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Chemical composition and biological activity of a new type of Brazilian propolis: Red propolis

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Abstract

Propolis has been used as a medicinal agent to treat infections and promote wound healing for centuries. The aim of the present study was to test the antimicrobial, antioxidant, and cytotoxic activities of a new type of Brazilian propolis, popularly called red propolis, as well as to analyze its chemical composition. The antimicrobial activity against *Staphylococcus aureus* ATCC 25923 and *Staphylococcus mutans* UA159 was evaluated and the chloroform fraction (Chlo-fr) was the most active with lower MIC ranging from 25 to 50 μg/ml. The hexane fraction (H-fr), having the highest concentration of total flavonoids, showed the best sequestrating activity for the free radical DPPH. The ethanolic extract of propolis (EEP) showed cytotoxic activity for the HeLa tumor cells with an IC_{50} of 7.45 μg/ml. When the EEP was analyzed by GC–MS, seven new compounds were found, among which four were isoflavones. Our results showed that the red propolis has biologically active compounds that had never been reported in other types of Brazilian propolis.

Keywords: Propolis; Antibacterial activity; Antioxidant activity; Cytotoxic activity; Chemical analysis; Isoflavones

1. Introduction

Propolis has been used in folk medicine for centuries. Pharmacological activities such as antimicrobial, anti-inflammatory (Khayyal et al., 1993; Park et al., 1998; Kujumgiev et al., 1999; Marcucci et al., 2001), anticariogenic (Koo et al., 2000a,b; Duarte et al., 2003, 2006; Hayacibara et al., 2005), anticarcinogenic and antioxidant (Burdock, 1998; Chen et al., 2003; Nagai et al., 2003; Aso et al., 2004; Ishikawa et al., 2004; Kumazawa et al., 2004) have been described. However, its chemical composition and pharmacological activity might vary widely from region to region (König, 1985; Greenaway et al., 1990; Park et al., 2002), and the medical applications of propolis have led to an increased interest in its chemical composition as well as its origin (Bankova et al., 1989; Park et al., 2002).

Brazilian propolis has been widely studied to elucidate its several biological properties. Thus, to date, 12 types of Brazilian propolis have been characterized and classified into types 1–12 (Park et al., 2000). These propolis varieties exhibited multiple inhibitory effects on critical virulence factors involved in the formation and development of mutans streptococci biofilm communities, such as cell adherence, GTFs activities, acid production, and F-ATPase activity (Koo et al., 1999, 2000a,b; Duarte et al., 2003, 2006).

A new type of Brazilian propolis, popularly known as "red propolis" demonstrated antioxidant and antimicrobial activities in preliminary *in vitro* assays in our laboratory, suggesting that further studies are needed to investigate its biological properties and chemical composition. Based on the wide use of propolis in folk medicine as treatment agent, our study was aimed at evaluating the biological activities of Brazilian red propolis ethanolic extract (EEP), its hexane (H-fr) and chloroform (Chlo-fr) fractions, as well its chemical composition.

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2. Material and methods

2.1. Propolis samples and fractionation

Samples of raw red propolis produced by *Apis mellifera* bees were collected in a northeastern region of Brazil, mangrove area, state of Alagoas, in March 2005, and classified as a new type of Brazilian propolis when compared to Park's classification (Park et al., 2000). The propolis sample (100 g) was extracted with ethanol 80% (v/v) (450 ml) in water bath, at 70 °C, for 30 min and then filtered to obtain its ethanolic extract (EEP). The EEP was further fractioned using a liquid–liquid extraction technique with hexane and chloroform solvents. The fractions obtained were monitored by thin layer chromatography (TLC), using the anisaldehyde reagent (4-methoxy-benzaldehyde, acetic acid, sulphuric acid/1.0:48.5:0.5), followed by incubation at 100 °C for 5 min. Fluorescent substances were visualized under UV light at the wavelengths of 254 and 366 nm (Tanaka et al., 2005). The ethanolic extract of propolis (EEP) and its hexanic (H-fr) and chloroform fractions (Chlo-fr) were concentrated in a rotaevaporator at 70 °C to obtain a yield of 58.2% (w/w), 11.1% (w/w) and 28.5% (w/w), respectively. The most polar fraction was submitted to the susceptibility test, as described below (Section 2.5), showing no biological activity.

