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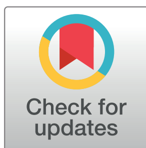

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RESEARCH ARTICLE

Chemical characterization and biological activity of six different extracts of propolis through conventional methods and supercritical extraction

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Abstract

Propolis is a natural product with many demonstrated biological activities and propolis extract has been used in the food, pharmaceutical and cosmetics industries. Different works have showed the variations in the chemical composition, and consequently, on the biological activity of the propolis that are associated with its type and geographic origin. Due to this study evaluated propolis extracts obtained through supercritical extraction and ethanolic extraction (conventional) in three samples of different types of propolis (red, green and brown), collected from different regions in Brazil (state of Bahia). Analyses were performed to determine the humidity, water activity, the content of total ash, proteins, lipids and fiber in raw propolis samples. The content of phenolic compounds, flavonoids, *in vitro* antioxidant activity (DPPH), catechin, ferulic acid and luteolin and antimicrobial activity against two bacteria (*Staphylococcus aureus* and *Escherichia coli*) were determined for all extracts. For the green and red ethanolic extracts the anti-leishmanicidal potential was also evaluated. The physicochemical profiles showed agreement in relation to the literature. The results identified significant differences among the extracts (p>0.05), which are in conformity with their extraction method, as well as with type and botanical origin of the samples. The extraction with supercritical fluid was not efficient to obtain extracts with the highest contents of antioxidants compounds, when compared with the ethanolic extracts. The best results were shown for the extracts obtained through the conventional extraction method (ethanolic) indicating a higher selectivity for the extraction of antioxidants compounds. The red variety showed the largest biological potential, which included the content of antioxidants compounds. The results found in this study confirm the influence of the type of the raw material on the composition and characteristics of the extracts. The parameters analysis were important to

characterize and evaluate the quality of the different Brazilian propolis extracts based on the increased use of propolis by the natural products industry.

Introduction

Propolis is a resinous material produced by bees (*Apis melífera L.*), primarily from plants, as a sticky exudate from leaf and flower buds, shoots, stems and fruit [1]. In Brazilian territory, 13 different types of propolis have been categorized based on physical-chemical characteristics [2–4]. *Baccharis dracunculifolia* is a type of common plant that produces green propolis, which is rich in compounds with prenylated phenylpropanoids, triterpenoids, benzoic acid and chlorogenics [5]. The *Dalbergia ecastophyllum (L.) Taub. (Fabaceae)* species are mangrove natives used by bees to produce the red propolis. Brown propolis is produced by the *Copaifera* species and mainly contains flavonoids and terpenes [6–8].

Bees use propolis to fill gaps and narrow spaces in their hives and to prevent microbial contamination in the egg deposition nests and respiratory outlets. The antimicrobial properties of propolis are important for maintaining a healthy hive environment for the bee colony [9].

In terms of chemical composition, propolis is generally composed of 50% resin, 30% wax, 10% essential oils, 5% pollen, and 5% other substances, including the derivatives of cinnamic acid, phenolic acids, substituted benzoic acids, amino acids and flavonoids [10–11]. The biological effects and chemical composition of propolis depend on various factors, such as the types of vegetable sources, the geographic origin, the season of the year and the time of collection [12–13].

Ethanol has been the most used solvent to obtain low-wax propolis extracts rich in biologically active compounds [14–16]. New methods of extracting the biocompounds of propolis have been studied in order to replace the conventional ethanolic extraction method [17–20] One of the most promising methods of extraction is with supercritical fluids [21]. This method has been demonstrated to be effective for application in chemical processes, petrochemical, pharmaceutical and food processing. This method is considered to be a clean technology and has the capacity to retain the antioxidant properties of the obtained extracts through its use of low temperatures, which is an important characteristic for the pharmaceutical and food industries [21–22].

Several studies have shown the antimicrobial [23–25], anti-inflammatory [26], cytotoxic [27–28] and antiparasitic properties [29] as well as the immunomodulatory [30–31] and anti-leishmanicidal effects of different propolis extracts from different sources, including red propolis from Alagoas, and green propolis from Paraná, Bahia and Minas Gerais in Brazil [18–19]. Propolis has been widely used in several disease models, showing great potential in protective immune response against leishmaniasis [32–33]. Miranda et al. [34] showed that nitric oxide and Brazilian propolis extract combined accelerates tissue repair by modulating cell migration, cytokine production and collagen deposition in experimental leishmaniasis, highlighting a new therapeutic option that can be considered for further *in vivo* investigations as a candidate for the treatment of cutaneous leishmaniasis.

In view of these findings, it is of extreme interest for the industry to search for new technologies that ensure the preservation of extracts and their active biological compounds. Furthermore, propolis extracts are constituted by biologically active components with promising biological activities and can be investigated for the formulation of new drugs. Considering the various applications of propolis, the objective of this study was to perform the chemical

characterization, evaluate the antioxidant capacity and antimicrobial activity of propolis extracts obtained by two methods of extraction (ethanolic and supercritical) from three samples (brown, green and red) collected in different geographical regions of Brazil (state of Bahia). The anti-leishmanicidal effects of the green and red propolis extracts obtained by the ethanolic extraction in the infection by *Leishmania braziliensis* were also studied.

Materials and methods

Obtaining and processing of propolis samples

Approximately 1000g of samples of green and brown propolis from Vitória da Conquista (Barra do Choça -14.863131, -40.552506) and red propolis samples from Canavieiras (-15.669756, -38.952456) state in Bahia, Brazil, were donated by the company Apis Jordans (S1 Table). The samples of propolis (red, green and brown) were crushed in a grinder (Philips–Brazil) in order to obtain an adequate granulometry (approximately 0.300 mm) to increase the surface area and homogenize the start material in the extraction processes. The samples were kept in a refrigerator at -20˚C in bottles (small quantities 25 g) protected with laminated paper in inert atmospheric conditions (N_2) to avoid degradation of the material.

