain the prenylated cinnamic acids found in BGP: *C*-prenylation of aromatic ring is said to be the key step for the total synthesis of Artepillin C and its derivatives, but current methodologies do not provide satisfactory results.[43-45] Despite the presence of Artepillin C and other derivatives in different Asteraceae genera (such as *Fluorensia* and *Rlhania*), BGP and its main resin source contain large amounts of prenylated cinnamic acids, which itself justifies the use of the natural product to obtain good yields of this class of bioactive substances.[18]

The present study reports the isolation of Artepillin C and other prenyl-derivatives from BGP through sequential flash column chromatography coupled with RP-HPLC-UV purification in a semi-preparative scale using the hexane extract of propolis prepared by Soxhlet. Baccharin served as a precursor for obtaining Drupanin by the alkaline hydrolysis of its ester group due to the absence of the monoprenylated compound in non-polar extracts. The investigation also includes the quantification of all isolated substances in both hexane and ethanol extracts of BGP in order to correlate the presence of prenylated substances with the antioxidant properties from the crude extracts, evaluated by the radical scavenging assay using DPPH (2,2-diphenil-1-picrylhydrazyl).

2. Experiment

2.1. Propolis

BGP samples were collected *in natura* from three local producers in the state of Rio de Janeiro in 2012, namely from Mesquita (GP1), Carmo (GP2), and Paracambi (GP3). The crude samples were obtained by scraping. After cleaning the samples (pieces of wood, leaves, and insects), they were frozen in a domestic freezer (-5°C). With the frozen samples, a semi-industrial blender (Cadence, Thunder Blender LIQ600) was used to pulverize it to a homogeneous green powder, which was sieved and stored under refrigeration until the extracts were prepared.[46,47]

Soxhlet extractors were used to extract the samples and 3.00 g of the pulverized GP1-3 samples were placed in a cellulose cartridge and submitted to continuous extraction

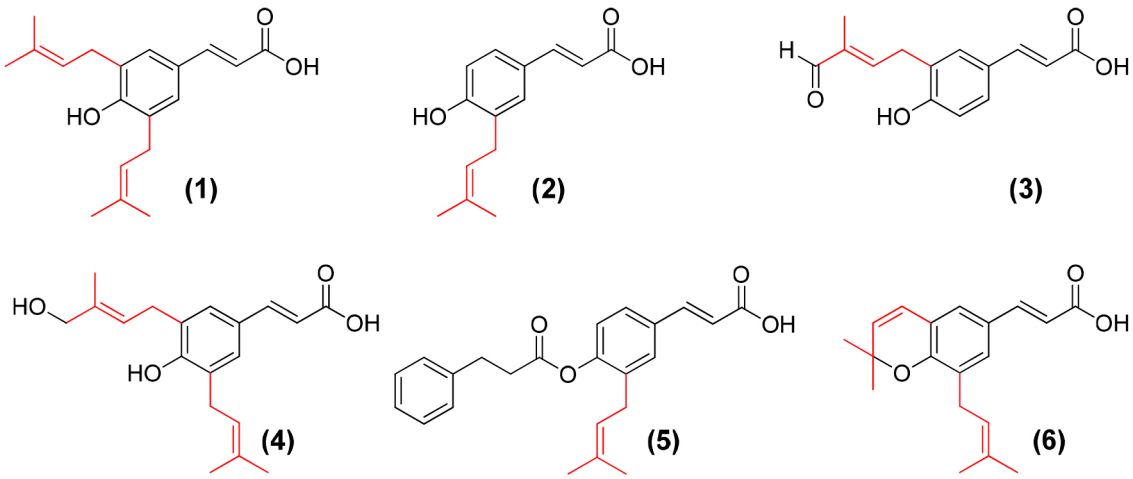

Figure 1. Prenylated compounds found in both BGP extracts: (*E*)-3,5-diprenyl-4-hydroxycinnamic acid (**1**), (*E*)-3-prenyl-4-hydroxycinnamic acid (**2**), (*E*)-3-[4-hydroxy-3-((*E*)-3-formyl-2-bytenyl)phenyl] cinnamic acid (**3**), (*E*)-3-[(*E*)-4-hydroxy-3-methylbutenyl)-5-(prenyl)-4-hydroxycinnamic acid (**4**), 3-prenyl-4-[(3-phenylpropanoyl)-oxy]-cinnamic acid (**5**) and (*E*)-2',2'-dimethilcromen-9-prenyl-7-cinnamic acid (**6**).