2.2. Total polyphenol and flavonoid contents

Total polyphenol content in EEP, H-fr, and Chlo-fr was determined by the Folin–Ciocalteau colorimetric method (Singleton et al., 1999). Extract solutions (0.5 ml) were mixed with 2.5 ml of the Folin–Ciocalteau reagent (1:10) and 2.0 ml of 4% Na_2CO_3. Absorbance was measured at 740 nm after 2-h incubation at room temperature, in the dark. EEP and its fractions were evaluated at the final concentration of 90 μg/ml. Total polyphenol contents were expressed as mg/g (gallic acid equivalents).

Total flavonoid contents in the extracts were determined using a method described by Park et al. (1995), with minor modifications. For this, 0.5 ml of EEP, H-fr or Chlo-fr solution, 4.3 ml of 80% ethanol, 0.1 ml of 10% $Al(NO_3)_3$ and 0.1 ml of 1 M potassium acetate was added. After 40 min at room temperature, the absorbance was measured at 415 nm. EEP, H-fr and Chlo-fr were evaluated at the final concentration of 2 mg/ml. Total flavonoid contents were calculated as quercetin (mg/g) from a calibration curve.

2.3. Reversed-phase high performance liquid chromatography (RP-HPLC)

EEP, H-fr and Chlo-fr analyses were performed by RP-HPLC with a chromatograph equipped with Shimadzu ODS-A column (RP-18, column size 4.6 mm × 250 mm; particle size, 5 μm) and photodiode array detector (SPD-M10AVp, Shimadzu Co.). EEP and its fractions were dissolved in ethanol (10 mg/ml) and filtered with a 0.22 μm filter (Millipore) prior to 20 μl injected into the HPLC system. The column was eluted by using a linear gradient of water (solvent A) and methanol (solvent B), starting by 40% B and increasing to 60% B (45 min), held at 90% B (45–75 min), and decreasing to 30% B (75–85 min) with a solvent flow rate of 1 ml/min. Chromatograms were recorded at 260 nm as described by Park et al. (2004). The following authentic standards of phenolic acids and flavonoids (Extrasynthese Co.) were examined: ρ-coumaric, ferulic acid, cinnamic acid, gallic acid, quercetin, kaempferol, kaempferide, apigenin, isorhamnetin, rhamnetin, sakuranetin, isosakuranetin, hesperidin, hesperetin, pinocembrin, chrysin, acacetin, galangin, myricetin, tectochrysin and artepillin C.

2.4. Gas chromatography–mass spectrometry (GC–MS)

EEP analyses were performed after methylation of the extracts, as described by Markham et al. (1996). Aliquots of 400 μl (10 mg/ml) of EEP were placed into glass vials. A solution of CH_2N_2 (400 μl) was added to each of the sample solutions. Samples were refrigerated for 4 h to allow complete methylation. Samples of the methylated solutions were analyzed by GC–MS using a 30 m × 0.25 mm i.d. CBP5 column installed in a GC 17A (Shimadzu Co.) instrument interfaced with a QP 5000 mass selective detector operated in scanning mode (*m/z* 40–400). GC–MS analysis was temperature programmed from 50 °C (0.3 min hold) to 285 °C (15 min hold) at 6 °C/min. Samples were injected with an AOC-17 autoinjector using a splitless injection technique (0.6 μl injection volume). Carrier gas (He) flow was set at 1.0 ml/min. The GC–MS peaks were identified by comparison with data from the literature and the profiles from the Wiley 138 and Nist 98 libraries.

2.5. Susceptibility test

The antimicrobial activity of EEP, H-fr, and Chlo-fr was determined by their minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) against *Staphylococcus aureus* ATCC 25923 and *Streptococcus mutans* UA159, in accordance with the Clinical and Laboratory Standards Institute, CLSI (2007) guidelines and a technique described by Koo et al. (2000a). To determine MIC, the starting inoculum was $1–2 \times 10^8$ CFU/ml, and the test extract concentrations ranged from 12.5 to 1600 μg/ml. Ethanol (final concentration: 0.6%, v/v) was used as the control vehicle. MIC was defined as the lowest concentration of extracts that could inhibit bacterial growth (no visible growth) (Koo et al., 2000a). To determine MBC, an aliquot (50 μl) of all incubated tubes with concentrations higher than MIC was sub-cultured on BHI agar, supplemented with 5% defibrinated sheep blood, using a Spiral plater (Whittley Automatic Spiral Plater®). MBC was defined as the lowest concentration that allowed no visible bacterial growth in agar (Koo et al., 2000a). Triplicate of three separate experiments were conducted for each concentration of the extracts tested.