Characterization of raw propolis

The analysis of humidity, protein and total ash contents were performed following the official methods of the Association of Official Agricultural Chemists (AOAC) [35]. The fiber content was obtained using an automatic fiber analyzer (A-220, ANKON, New York–USA) and was based on the Van-Soest et al. [36] method. The quantification of the water activity was performed using a decagon LabMaster (Novasina, Lachen–Switzerland), with a CM-2 electrolytic cell (25˚C). Total lipids were extracted and quantified using the cold extraction method described by Bligh & Dyer [37]. All of the analyses were executed in triplicate.

Obtaining propolis extracts by conventional extraction (ethanolic)

The ethanolic extracts of propolis were made by adding 15 mL of ethanol (80%) to 2 g of crushed and homogenized propolis [38]. The extraction was performed at 70˚C for 30 minutes under constant agitation in a Shaker incubator (MA 420/Marconi–Brazil), at 710-rpm. After that step, the extract was centrifuged (Centrifuge SIGMA 2–16 KL, USA) at 8800 rpm at 5˚C for 10 minutes and the supernatant was transferred to glass test tubes (15x160 mm). 10 mL ethanol (80%) was added to the residue in the centrifuge tube, and the centrifugation was repeated [5,21]. All the extracts were maintained in inert atmospheric conditions (N_2) and at a temperature of 5˚C to avoid degradation.

Obtaining propolis extracts by supercritical fluid extraction (SFE)

The extracts were obtained using a SFT-110 Supercritical Fluid Extractor (Supercritical Fluid Technologies, Inc.–USA). The equipment is composed of a high-pressure bomb (capacity of up to 10,000 psi), an extraction cell (capacity of 100 mL), an oven (containing a pre-warmer), a static/dynamics valve and restrictor valve, a flow meter, a flux totalizer (ITRÓN, ACD G1.0, Argentina) and a CO_2 cylinder (White Martins–Brazil). A CO_2 cylinder with a fishing tube was used to ensure that only CO_2 in its liquid state was used in the system, a requirement of the SFT-110.

In each experiment, the extraction cell comprised 5 g of ground propolis sample with 1% ethanol as co-solvent (m/m), wool, and glass pearls. The extraction conditions were as follows: S/F, 110 (mass of $CO_{2[solvent]}$/mass of $propolis_{[solute]}$); co-solvent, 1.0% ethanol (m/m);

pressure, 350 bar; temperature, 50˚C; CO_2 flow, 6 g·min^{-1}. The extraction time was about 60 min [5,39,40].

At the end of the extraction, the vials containing the extracts were covered with aluminum foil and kept in inert atmospheric conditions (N_2) and at a temperature of 5˚C to avoid degradation of the material.

Determination of total phenolic compounds

The analysis to determine the total phenolic compounds in the propolis extracts were performed using the spectrophotometric Folin-Ciocalteu method described by Woisky and Salatino [41], using gallic acid as a standard. Ethanol was used to dissolve the extracts in order to obtain a concentration of 0.1 mg.min^{-1}. Afterwards, 0.5 mL of the aliquot of the extract was taken and mixed with 2.5 mL of aqueous Folin-Ciocalteau solution (10%) and 2.0 mL of sodium carbonate at 7.5%. The solution was placed in a thermo-regulated bath at 50˚C for 5 minutes, and then the absorbance was measured in a spectrophotometer (Lambda 25 UV/vis Systems–PerkinElmer, Washington-USA) at 765 nm. The results of the concentrations of total phenolics were compared to a standard curve of gallic acid (gallic acid equivalents EGA) (mg EGA/g) under the same conditions. All the analyses were executed in triplicate.

Determination of flavonoid content

The flavonoid content determination of the brown, green and red propolis extracts was performed using a spectrophotometer (Lambda 25 UV/vis Systems–PerkinElmer, Washington-USA) at 415 nm. The solution was prepared using aluminum chloride at 2.0% in methanol [42] in a 1:1 solution. The same procedure was performed using known solutions of quercetin standard to elaborate a standard curve. Furthermore, a blank sample was prepared under the same conditions and the quantity of flavonoid content was expressed as quercetin equivalents (EQ) (mg EQ/g). All the analyses were executed in triplicate.

Determination of antioxidant activity (2,2-Diphenyl- 1-picrylhydrazyl–DPPH)

The evaluation of the antioxidant activity of the extracts was performed using 1,1-diphenyl-2-picrilidrazil (DPPH) according to the methodology described by Yen and Wu [43]. The extracts were diluted to five concentrations (90–190 μg.mL^{-1}) in triplicates.

Afterwards, 1.0 mL of each dilution was transferred to a test tube containing 3.0 mL of ethanolic solution of DPPH (0.004%). After 30 minutes of incubation in the dark at room temperature, the reduction of the free radical DPPH was measured by reading the absorbance using a spectrophotometer (Lambda 25 UV/vis Systems–PerkinElmer, Washington-USA) at 517 nm. A blank sample was prepared using ethanol instead of the sample. Eq 1 was used to calculate the capacity to sequestrate free radical expressed as a percentage of the radical oxidation inhibition. The IC_{50} value (necessary concentration of the extract to sequestrate 50% of DPPH radical) was calculated through the line equation based on the concentrations of extracts and its respective percentages of radical DPPH sequestration.

$$\%\text{sequestration} = 100 - [(\text{final absorbance of sample x } 100)/\text{blank absorbance}] \quad (\text{Eq } 1)$$