using 150.0 mL of each solvent: hexane P.A. (HEP1-3) followed by ethanol P.A. 95% (EEP1-3). The extractions lasted six hours. A rotary evaporator was used to remove the solvent. After overnight refrigeration, both crude extracts were solubilized with 30.0 mL of methanol to improve wax separation. Vacuum filtration was used to separate wax from propolis extracts and, after the solvent evaporated, pure extracts containing the bioactive prenylated cinnamic acids found in propolis were stored at room temperature.

2.2. Total Phenolic (TP) and Flavonoid (TF) Contents

Colorimetric assays for total phenolics and flavonoids were performed according to the methodology previously described by Salgueiro and Castro (2016),[46] with Folin-Ciocalteau (TP) and $AlCl_3$ (TF) reagents, using a UV-Mini 1240 (Shimadzu) spectrophotometer. Hexane (HEP1-3) and ethanol extracts (EEP1-3) were solubilized in spectroscopic methanol at a concentration of 1.0 $mg.mL^{-1}$. Analysis was carried out using ultrapure water (Milli-Q) and spectroscopic methanol as blanks, respectively. Results were obtained from calibration curves prepared with gallic acid (VETEC-RJ, 99%) for TP (E_{GA}.100 mg^{-1} of extract) and quercetin (SIGMA, 98%) for TF (E_Q.100 mg^{-1} of extract). The analyses were performed in triplicates.

2.3. RP-HPLC–PDA Analysis Conditions

A Shimadzu Prominence (Kyoto, Japan) liquid chromatography instrument was used with the LC Solution software consisting of an SIL-20A prominence autosampler, a column oven and a SPD-M20A diode array detector. Each sample (1.0 $mg.mL^{-1}$ in MeOH) was filtered through a 0.45 mm filter before being injected (20 µL) onto a RP-18 column (250 mm x 4.6 mm x 5 µm, Betasil-Thermo) and kept at 40 °C. The mobile phase consisted of two channels, channel (A) 1% acetic acid in water and channel (B) pure methanol. The flow rate was set at 1.0 $mL.min^{-1}$ and the column gradient program started at 35% B by 2 minutes, and ramped linearly over the course of 20 min at 80% B. The system was then linearly increased for 25 min to 95% B, where it was kept for up to 3 minutes. After the gradient returned to 35% B, it was kept there for 2 additional minutes. The detection was monitored at 280 and 315 nm and the components were identified by comparing retention times (T_R) and respective ultraviolet absorption curves with standards isolated during this study. The samples were previously filtered through a 0.45 mm filter. Quantitative analyses were carried out by external calibration and samples were analyzed in triplicates.