2.6. Free radical-scavenging activity on DPPH

The effect of DPPH radical-scavenging was evaluated in accordance with the method of Chen et al. (2003) with a slight modification. The assay mixture contained 0.5 ml of EEP, H-fr or Chlo-fr solution, 3.0 ml of ethanol P.A, and 0.3 ml of

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0.5 mM DPPH ethanolic solution. Absorbance was recorded at 517 nm after 40 min of incubation at room temperature, in the dark. Results were expressed as percentage decrease with respect to control values. EEP, H-fr and Chlo-fr samples were evaluated at final concentration of 90 μg/ml, and α-tocopherol and BHT at the same concentration were used as the reference samples.

2.7. Cytotoxic activity determination in vitro

The HeLa strain of tumor cells, cultivated in a DMEM (Dulbecco's Modified Eagle's Medium—Gibco/Life Technologies) culture medium, supplemented with 10% (v/v) of bovine fetal serum (Cultilab, Campinas, SP), was kept in an oven at 37 °C with a tension of 5% of CO_2 and 95% of O_2. Penicillin and streptomycin (Amresco) were used as antibiotics. After trypsinization, the cells were suspended in DMEM containing 10% (v/v) of bovine fetal serum and distributed onto 24-well plates at the density of 2.5×10^4 cells/well and incubated at 37 °C. After reaching 60–70% of confluence, they were exposed to the vehicle or to EEP at various concentrations (2–200 μg/ml) for a period of 24 or 48 h. After the exposure time, 100 μl of methylthiazolyltetrazolium chloride solution (MTT) (5 mg/ml in PBS) was added to the wells and the cells incubated for another 3 h. Culture medium was removed, each plate was washed twice with PBS, and 200 μl of isopropanol solution acidified in HCl 0.04 M was added. Cellular viability was determined by absorbance at 560 nm with reference to 655 nm (Mosmann, 1983). The data obtained in the cell proliferation and cytotoxicity assays were analyzed using the GraphPad Prism program (GraphPad Software, San Diego, CA).

3. Results and discussion

The chemical composition of propolis is dependant on its geographical location; as a result, its biological activity is closely related to the vegetation native to the site of collection (Park et al., 2002; Christov et al., 2005). The present study investigated a new type of Brazilian propolis, denominated red propolis, collected in northeastern Brazil. Its intense red color and chemical composition make it different from the 12 types of Brazilian propolis classified by Park et al. (2000).

The GC–MS analysis of the red propolis allowed the identification of 20 compounds (Table 1). The following compounds were identified for the first time in Brazilian propolis samples: methyl *o*-orsellinate (6), methyl abietate (11), 2,4,6-trimethylphenol (15), homopterocarpin (13), medicarpin (14), 4′,7-dimethoxy-2′-isoflavonol (16) and 7,4′-dihydroxyisoflavone (17). At least four isoflavones, never before reported in propolis, could be observed; such isoflavones as homopterocarpin (13), medicarpin (14) and 4′,7-dimethoxy-2′-isoflavonol (16) presented the most abundant compounds by the GC–MS technique (Fig. 1). These isoflavones have been reported as having antimicrobial, antifungal, anticancer and antioxidant activity (Soby et al., 1997; Militao et al., 2005, 2006; Wang et al., 2000; Rufer and Kulling, 2006). The isoflavones are typical compounds of the legume family and thus these compounds may be useful as chemical markers of this new type of Brazilian propolis.

Table 1
Compounds identified in Brazilian red propolis by GC–MS of EEP from Brazilian red propolis (RT: retention time, min)