Chromatographic analysis of the propolis extracts

Catechin (1), p-coumaric acid (2), trans-ferulic acid (3), luteolin (4) and formononetin (5) were identified and quantified from the propolis extracts. First, 10 mg.min^{-1}of propolis extracts obtained in the different extraction methods were prepared and dissolved in ethanol,

then placed in ultrasonic bath (TECNAL–São Paulo, Brazil) for 30 minutes. A filter of cellulose ester membrane 0.45μM (Micropore) was used to filter the samples, prior to injection on a High-Performance Liquid Chromatograph (HPLC). The chromatographic analysis were accomplish using the HPLC EZChrom Elite system, which consists of a VRW HITACHI L-2130 pump, supplied with an automatic injector and diode arrangement detector (DAD) VRW HITACHI L-2455, and a VRW HITACHI L-2300 oven. The method used to promote the chromatographic separation was adapted from Daugsch [44] and Machado et al. [39]. A LiChroCART Purospher StaR RP18-e (75 mm x 4 mm i.d.) (3 μm) column (Merck, Darmastad, Germany) was used together with a LiChroCART 4–4 LiChrospher 100RP18 (5 μm) pre-column from Merck.

HPLC was performed with an elution gradient using a mobile phase of aseptic acid 5% (aqueous phase) and methanol (organic phase) in different proportions and the total time of the experiment was 70 minutes. The volume of injection was of 10 μL and the chromatographic acquisition was defined at 290 nm (DAD). To ensure the reliability of the results, a validation was done according to the National Health Surveillance Agency (ANVISA) [45] and National Institute of Metrology, Standardization and Industrial Quality (INMETRO) [46] methodologies. This step was performed in accordance to the parameters of selectivity, linearity, precision, accuracy, detection limits and quantification limits.

Antimicrobial activity of the propolis extracts

The Minimum Inhibitory Concentration (MIC) was used to obtain the antimicrobial activity, based on CLSI/NCCLS M7-A6 documents [47]. The strains used were *S. aureus* (ATCC 29213) and *E. coli* (ATCC 25922).

The bacterial samples, which were obtained from frozen stocks stored at -20˚C, were seeded on brain heart infusion (BHI) agar and incubated in a bacterial incubator (Thermo Scientific, Massachusetts, EUA) at 37˚C for 24 h and then cultured on BHI agar plates to prepare the inoculum. The initial inoculum was 1–2 x 10^5 $CFU.mL^{-1}$ and the extract concentration varied from 3.1 to $1600 μg.mL^{-1}$, with the aim of determining the MIC. The tests were performed in triplicate. The analysis were defined as the minimum concentration of an extract with the capacity to inhibit bacterial growth [48].

Ethics statement

Male BALB/c mice aged 6–8 weeks were obtained from the animal care facility at CPqGM/FIOCRUZ, located in the city of Salvador, Bahia-Brazil. All animal experimentation were conducted in accordance with the Guidelines for Animal Experimentation as established by the Brazilian Council for Animal Experimentation Control (CONCEA). The present study received approval from the local institutional review board (CEUA) (protocol: CEUA-015/2015-CPqGM/FIOCRUZ).

Parasites

In this study, a strain of *Leishmania Viannia braziliensis* (MHOM / BR / 01 / BA788) was used. The promastigotes were cultured for seven days in Schneider's insect medium supplemented with 10% inactive fetal bovine serum (FBS), 100 $U.mL^{-1}$ penicillin, 100 $mg.mL^{-1}$ streptomycin, and 2 mML-glutamine in 25 cm^2 flasks.

Macrophage toxicity assay

BALB/c mice femurs and tibia were used to obtain bone marrow-derived murine (BMM) cells that were cultured at 37˚C under 5% CO_2 for 7 days in RPMI medium supplemented with

20% FBS, 100 U.mL^{-1} penicillin, 100 mg.mL^{-1} streptomycin, and 2 mM L-glutamine. Thereafter, 30% of a L929 cell culture supernatant was used as a source of macrophage colony stimulating factor. After differentiation, the BMMs (10^5 per well) were plated in 96-well plates and cultured at 37˚C under 5% CO_2 in RPMI-supplemented medium for 24 hours. The uninfected macrophages were treated with the ethanolic propolis extracts at varying concentrations (5, 10, 20, 40, 80 and 160 μg.mL^{-1}) at 37˚C for 48 h. To finalize the procedure, the cells were reincubated for another 4 h with supplemented RPMI medium containing 10% Alamar Blue. The absorbance was then read at 570 nm and 600 nm using a spectrophotometer (SPECTRA Max 190).

Macrophage infection

BMM monocytes were isolated as described above and 2×10^5/cells per well were seeded in 96-well plates. Macrophages were infected (10:1) with stationary-phase *Leishmania* (V.) *braziliensis* (MHOM/BR/01/BA788) promastigotes for 24 h and the treated with varying concentrations (10, 25, 50, 75 and 100 μg/mL) of green and red ethanolic propolis extracts for 48 h. The media was replaced with 0. 2 mL of supplemented Schneider medium. Cells were then cultured at 24˚C for an additional five days and the number of viable parasites were determined by direct counting. Amphotericin B (0.25 μg.mL^{-1}) was used as a positive control.

Statistical analysis

The program Statistica16.0 from StatSoft (Tulsa, OK, USA) was used for the statistical analysis of the results and to identify significant differences between the means. An ANOVA one-way was used to identify the differences between the concentrations of phenolic compounds, flavonoids, antioxidant activity, and the concentration of the compounds by HPLC in the extracts obtained through the two extraction methods for the three propolis samples (green, red and brown). In addition, the same test was applied to evaluate the differences between the characterization analyzes of raw propolis samples. Results are expressed as mean ± standard error of the mean (n = 3). In all statistical procedures, the level of significance was set at $p<0.05$.