2.4. Fractionation of HEP_1 through flash column chromatography

The hexane extract was selected because of its high concentration of the main prenylated compounds when compared to the respective ethanol extracts. The crude HEP1 extract was first pre-fractionated using flash chromatography with 70.0 g of silica gel 60 (230 – 240 mesh) in a glass column (60.0 cm x 3.0 cm) using 150.0 mL of $CHCl_3$ as solvent. The solution containing the extract (300.0 mg) was combined with a small amount of silica, and after evaporation, the silica-HEP_1 mixture was applied to the top of the column. The fractionation was performed according to the elution gradient previously described by Hattori and colleagues (2011)[48] with some modifications, using 100.0 mL of a mixture containing $CHCl_3$-Acetone in the following order: 100% $CHCl_3$ – $CHCl_3$-Acetone (1%, v/v) – $CHCl_3$-Acetone (3%, v/v) – $CHCl_3$-Acetone (5%, v/v) – $CHCl_3$-Acetone (7%, v/v) – $CHCl_3$-Acetone (10%, v/v) – $CHCl_3$-Acetone (15%, v/v) - $CHCl_3$-Acetone (20%, v/v) – 100% Acetone. A total of 108 fractions were collected (10.0 – 15.0 mL) at the end of elution, and they were mixed according to chromatographic similarity evaluated by TLC using silica gel in an aluminum plate eluted with Hexane-Acetone (20%) as mobile phase, followed by RP-HPLC-PDA analysis. The main fraction containing the rich mixture of prenylated compounds was obtained an eight of 90.0 mg (30%).

2.5. Isolation of prenylated substances by semi-preparative RP-HPLC-UV

The fraction containing the main prenylated compounds (90.0 mg) was purified by RP-HPLC-UV in a semi-preparative scale using a Shimadzu LC-10AS liquid chromatograph equipped with a UV-vis detector and a Rheodyne injector, controlled by the LCSolution software (Shimadzu). The fraction was first solubilized in 5.0 mL of spectroscopic methanol and then purified by injecting successive 0.5 mL injections of filtered solution into a C -18 reverse phase column (250 mm x 10 mm x 5 um, Luna-Phenomenex). The flow rate was set at 4.0 $mL.min^{-1}$ with the following mobile phase system: 1% acetic acid in water (Solvent A) and pure acetonitrile (Solvent B) in a linear gradient (75 – 100% B) for 17 minutes. The effluent was monitored at 315 nm and the peak fraction was collected according to the elution profile. The fractions were collected in glass tubes and evaporated to dryness to yield three prenylated compounds in order of retention time as follows: Artepillin C (1. 25 mg. T_R 6.0 minutes), Baccharin (5. 15 mg. T_R 9.0 minutes), and Culifolin (6. 5.0 mg. T_R 11.0 minutes). The identity of these compounds was confirmed using spectroscopic data obtained by ^1H and ^{13}C NMR analysis through comparison with previously reported spectroscopic data found in the literature and it's available as a supplementary information.[48-51]

2.6. Preparation of Drupanin (2)

Since Drupanin (2), the mono-prenylated cinnamic acid of propolis, was not found in hexane extracts, it was obtained from Baccharin (5), isolated from HEP1. A non-aqueous

alkaline medium was used for the hydrolysis of the ester group, as described by Theodorou and colleagues (2018).[52] A semi-synthesis method was used to obtain the product, which was then analyzed quantitatively for ethanol extracts (EEP1-3) and antioxidants.

A solution of Baccharin (15 mg, 0.038 mmol) was prepared using 0.5 mL of CH_2Cl_2 and then mixed with 0.3 mL of NaOH 3N in methanol. The volume of the reaction medium was increased to 2.0 mL using a CH_2Cl_2-MeOH (9:1, v/v) solution. The hydrolysis reaction was carried out for 1 hour at room temperature until the complete formation of products was observed, as monitored by TLC. Following the removal of solvents, the residue was diluted with 0.5 mL of water and extracted with ethyl acetate (2x 10.0 mL). The aqueous solution was then acidified with HCl to pH 2 and extracted with ethyl acetate (2x 10.0 mL). The combined organic layers were dried with Na_2SO_4, and the solvent was removed by vacuum to give 7.1 mg (80%) of 2.

2.7. Antioxidant assays

Propolis extracts (HEP1-3 and EEP1-3) and isolated substances were tested for radical scavenging activity against the free radical DPPH (2,2-diphenyl-1-picrylhydrazyl) according to the methodology previously described by Salgueiro and Castro (2016).[46] The DPPH assay was performed by mixing 71.0 µL of each extract or isolated compound (10.0 – 100.0 µg.mL^{-1}) with 29 µL of a solution containing the free radical DPPH at 0.3 mM in a 96-well microplate. Mixtures were incubated for 30 minutes in the dark and then the absorbance was measured at 520 nm using a microplate reader ELISA 680 (Bio-Rad, Brazil). The results were expressed as EC_{50} (µg.mL^{-1}), which means the concentration of antioxidants required to decrease free radical activity by 50%, and the results were obtained by a linear regression using each concentration measure. The flavonoids kaempferol and naringenin were used, respectively, as positive, and negative controls.