Peak no.	RT	Compounds
1	9.88	Butanedioic acid, dimethyl ester
2	12.46	Hydroxy-butanedioic acid, dimethyl ester
3	15.31	*m*-Guaiacol
4	16.82	1-Methoxy-4-(1-propenyl)-benzene
5	19.67	Methyleugenol
6	21.27	methyl *o*-orsellinate
7	23.14	1,2,3-Trimethoxy-5-(2-propenyl)-benzene
8	24.21	Methoxyeugenol
9	30.26	Hexadecanoic acid, methyl ester
10	33.38	10-Octadecenoic acid, methyl ester
11	36.40	Methyl abietate
12	37.11	Benzoic acid
13	40.41	Homopterocarpin
14	41.39	Medicarpin
15	41.74	2,4,6-Trimethylphenol
16	43.79	4′,7-Dimethoxy-2′-isoflavonol
17	44.41	7,4′-Dihydroxyisoflavone
18	44.86	2*H*-1-Benzopyran-7-ol
19	45.66	2,2,6-Beta-trimethyl-bicyclo(4.3.0)non-9(1)-en-7.alpha.-ol
20	46.37	1,1,2-Trimethyl-3,5-bis(1-methylethenyl)-, (2.alpha., 3.alpha., 5.beta.)-cyclohexane

HPLC analysis was used to determine the chemical profile of EEP, H-fr and Chlo-fr, showing a complex chemical composition with various peaks at different retention times; however, H-fr presented few compounds and no similarity to EEP (Fig. 2). Only one flavonoid (quercetin), one isoflavone (daidzein), and one phenolic acid (ferulic acid) could be identified in EEP and Chlo-fr. No compound used as standard and commonly found in other types of propolis was identified, thus demonstrating that this really concerns a new type of Brazilian propolis.

When the chemical profile of red propolis (EEP) was compared with the 12 types of Brazilian propolis classified by Park et al. (2000), a profile consisting of substances of a more polar nature was clearly observed. In propolis samples from Venezuela originating from resins of *Clusia minor* and *Clusia major*, Tomás-Barberán et al. (1993) found isoprenylated benzophenones as the majority compounds. The chemical profile obtained by these authors through the HPLC technique was

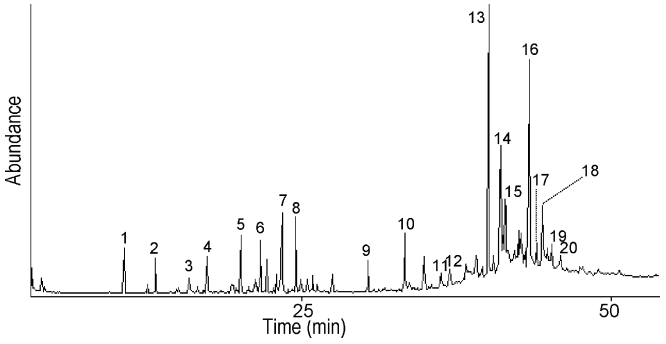

Fig. 1. GC–MS profile of EEP from Brazilian red propolis.

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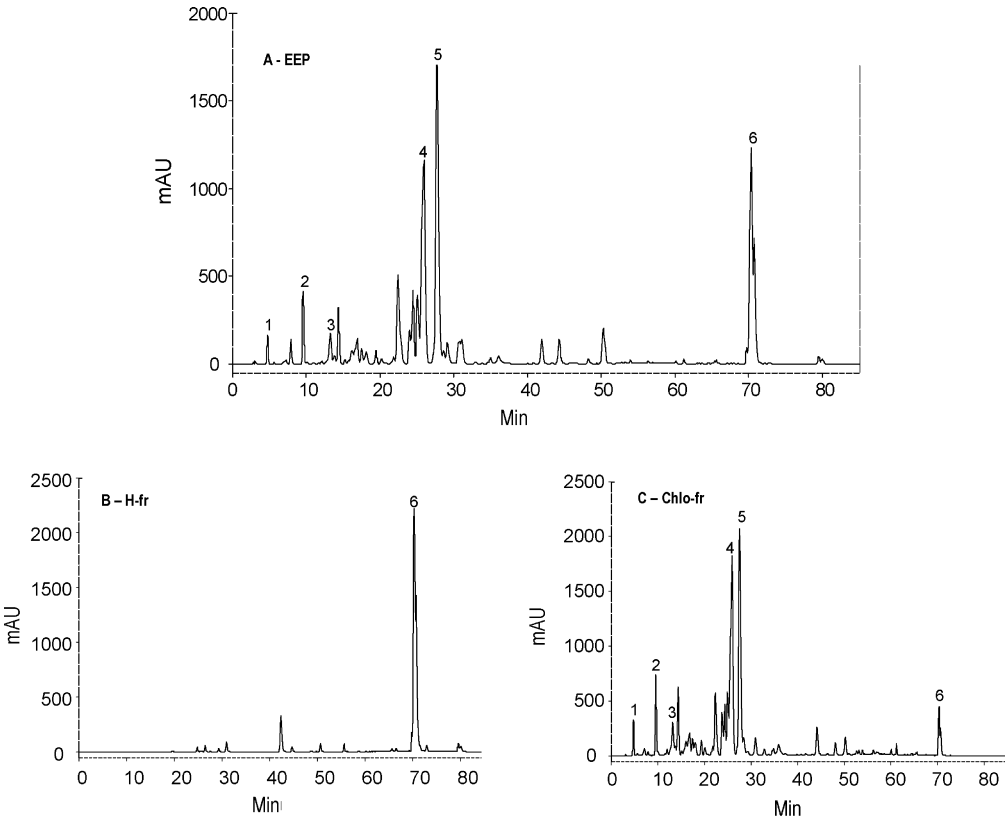