In relation to the results of infection, GraphPad Prism Software 5.0 (GraphPad, San Diego, CA) was used for the analyses. For *in vitro* experiment using cells, the Kruskal-Wallis nonparametric test with Dunn's posttest were used for multiple comparisons. Linear trend ad hoc analysis were used to evaluate the statistical significance between the groups, considered when $p<0.05$. Data are presented as the mean ± standard deviation (SD) from experiments performed in quintuplicate.

Results and discussion

Characterization of raw propolis samples

Analyzing the physicochemical composition of propolis is important for determining the quality of this material when it is considered for use in industrial areas, such as the food, cosmetics and pharmaceutics industries. The results of the physicochemical characterization of the three different raw propolis extracts analyzed in this study are found in Table 1. Significant differences were found in the analyses of humidity, water activity and lipids.

The brown variety showed a humidity value of 8.03%, which was slightly out of the required standards for the humidity (a maximum of 8%) [26]. The green and red propolis were demonstrated to be within the standard required.

In relation to the water activity, the samples demonstrated a value of 0.765% for red, 0.803% for green and 0.876% for brown propolis. The values are in agreement with the results

Table 1. Determination of the content of humidity, water activity, total ash, raw protein, total lipids and fiber of brown, green and red propolis samples.

Sample	Humidity (%)	Water activity (%)	Total Ash (%)	Protein (%)	Lipids (%)	Fiber (%)
Brown	8.03±0.12[a]	0.876±0.006[a]	1.35±0.19[a]	2.49±0.08[a]	11.04±0.12[b]	70.82±5.91[a]
Green	6.30±0.30[b]	0.803±0.003[b]	1.44±0.10[a]	2.31±0.08[a]	8.19 ±0.64[c]	70.02±6.86[a]
Red	7.64±0.12[a]	0.765±0.003[c]	1.43±0.05[a]	2.12±0.09[a]	15.61±1.01[a]	68.72±2.89[a]

Values showing the same letter on the same column do not show significant difference (p>0.05) through the Tukey test at a 95% confidence level.

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of humidity, where the samples with higher humidity showed higher water activity. The water activity and the humidity are the parameters that permit the determination of conservation, microbial propagation and the occurrence of chemical reactions of the products [49].

Concerning the results of total ash, the green and red propolis showed similar values compared with the brown propolis. The values found for the analysis of ash proved to be slightly lower that the values found by Machado et al. [5] for brown propolis from the state of Santa Catarina (1.73%).

The importance of the determination of total ash in propolis material were due to the possibility of commercialization in a powder form, where this analysis can identify any adulteration [50]. The samples agree with the limit established by Brazilian legislation (a maximum 5%) [51].

The protein content values found in the samples showed no significant difference (Table 1). According to Bogdanov et al. [52], the content of protein in the composition that determines quality of the sample is above 0.7%. Therefore, compared with results from this work, the propolis samples were considered quality according to the literature.

The results for the lipid analysis showed that the red variety of propolis had 15.61%, which was 47.54 and 29.27% more lipid compared to the same analysis of the brown and green varieties, respectively. These values proved to be below the values found by Machado et al [5], for red propolis (65.74%) originating from Sergipe.

The fiber content values found in the samples showed no significant difference, consistent with previous results from the literature [5].

The variation found between the samples studied and with those from other studies, including the significant differences between the samples for the humidity, water activity and lipids, can be explained by the type of propolis, the flora of the region and the period of collection [50].

Determination of content for phenolic compounds, flavonoids and antioxidant activity of ethanolic and supercritical extraction

The results for the phenolic, flavonoid analysis and antioxidant capacity of the extracts from different samples of propolis obtained through conventional (ethanolic) methods and supercritical extraction are found in Table 2. The results showed significant differences (p>0.05) for the extracts analyzed (Table 2) when comparing the extraction method for the same sample, as well as for the extracts obtained by the same method and samples of different types.

The variations identified among the samples were already expected, considering that propolis of different types exhibit very different chemical profiles [51–52]. Furthermore, the method of extraction and solvent can change the chemical composition of propolis extract [53]. The results found in this study confirm the influence of the type and origin of the raw material [54], as well as the extraction method [55], in the composition and characteristics of the extracts. Serra Bonvehí and Ventura [56] investigated fifteen propolis samples from various botanic and geographic origins, verifying significant differences in their contents of polyphenols, flavonoids and active components.

Table 2. Determination of the content of total phenolics (mg EAG/g), flavonoids (mg EQ/g) and the antioxidant activity by DPPH (IC_{50}) of extracts of three different samples obtained by ethanolic (EtOH) and supercritical (SFE) extraction.

Samples	Phenolic compounds (mg EAG/g)	Flavonoids (mg EQ/g)	DPPH (IC_{50})
Brown EtOH	249.28±0.01[a]	29.67±0.01[a]	159.74±0.03[a]
Brown SCO_2	113.41±0.01[b]	102.02±0.01[b]	371.12±0.01[b]
Green EtOH	374.10±0.01[c]	131.69±0.01[c]	133.25±0.02[c]
Green SCO_2	174.31±0.02[d]	96.86±0.01[d]	263.92±0.02[d]
Red EtOH	481.59±0.02[e]	186.96±0.01[e]	89.90±0.02[e]
Red SCO_2	171.33±0.01[d]	103.30±0.09[b]	141.81±0.01[f]

EtOH–Extract obtained by ethanolic extraction; SCO_2: Extract obtained by supercritical extraction; IC_{50}: Lower values of IC_{50} indicate higher activity of radical elimination.