Results and Discussion

Table 1 shows the crude yields of each extraction, as well as the total phenolic compounds (TP and TF) and the antioxidant capacity (DPPH/EC_{50}). The chemical profile of the hydroalcoholic extract of BGP, obtained by dynamic maceration, was previously described by Salgueiro and Castro (2016), along with its phenolics, flavonoids, and antioxidant properties.

In comparison to Salgueiro and Castro (2016)'s characterization of the same BGP samples, Soxhlet ethanolic extracts exhibit a higher yield than their respective dynamic maceration extracts previously reported. The same results were also observed for the contents of total phenolics and flavonoids, as well as for the radical scavenging activity, with the Soxhlet extracts showing better results than the respective maceration extracts. In this study, the TF results for the ethanolic extracts were significantly superior to those previously obtained by Salgueiro and Castro (2016). It is relevant to mention that the continuous extraction method was used to increase the concentration of prenylated phytochemicals in the propolis for later quantification and isolation by HPLC.

The characterization of hexane extracts from these GP samples was not reported in the previous study. In this study, sequential extractions of propolis were performed in order to examine the effects of the polarity of solvents used in preparing the extracts. In general, ethanol extracts (**EEP1-3**) had a higher mass yield in comparison to the respective hexane extracts (**HEP1-3**). Propolis substances have a higher solubility in polar solvents such as alcohols, and it can explain the higher yields of the second extraction. The sample **GP2** (Carmo – RJ) produced the lowest crude yields for both **HEP2** (7.50%) and **EEP2** (38.22%). The samples **GP1** (Mesquita – RJ) and **GP3** (Paracambi – RJ) produced extracts with similar mass results for both hexane (20.29% for **HEP1**, and 22.47% for **HEP3**) and ethanol extracts (61.69% for **EEP1**, and 61.98% for **EEP2**). This observation can be attributed to the presence of flora in these regions that permits the production of propolis. Ethanol extracts obtained by sequential extraction showed a higher concentration of phenolic substances evaluated by colorimetric methods.

TP results ranged from 1.73 mg E_{GA}.100 mg^{-1} of extract (**HEP3**) to 12.84 mg E_{GA}.100 mg^{-1} of extract (**EEP3**), and the TF results varied from 1.45 mg E_Q.100 mg^{-1} of extract (**HEP1**) to 16.82 mg E_Q.100 mg^{-1} of extract (**EEP3**). Both

Table 1. Extraction yields, Total phenolics (TP) and flavonoids (TF), and antioxidant properties for all GP extracts.

Extract	Yield (%)	TP [a]	TF [b]	EC_{50} [c]
HEP1	20.29 ±1.38	01.90 ±0.037	01.45 ±0.004	84.94 ±1.5255
EEP1	62.09 ±1.53	08.90 ±0.013	12.73 ±0.001	22.46 ±0.4708
HEP2	07.50 ±0.95	01.91 ±0.035	02.32 ±0.002	80.24 ±0.8168
EEP2	38.22 ±1.16	11.92 ±0.011	15.69 ±0.005	18.62 ±0.2951
HEP3	22.47 ±1.22	01.73 ±0.037	01.55 ±0.001	74.48 ±1.0374
EEP3	61.98 ±1.87	12.84 ±0.006	16.82 ±0.005	16.49 ±0.7849

a- mg E_{GA} 100 mg^{-1} of extract; b- mg E_Q 100 mg^{-1} of extract; c- EC_{50} in µg.mL^{-1}. TP – total phenolics, TF – total flavonoids.; Data expressed as mean ± standard deviation, n=3

TP and TF extract contents depend on the flora available for resin collection, so the chemical composition of the extracts may vary depending on the production region. Despite the difference in extraction yield, the sample GP2 (Carmo - RJ) showed no significant difference in TP and TF extract content when compared to other samples. A similar effect on propolis extraction was described by Cunha and colleagues (2004)[53] for GP extracts from the state of São Paulo, in which no significant correlation was found between the phenolic profile (TP and TF) and the global yield of ethanol extracts using different extraction methods.