Fig. 2. HPLC chromatograms of ethanolic extract of red propolis and its fractions. (A) EEP; (B) H-fr; (C) Chlo-fr. 1, Ferulic acid (EEP = 7.4 mg/g; Chlo-fr = 9.1mg/g); 2, daidzein (EEP = 4.5 mg/g; Chlo-fr = 6.2 mg/g); 3, quercetin (EEP = 9.1 mg/g; Chlo-fr = 10.1mg/g); 4, UV λ 230, 242, 372 nm; RT = 26.2 min; 5, UV λ 243, 323 nm; RT = 27.8 min; 6, UV λ 227, 247, 359 nm; RT = 70.2 min.

composed of few compounds with low polarity, similar to those found in the type 6 propolis classified by Park et al. (2000). Based on these evidences, the botanical origin of Brazilian red propolis appears to be different from that of the Cuban red propolis, whose source is resin from *Clusia rosea*, rich in isoprenylated benzophenones (Hernandez et al., 2005). In the vicinity where the Brazilian red propolis was collected, 20 plants were found in a magrove area and considered as a probable source of resin for propolis production. However, only one plant showed resin with a profile similar to that of the red propolis, and it was identified as species of Leguminosae family (*Dalbergia ecastophyllum*), which might have resulted in isoflavones presence in this propolis. The Brazilian red propolis has a similar composition to that of a specific type of Cuban red propolis produced in the province of Pinar Del Rio, having no benzophenones, but having various isoflavones, such as medicarpin and homoterocarpin (Piccinelli et al., 2005). Trusheva et al. (2006) also observed the presence of isoflavonoids in red propolis. Nevertheless, Nepalese propolis has been reported as having various biologically active neoflavonoids, in addition to having such isoflavones as medicarpin and (+)−vesticarpan (Awale et al., 2005).

The total phenolic compound content found in EEP for red propolis (232 mg/g) (Table 2) was higher than that ever found for Brazilian propolis samples (Woisky and Salatino, 1998; Kumazawa et al., 2004). These values are similar to the phenolic compound concentrations present in temperate climate propolis originating from the species *Populus* sp., a resin-producing plant rich in polyphenols (Ahn et al., 2004; Kumazawa et al., 2004). The Chlo-fr presented a concentration of 324 mg/g, about twice as much as that found in H-fr, demonstrating the existence of phenolic compounds of different polarities in the red propolis tested (Table 2). The low flavonoid concentration observed in EEP and in Chlo-fr was similar to that normally found for Brazil-

Table 2
Values of total polyphenol, flavonoids, antioxidant activity of DPPH (%), MIC and MBC of EEP and fractions (hexanic-H-fr and chloroformic-Chlo-fr)

	EEP	H-fr	Chlo-fr
Total polyphenol (mg/g)[a,b]	232 ± 22.3	167 ± 2.2	324 ± 4.2
Flavonoids (mg/g)[a,c]	43 ± 1.0	158 ± 0.9	10 ± 0.6
Antioxidant activity of DPPH (%)[a,d]	57 ± 3.2	78 ± 1.3	55 ± 1.3
MIC[e]			
Staphylococcus aureus ATCC 25923	50–100	–	25–50
Staphylococcus mutans UA159	50–100	–	25–50
MBC[e]			
Staphylococcus aureus ATCC 25923	200–400	–	100–200
Staphylococcus mutans UA159	200–400	–	100–200

[a] Means ± S.D. ($n = 3$).
[b] Gallic acid equivalent.
[c] Quercetin equivalent.
[d] Standards: butylated hydroxytoluene (15.4% ± 2.5) and α-tocopherol (95.9% ± 0.5).
[e] The MIC and MBC are expressed in μg/ml; (–) absence of inhibition.