Statistical analysis: Values showing the same letter on the same column did not show significant difference ($p>0.05$) using the Tukey test at a 95% confidence level.

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The main chemical classes present in propolis are flavonoids, phenolics, and aromatic compounds [57]. The content of phenolic compounds varied from 113.41±0.01 (Brown SCO_2) to 481.59±0.02 mg EAG/g (Red EtOH), whereas the content of flavonoids varied from 29.67 ±0.01 (Brown EtOH) to 186.96±0.01 mg EQ/g (Red EtOH) among other samples, and the antioxidant capacity varied from 371.12±0.01 (Brown SCO_2) to 89.90±0.02 (Red EtOH) (IC_{50}).

For the major procedures analyzed, the ethanolic extraction yielded the best results. The ethanolic extraction of red propolis showed 48% more phenolic compounds compared to the brown propolis and 23.89% more than the green variety. Comparing the supercritical extractions with regards to phenolic compounds, the green propolis yielded 1.7% more compared to the red propolis and 34.9% more than the brown propolis.

The results found by Tei et al. [58], for five green propolis samples from Paraiba (Brazil) and five samples from Minas Gerais (Brazil) had 70.9% less phenolic compounds compared with the results found in this study.

Frozza et al. [59] demonstrated 68.53% less phenolic compounds in red propolis and Machado et al. [5], showed 13.61% less for brown propolis from Paraná (Brazil) extracted using the supercritical fluid extraction method.

The values identified in this study for the red and green samples appeared to be higher than the values found in the literature. These results are justified by the fact that the samples were from different origins [60].

Regarding the flavonoid analysis, the red propolis extracted by the ethanolic method indicated a difference of 84.13% more compared with the brown propolis extracted by the same method, while for the green propolis, the difference was 29.56%. Among the supercritical extracts, the total flavonoid content ranged from 1.24% (brown)– 6.23% (green) to red propolis.

The green sample tested in this present study had 64.46% more flavonoids compared with the results identify by Machado et al. [5] for green propolis originating from Minas Gerais extracted by the same method (Ethanolic extraction) and 74.17% more flavonoid compounds compared to the same sample extracted by supercritical extraction. Alencar et al. [61] also found lower values of flavonoid content for ethanolic extracts of red propolis from Sergipe.

Lower IC_{50} values indicated a higher radical scavenging activity; the brown and green propolis extracted by conventional methods demonstrated 77.68% and 48.22% less antioxidant activity when compared with the red propolis, respectively. In respect to the supercritical method, the green propolis showed 86.10% less antioxidant activity in relation to the red type. Frozza et al [59] found an IC_{50} value of 270.13 for red propolis from the northeast of Brazil,

showing that the red propolis studied required less mass to inhibit 50% of DPPH radical formation.

Comparing the results presented in Table 2 in relation to the extraction method, it is possible to notice a significant difference ($p>0.05$) between the values for the phenolic, flavonoid and antioxidant activity (DPPH), where the ethanol extraction presented the best results between the samples and in the samples of different types. These results demonstrate the importance of the extraction method in the composition of the extract.

Similar results were observed by Zordi et al. [62], who determined that the highest concentrations of antioxidant compounds from ethanolic extracts of Italian propolis were obtained, when compared with the extracts obtained by the SFE process under different conditions and using SCO_2. Machado et al., [5] and Silva et al. [40] also found higher values of total phenols and flavonoids in ethanolic extracts of Brazilian propolis, in relation to the supercritical extracts.

Considering the different types of processes used around the world to obtain propolis extracts, ethanol is the first choice of solvent, especially due to the affinity of its chemical characteristics with the matrix. Other solvents such as ethylic ether, water, methanol and chloroform can also be used for the extraction of specific classes of propolis constituents [62–63]. According to Biscaia et al. [64], low concentrations of flavonoid, phenolic, and antioxidant activity were shown in the extracts obtained by SFE (SO_2) and can be explained by the fact that unwanted substances such as resin, wax and other materials that are present in propolis in high concentrations can interfere with the biological potential of the extracts. The wax and other organic wastes are removed during the process of ethanolic extraction [65].

SFE extraction is currently an alternative to conventional processes, presenting numerous advantages. Although some studies show advantages in the use of SFE to obtain ecologically clean extracts and with greater biotechnological potential [17,66,67], in this study the conventional extraction was more efficient. Most polar phenolic compounds are practically insoluble in pure CO_2, but are sufficiently soluble in a CO_2+ethanol mixture or in a CO_2+ethanol+water mixture, allowing for their separation on the basis of molecular weights and polarity. Monroy et al. [55] used green propolis from southeastern Brazil to obtain extracts concentrated in phenolic compounds using supercritical carbon dioxide as an anti-solvent to selectively fractionate ethanolic and hydroalcoholic extracts of green propolis by precipitation in four separators in series.

In general, red propolis presented the best levels of antioxidant compounds, regardless of the extraction method used. Red propolis has been classified as a separate type based on its unique chemical composition, particularly rich in isoflavonoids [68]. Furthermore, ethanolic extraction was more efficient to obtain extracts with higher antioxidant capacity. Extraction with ethanol is particularly suitable to obtain dewaxed propolis extracts rich in polyphenol components [14,39].

Hatano et al. [69] also studied the red propolis (from Shandong–China). Extracts obtained by ethanolic extraction showed strong antioxidant activity. The total polyphenol content, the flavonoid content, DPPH radical scavenging activity values were 433.8 $mg.g^{-1}$ of extract, 129.6 $mg.g^{-1}$ of extract, and 98.8%, respectively.