Comparing the antioxidant results displayed in Table 1, **HEP1** has the lowest antioxidant capacity in radical scavenging using DPPH (89.94 $\mu g.mL^{-1}$), while **EEP3** showed the highest result among the extracts: DPPH – 16.49 $\mu g.mL^{-1}$. This difference can be explained by comparing the total phenolic contents of the extracts: ethanol extracts showed the best results in the TP assay and also showed superior radical scavenging activity using DPPH in comparison to the respective hexane extracts. The present study focused on the extraction efficiency of prenylated compounds present in GP with the goal of isolating and characterizing these bioactive compounds. A high concentration of waxes, terpenes, and resinous substances makes the propolis matrix lipophilic and poorly soluble in water.

The BGP samples used in this study were selected based on the total Artepillin C content reported by Salgueiro and Castro (2016). The main prenylated compounds found in BGP are less hydrophilic due to the extension of the isoprenoid moiety. In this case, extraction with non-polar solvents may increase the extraction yield of these components without interfering with more polar substances and resins commonly extracted with ethanol. Furthermore, non-polar extracts of propolis and its main botanical source (B. *dracunculifolia* DC) contain high levels of these derivatives as described in the literature. These observations confirm the effect of this type of solvent on the structure of prenylated cinnamic acids.[21,51]

Both hexane and ethanol extracts were analyzed using RP-HPLC-PDA for chemical profiles, and prenylated compounds were quantified using RP-HPLC-UV after being isolated . The main difference observed between hexane and ethanol extracts (Figure 2A) was the presence of more polar substances in the ethanol extract (T_R 2.5 – 15.0 minutes) and lesser concentrations of non-polar substances