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ian green propolis (Kumazawa et al., 2004). The results obtained for H-fr showed that almost all the phenolic compounds present (167 mg/g) are compounds of the flavonoid class (158 mg/g) (Table 2), since they formed stable complexes with the aluminum cation and could be determined at 415 nm (Woisky and Salatino, 1998). A brown coloring (UV) and intense blue fluorescence (hot anisaldehyde solution) could be verified for H-fr using TCL plates, suggesting the presence of flavonoids in such fraction.

This new type of Brazilian propolis also demonstrated a notable antimicrobial activity against the microorganisms *Staphylococcus aureus* ATCC 25923 and *Staphylococcus mutans* UA159. The results obtained for MIC and MBC shown in Table 2 revealed potential antimicrobial activity against EEP and Chlo-fr. H-fr showed no antimicrobial activity and the MIC value for Chlo-fr was between 25 and 50 μg/ml for both microorganisms tested. As expected, the bactericide concentration for all the extracts was four times higher than MIC. Showing MIC values between 25 and 400 μg/ml, EEP and Chlo-fr demonstrated potent antibacterial activity at lower concentrations than those used in previous studies focusing on Brazilian propolis, reported as having strong antimicrobial activity (Koo et al., 2000a; Hayacibara et al., 2005). A previous *in vitro* study reported EEP obtained from the Brazilian red propolis as having potential antibacterial activity against *Staphylococcus mutans* Ingbritt 1600, with MIC ranging from 50 to 100 μg/ml (Castro et al., 2003). The best antimicrobial activity was found for the Chlo-fr showing the highest concentration of total polyphenol (324 mg/g).

The sequestrating activity of the free radical DPPH of EEP, H-fr and Chlo-fr are presented in Table 2. The sequestrating capacity model of the radical DPPH is a simple method of assessing the antioxidant activity of pure compounds, or in a mixture. The extracts used and the reference substances (BHT and α-tocopherol) were assessed at a final concentration of 90 μg/ml. As shown in Table 2, the H-fr demonstrated strong sequestrating activity of the free radical DPPH (78%), against 57% and 55% of EEP and Chlo-fr, respectively. The H-fr fraction was the one that presented the highest flavonoid contents, compounds recognized as having free radical sequestrating action (Furusawa et al., 2005). The Pearson correlation between antioxidant activity and flavonoids contents demonstrated a high positive correlation ($r = 0.98$). Many studies have reported antioxidant activity for flavonoids, which is due to their ability to reduce free radical formation and to scavenge free radicals (Rice-Evans et al., 1996; Pietta, 2000; Heim et al., 2002; Ahn et al., 2004). On the other hand, when a correlation between antioxidant activity and phenolic contents was analyzed, a higher negative value could be observed (−0.82), demonstrating that in case of this propolis the free radical scavengers was related to the flavonoid content. Kumazawa et al. (2004) verified the low sequestrating capacity of Brazilian propolis from the southeast of Brazil (around 40%), however, the sequestrating activity of a pure compound isolated from this propolis (artepillin C), was around 80%.

The *in vitro* cytotoxic activity of EEP for the HeLa tumoral cells presented an IC_{50} value of 7.45 μg/ml. Cuesta-Rubio et al. (2002) assessed the activity of isoprenylated benzophenone nemorosone isolated from Cuban propolis and found an IC_{50} of 1.6 μg/ml, using the same tumor strain as that tested in the present study. In accordance with the recommendations of the National Cancer Institute, USA, a product is considered active when the IC_{50} is attained with a concentration lower than 4 μg/ml (Cordell et al., 1993). These recommendations are, however, valid for pure compounds, and bioguided fractionation is needed to isolate and identify the active compounds of this new type of Brazilian propolis.

The Brazilian red propolis is a promising source of new bioactive compounds first reported in propolis. As the fractions were more active than the EEP, the biological properties of this propolis are not considered a synergic effect among the various compounds, suggesting the need for isolation and identification of the various bioactive compounds responsible for the antioxidant, antimicrobial and anticancer activities.

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