Quantification of catechin, ferulic acid and luteolin in ethanolic and supercritical extracts by HPLC. The results regarding the quantitative analysis of catechin (polyphenol), ferulic acid/congeners (aromatic acid) and luteolin (flavonoid) are shown in Table 3. Those three compounds mentioned above were found in the ethanolic extracts of brown and green propolis at different concentrations. In the ethanolic sample of red propolis, only trans ferulic acid was found.

Table 3. Determination of the content of chatequin, trans ferulic acid and luteolin of red, green and brown propolis extracts obtained by ethanolic extraction (EtOH) and by SFE (SCO_2).

Samples	Chatequin (mg/g)	Trans feluric acid (mg/g)	Lutenoin (mg/g)
Brown EtOH	49.39	0.10	5.24
Brown SCO_2	<LD	<LD	<LD
Green EtOH	76.70	0.50	4.25
Green SCO_2	<LD	<LD	<LD
Red EtOH	<LD	0.60	<LD
Red SCO_2	<LD	<LD	<LD

EtOH–Extracts obtained by ethanolic extraction; SCO_2 –Extracts obtained by SFE (CO_2 as supercritical fluid); <LD: below detection levels.

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None of the compounds investigated were quantified for extracts obtained by extraction with supercritical fluid and the best extraction efficiency with ethanol was also demonstrated (Table 3). It is known that extraction method influences the obtained extract, and different extracts from the same propolis sample may exhibit dissimilar properties. The yield and selectivity for some compounds are directly affected by the extraction method [70–72].

Zordi et al., [62] indicated the use of supercritical CO_2 could be as a pre-treatment of the raw propolis to facilitate the additional extraction with ethanol. However, Machado et al., [39] showed the positive influence of the supercritical extraction to obtain two compounds in samples of Brazilian green propolis (Artepillin C and p-coumaric acid).

Regarding the supercritical extraction, all of the compounds analyzed were demonstrated to be below detection levels. Fig 1 shows the chromatogram of a green propolis samples obtained by ethanolic extraction.

The content of chatequin varied from 49.39 (Brown EtOH) to 76.70 (Green EtOH) mg/g. The trans ferulic acid amount varied from 0.109 (Brown EtOH) to 0.60 (Red EtOH), whereas the luteolin compound varied from 4.25 (Green EtOH) to 5.24 (Brown EtOH).

Compared with literature data, these compounds are commonly found in different types of propolis from various regions around the world (Table 4). Our results scientifically prove that the chemical composition (biological activities) of the different propolis around the world depends on the geoclimatic conditions and the botanical source of substrate (exudates/ pollen) that bees use for production of this material, differentiating the types of propolis and their chemical characteristics. The concentration of flavonoids, phenolic compounds, aromatics

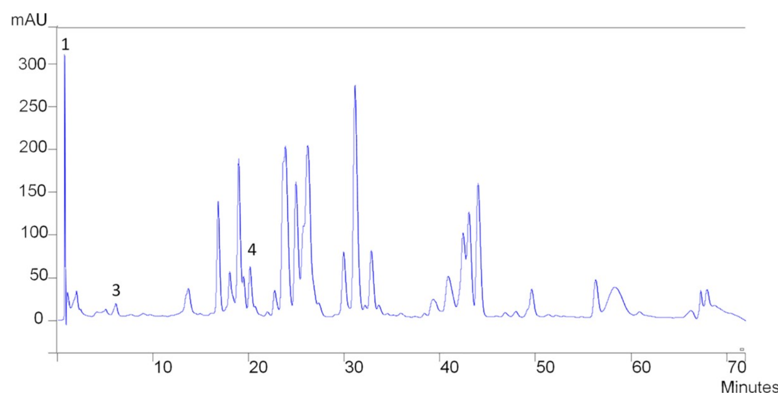

Fig 1. Chromatograms of green propolis ethanolic extract–(1) chatequin, (3) trans-ferulic acid and (4) luteolin.

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Table 4. Bibliographical review of recent studies on the quantification of the compounds catechin, ferric acid and luteolin in extracts of propolis from different regions.

Literature	Propolis	Origin	Chatequin	Trans ferulic acid	Luteolin	Method
Righi et al., [68]	Green/ Black	Brazil	+	+	+	HPLC/DAD/ESI/MS
Fernandes-Silva et al., [73]	Green	Brazil	-	+	-	CG-MS
Mendonça et al., [74]	Red	Brazil	+	+	-	HPLC/ LC-Orbitrap-FTMS
Hatano et al., [69]	Red	China	-	+	-	HPLC/PDA
Cao et al., [75]	-	China	-	+	+	Capillary electrophoresis system
Cui-ping et al., [76]	-	China	+	-	-	HPLC
Yang et al., [77]	-	China	-	-	+	LC-DAD
Hegazi and El Hady [78]	-	Egypt	-	+	-	GC/MS
Mohdaly et al., [79]	-	Egypt	+	+	+	HPLC
Kasiotis et al., [80]	-	Greece	+	+	+	HPLC-MS
Croci et al., [81]	-	Israel and Romenia	-	+	-	HPLC/DAD
Popova et al., [82]	-	Poland	-	+	-	GC/MS
Doganli [83]	-	Turkey	+	+	+	UPLC- ESI_MS/MS

Positive sign (+) means that the compound has been identified by the respective authors. Negative sign (-) means that the compound has not been identified by the respective authors.

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and even compounds not yet determined by the scientific community determine the characteristics of different propolis samples (Table 4). The amount (mg.g^{-1} of extract) observed in this study correlates with values observed in the literature, and the different concentration, presence or not of a compound in the propolis extracts (of different regions) reinforces the previously explained results.

Analysis of the antimicrobial activity of extracts

The results of the MIC determination for the different (EtOH and SCO_2) extracts of propolis (brown, green and red) tested are found in the Table 5.