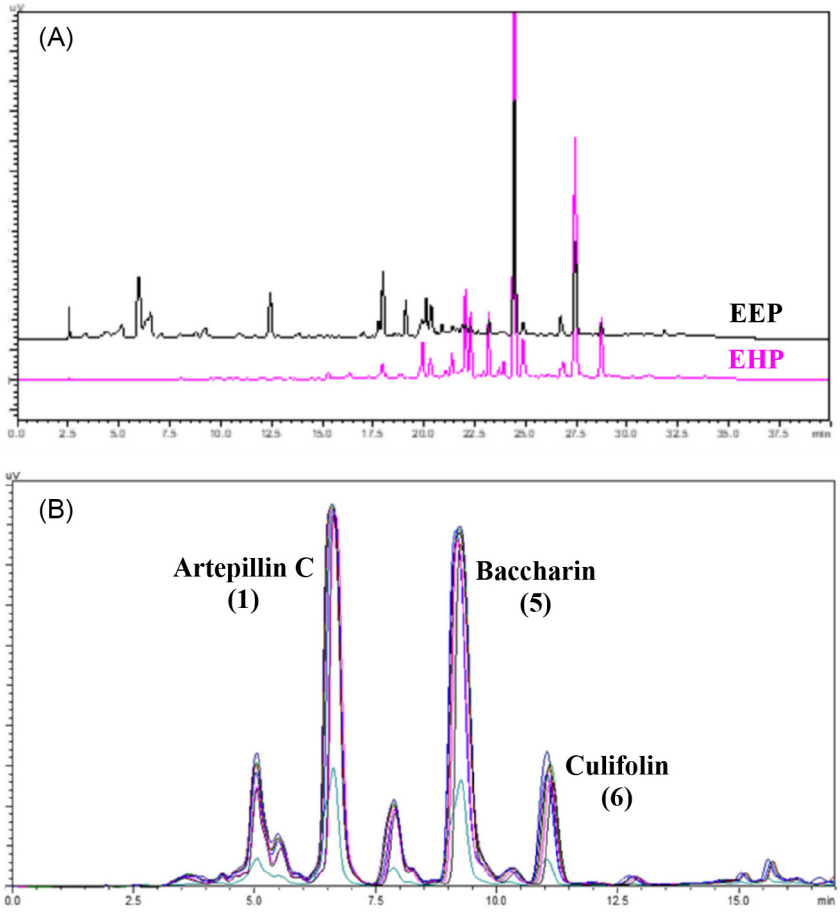

Figure 2. (**A**) Analytical Chromatograms (RP-HPLC-PDA) obtained for **EEP1** (black) and **HEP1** (pink) at a concentration of 1.0 $mg.mL^{-1}$. (**B**) Overlay of the semi-preparative chromatograms (RP-HPLC UV) following 10 successive injections of the main fraction containing prenylated compounds

(T_R 15.0 – 30.0 minutes), which could explain the TP and TF assays results in Table 1. The two components with the highest concentrations were first as Artepillin C (T_R 24.5 minutes) and second Baccharin (T_R 27.4 minutes) as determined by comparison with the retention time and the respective absorption spectra (UV-Vis) found in the literature. Based on these observations, hexane extraction proved to be the most viable method for isolating prenylated compounds. In order to isolate the main compounds identified in **HEP1**, 300.0 mg of crude extract was pre-fractionated using flash chromatography column with a gradient of $CHCl_3$-Acetone (0 – 20%) to produce 90.0 mg of a main fraction rich in prenylated compounds and less resinous compared to crude hexane extracts. The fraction was submitted to RP-HPLC-UV in a semi-preparative scale by adapting the analytical method, and the upscale result was fast and efficient.

The final processing resulted in overlaid chromatograms (Figure 2B), indicating high reproducibility after successive injections of the extract, leading to the purification of 25.0 mg of Artepillin C (27%), 14.0 mg of Baccharin (15%) and 5.0 mg of Culifolin (5%) to pure compounds (98% by HPLC-PDA), which were fully characterized by ^1H and ^{13}C NMR, FTIR and EM (see supplementary information). The isolation of prenylated cinnamic acids resulted in pure compounds that were quantified in the crude extracts using RP-HPLC-PDA. Drupanin (**2**) was not found in the hexane extracts, so it was obtained by semi-synthesis from its respective ester Baccharin (**5**). A list of the analytical curve equations for each component is available as supplementary information. The isolated prenylated substances (**1**, **5** e **6**), as well as the semi-synthesis product (**2**), showed above 98% chromatographic purity.

Table 2 summarizes the quantified prenylated compounds in each extract. Baccharin and Culifolin were identified and quantified for the first time in these GP samples from Rio de Janeiro. The extracts prepared using **GP1** contained the highest contents of all prenylated substances, including Drupanin (**2**) in **EEP1**. The absence of a prenyl group in the chemical structure increases the interaction of the phenolic hydroxyl group with more polar solvents, while in the structure of Artepillin C, this effect is suppressed due to the steric hindrance caused by the addition of one more prenyl unit, making it more lipophilic. The side chains also affected the quantification of Culifolin and Baccharin, with lower concentrations found in the ethanol extracts due to the absence of a free hydroxyl group.

A radical scavenging assay using DPPH was performed in order to evaluate the antioxidant properties of the prenylated compounds obtained by isolation or semi-synthesis. Among the prenylated substances, the biomarker Artepillin C (**1**) exhibited the strongest antioxidant activity (EC_{50} 8.49 $\mu g.mL^{-1}$). The presence of two prenyl groups in its structure increases the steric hindrance effect, and it stabilizes the formation of a free radical, while the absence of one of its isoprenoid moieties in the structure of Drupanin (**2**) can explain the difference observed in activity (EC_{50} 50.14 $\mu g.mL^{-1}$). Positive control (Canferol) demonstrated greater radical scavenging activity (EC_{50} 6.11 $\mu g.mL^{-1}$) compared to the results for prenylated cinnamic acids. However, the extracts of sample **GP1** (Mesquita – RJ) with higher Artepillin C (**1**) content in both hexane and ethanol extracts failed to achieve the best results in radical scavenging using DPPH as shown by the results in Table 2. It suggests the synergism between the different components of Brazilian GP may be responsible for modulating its antioxidant properties, despite the isolated compound's antioxidant properties.

In addition, derivatives Baccharin (**5**) and Culifolin (**6**) did not exhibit significant antioxidant activity ($EC_{50} > 100\ \mu g.mL^{-1}$), which can be explained by the absence of a phenolic hydroxyl group, since it was derivatized to an ester function in the structure of Baccharin and an ether function in Culifolin, preventing them from showing radical scavenging activity. Both Baccharin (5) and Culifolin (6) showed similar radical-scavenging activity as the negative control Naringenin ($EC_{50} > 1000\ \mu g.mL^{-1}$). The presence of phenolic hydroxyls in isolated substances favors antioxidant activity as observed in a wide spectrum of phenolic acids found in diverse types of propolis such as chlorogenic acid (EC_{50} 5.85 $\mu g.mL^{-1}$), caffeic acid (EC_{50} 9.30 $\mu g.mL^{-1}$) and ferulic acid (EC_{50} 24.71 $\mu g.mL^{-1}$). The presence of these substances may also explain the higher antioxidant capacity found in ethanol and other polar GP extracts.[46] However, it is important to note that further investigations into the antioxidant properties of propolis and its prenylated compounds should follow other methods in order to gain a better understanding of the structure-antioxidant activity of green propolis and its main active components. [54]

Table 2. Quantification (mg.100 mg^{-1} of extract) of all prenylated substances in the BGP hexane and ethanolic extracts (**HEP1-3** and **EEP1-3**)

Extrato	Artepillin C (1)	Baccharrin (5)	Culifolin (6)	Drupanin (2)
HEP1	6.09 ±1.0721	7.87 ±0.0338	2.03 ±0.0341	nd
EEP1	4.08 ±1.0749	3.12 ±0.0368	0.53 ±0.0013	8.06 ±0.0982
HEP2	1.97 ±1.0721	5.63 ±0.0366	2.30 ±0.1421	nd
EEP2	1.26 ±1.0711	1.20 ±0.0225	0.15 ±0.0314	3.35 ±0.2538
HEP3	3.06 ±1.0704	7.04 ±0.0312	2.03 ±0.1723	nd
EEP3	3.47 ±1.0739	3.69 ±0.0276	0.74 ±0.0923	7.04 ±0.0124

Data expressed as mean ± standard deviation, n=3. nd – not detected.

Conclusion

This study provides an insight into the extraction profile of Brazilian GP based on the type of solvent used and the relation between the levels of phenolics and prenylated compounds. The two-step method for the isolation of prenylated compounds (pre-fractionation by flash chromatography followed by RP-HPLC-UV purification) led to the isolation of the three main prenylated compounds found in the hexane extracts of propolis: Artepillin C, Baccharin and Culifolin. For comparison, Drupanin was obtained through the semi-synthesis of Baccharin, and all of the substances were used to quantify both extracts. Artepillin C was the dominant component of the extracts, while Culifolin was a minor component. The antioxidant activity of isolated substances highlighted the importance of a free hydroxyl group in the structure for radical scavenging activity. As a result of the presence of two prenyl groups causing steric hindrance on the phenolic hydroxyl group, Artepillin C was the most active prenylated substance in the assays.

Supplementary Information

The spectroscopic data used in the characterization of the prenylated substances, as well as the analytical curves used to quantify crude propolis extracts, are available as supplementary information.

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