The extracts demonstrated activity against the Gram-positive bacteria *S. aureus* (ATCC 29213) and the Gram-negative bacteria *E. coli* (ATCC 25922). *S. aureus* is a bacterium found in the skin of approximately 15% of human beings and is responsible for generating infections and food contamination. *E. coli* is a bacterium that lives naturally in the gut of humans and some animals but in large amounts can cause problems such as intestinal and urinary tract infections, especially in individuals consuming contaminated food or water [84].

The antimicrobial activity of the propolis were higher against Gram-positive bacteria because of the flavonoids and aromatic compounds. These chemical compounds supposedly

Table 5. Determination of MIC (μg.mL^{-1}) of the extracts from different samples of propolis obtained by ethanolic extraction (EtOH) and by supercritical extraction (SCO_2).

Samples	*Staphylococcus aureus* ATCC 29213	*Escherichia coli* ATCC 25922
Brown EtOH	800–400	1600–800
Brown SCO_2	1600–800	1600
Green EtOH	400–200	1600–400
Green SCO_2	800–400	1600
Red EtOH	200	400
Red SCO_2	400	800

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act on the structure of Gram-positive bacterial cell walls, but the mechanism of this action is still unknown [85–86].

Scientists believe that the reason for propolis showing lower antimicrobial activity against Gram-negative bacteria is because of the multi-layered structure and higher fat content of the cell wall, which may be more resistant to propolis extracts [84,87–89].

Comparing the extraction method, the ethanolic extracts demonstrated a better antimicrobial activity compared to the supercritical extracts. The ethanolic extracts also demonstrated a higher content of total phenolic acids and flavonoids as well as better antioxidant activity. Jug et al. [90] evaluated the antibacterial and antifungal efficiency of propolis extracts obtained by different extraction methods and determined that the ethanolic extract had the best antimicrobial potential.

The red propolis appeared to have the best antimicrobial activity *in vitro* for the two bacterial strains tested, compared with the brown or green propolis. The red extract also showed the highest value of phenolic and flavonoid compounds, which may be associated with the better antimicrobial activity showed by this extract.

The extracts from the different samples tested exhibited a higher activity against Gram-positive bacteria instead of Gram-negative bacteria, which showed resistance to propolis extract, as expected [90–93]. Alencar et al. [61] demonstrated the antimicrobial activity of ethanolic and chloroform extracts of Brazilian red propolis from Alagoas state, against *S. aureus* ATCC 25923 (with a MIC of 50–100 EtOH extract and a MIC of 200–400) and *Staphylococcus mutans* UA159.

Studies show that antimicrobial activity occurs due to the complex synergistic effects between phenolic acids and flavonoids compounds as well as their derivatives, which are all present in propolis [94–96].

The determination of the MIC was relevant for appraising the quality of the extracts and products based on propolis [2, 97–99].

Taken together, our results showed that the red and green propolis extracts had the best values regarding phenolics, flavonoids and antioxidant capacity, as well as antimicrobial activity.

Effects of propolis extract on murine macrophages infected with *Leishmania in vitro*

In the next set of experiments, we tested if exposure to propolis extract reduced the intracellular viability of *Leishmania (V.) braziliensis*. Macrophages are the main mammalian host cell defense against *Leishmania* infection [100]. Therefore, we evaluated the macrophage viability before investigating the leishmanicidal effects of propolis extract on murine macrophages *in vitro*. According to results determined (Fig 2), the cell viability was unaffected by each concentration of the ethanolic propolis extract tested, except for the 160 $\mu g.mL^{-1}$ concentration, as measured by Alamar Blue assay. This finding agrees with previous reports obtained using different propolis extracts [33].

The treatment for leishmaniasis disease can be hard and painful, such that many patients give up on the treatment. In order to find an alternative path for the treatment of that disease, researchers are using natural products to fight these parasites. Some studies on the *in vitro* bioactivity of propolis have been performed against *Leishmania* species. Propolis from different geographical origins, and types (brown, green and red), have already demonstrated activity against the *L. amazonensis*, *L. braziliensis*, *L. infantum* and *L. major* [101–109].

The leishmanicidal effect of the two (green and red) ethanolic extracts of propolis were measured in BALB/c macrophages infected with *L. (V.) braziliensis*. All propolis extracts were demonstrated to reduce *L. (V) braziliensis* burden in a dose-dependent manner (Fig 3).

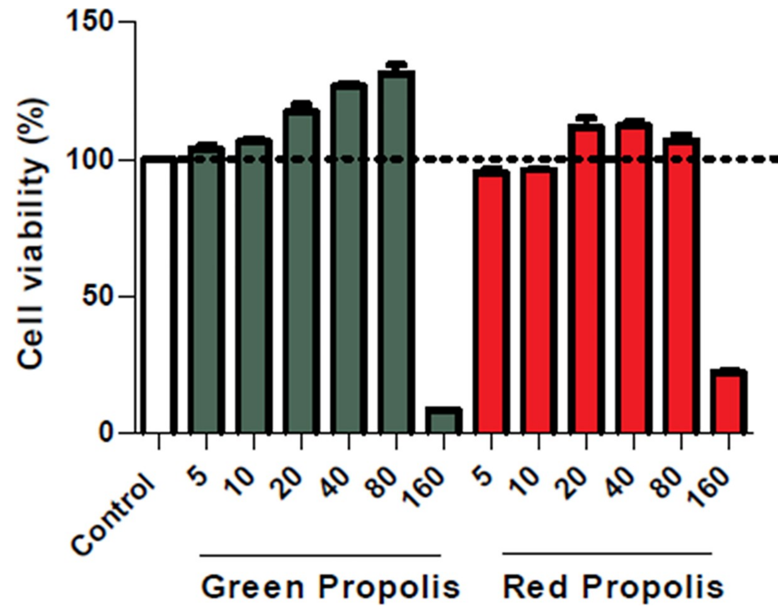

Fig 2. Cell cytotoxicity assessment by Alamar Blue. The data represents the viability of uninfected macrophages treated for 24 h with medium (Ctr) and the with the ethanolic extracts of propolis. The experiments were performed at least three times in quadruplicate for each experimental group. Data are shown as the mean +/- SD and are representative of three experiments.

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Rebouças-Silva et al. [33] demonstrated similar results with green propolis obtained from three different pharmaceutical preparations: dry, alcoholic, and glycolic extracts.

The red propolis extract demonstrated a better reduction when compared to the green extract, corroborating the results that red propolis had more effective antioxidant activity (Table 2). The 100 μg.mL^{-1} concentration showed almost the same effect as Amphotericin b, which was used as positive control. These results show that the red propolis from Bahia can be very cytotoxic against *L. (V) braziliensis*. Similar results were obtained by Santana et al. [69]

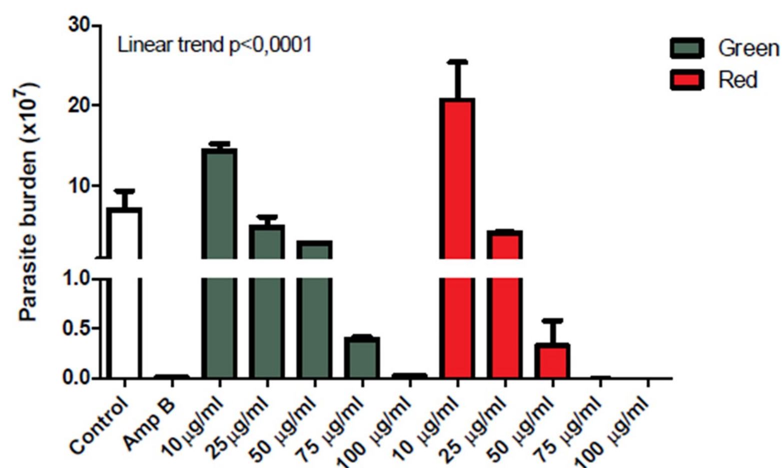

Fig 3. Reduction of the viability *L. braziliensis* promastigotes after treatment with extracts. Parasites were incubated with media alone or with ethanolic propolis extracts for 5 days. The viable parasites were counted daily with a Neubauer Chamber. The experiments were performed in quadruplicate for each experimental group (*p <0.05 and **p <0.01). Data are shown as mean +/- SD and are representative of two experiments.

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with brown hydroalcoholic extract of propolis from the semi-arid region of Piauí (Brazil). In another study with Cuban red propolis, the antiprotozoal property was evaluated and can be associated with the chemical composition. The samples showed 3.3–16.1 $\mu g.mL^{-1}$ against *L. infantum* [106].

Regueira-Neto et al. [104] evaluated the antileishmanial and cytotoxic activities of hydroethanolic red propolis samples collected from different Brazilian states (Pernambuco and Alagoas) and seasons whilst searching for possible activity differences. All extracts showed antileishmanial and cytotoxic activity. The propolis sample collected in Pernambuco during the rainy season showed to be more cytotoxic against protozoan parasites (*L. (V.) braziliensis* and *L. infantum*) and fibroblast cells and killed the parasites with lower concentrations than the sample collected in the dry season.

Ayres et al. [102] evaluated the effect of Brazilian red propolis gel (propain) alone or combined with glucantime on *L. amazonensis* infection. The red propolis containing high concentration of bioactive compounds (prenylated and benzophenones) showed to be the most active extract against *L. amazonensis*. Ethanolic extracts of propolis were capable to reduce parasite load as monitored by the percentage of infected macrophages and the number of intracellular parasites. The parasite load of macrophages was reduced by the extract (25 $\mu g.mL^{-1}$), presenting no direct toxic effects on promastigotes and extracellular amastigotes.

Several biological properties of propolis extracts have been widely investigated, and its antimicrobial activity was the most studied one. Besides, its anti-inflammatory, antitumor, antioxidant, antiprotozoal, immunomodulatory, radioprotective, regenerative (tissues), healing and anti-ulcerative activities and others have been investigated as well. Here, some properties were discussed.

The results of this work confirmed the antioxidant, antimicrobial and antiprotozoal properties of propolis extracts of various types collected in different regions in the state of Bahia (Northeastern Brazil).

It was also shown that the extraction method may influence the extraction of compounds present in propolis, and, consequently, in the biological activity of the extracts.

Conclusions

The wide diversity of the components present in raw material is evidenced by the characteristics of the raw material and the propolis extracts, and also by the compounds values obtained from two different methods. The results identified significant differences among the samples ($p>0.05$), which are in conformity with their place of origin. Ethanolic extraction was demonstrated to be the most efficient method for obtaining extracts with a high content of antioxidant compounds, such as phenolic and flavonoid compounds, which are associated with the biological potential of the propolis extract. Complex natural products such as propolis can result in different products, depending on the method used. Therefore, the viability of the process is related to the process yield and the product (extract) quality, in order to enhance the biological potential present in the raw material. Then, considering the advantages to achieve the concentration of biological active substances, the ethanolic extraction was best. Amongst the samples evaluated, the red propolis showed the higher biological potential, as well as the larger content of antioxidant compounds. These samples contained different constituents that may exert antimicrobial and antiprotozoal effects, which may be useful to the development of new drugs.

Supporting information

S1 Table. Identification of propolis samples from different regions of Bahia in Brazil analyzed in this study.
(DOCX)

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