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Isolation and analysis of bioactive isoflavonoids and chalcone from a new type of Brazilian propolis

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ABSTRACT

Activity-directed fractionation and purification processes were employed to identify isoflavonoids with antioxidant and antimicrobial activities from Brazilian red propolis. Crude propolis was extracted with ethanol (80%, v/v) and fractioned by liquid–liquid extraction technique using hexane and chloroform. Since chloroform fraction showed strong antioxidant and antimicrobial activities it was purified and isolated using various chromatographic techniques. Comparing our spectral data (UV, NMR, and mass spectrometry) with values found in the literature, we identified two bioactive isoflavonoids (vestitol and neovestitol), together with one chalcone (isoliquiritigenin). Vestitol presented higher antioxidant activity against β-carotene consumption than neovestitol. The antimicrobial activity of these three compounds against *Staphylococcus aureus*, *Streptococcus mutans*, and *Actinomyces naeslundii* was evaluated and we concluded that isoliquiritigenin was the most active one with lower MIC, ranging from 15.6 to 62.5 μg/mL. Our results showed that Brazilian red propolis has biologically active isoflavonoids that may be used as a mild antioxidant and antimicrobial for food preservation.

1. Introduction

Flavonoids and the isomeric isoflavonoids are important constituents in vegetables used for human consumption [1,2]. Isoflavonoids are plant secondary metabolites with multiple biological and pharmacological effects, such as antioxidant, antibacterial, antiviral, anti-inflammatory, etc. [3,4]. They occur widely in legumes but have also been found in a considerable number of non-legumes. Reynaud et al. [5] listed 35 non-leguminous families that produce isoflavones to which [6] added an additional 17 families. According to recent epidemiological investigations, isoflavonoids abundant in certain plant foods are more beneficial for human health than flavonoids [7]. Dietary consumption of foods and food additives containing isoflavone phytoestrogens has been associated with several beneficial properties to human health, such as prevention of coronary heart disease and osteoporosis, reduction of menopausal symptoms, and prevention of distinct cancer forms (e.g. breast, prostate, and colon cancer) [8,9].

In our previous studies, we reported the crude extract antioxidant and antimicrobial activities, the botanical origin of Brazilian red propolis, as well as the presence of the isoflavones, suggesting new biological potentialities for this novel type of propolis [10–12]. Nevertheless, the presence of isoflavonoids has never been reported in other types of Brazilian propolis. Trusheva et al. [13] isolated phenolic compounds from Brazilian red propolis; however, the authors did not use the bioassay-guided fractionation and found only the isoflavonoids isosativan and medicarpin with antimicrobial and antioxidant activities.

Propolis, a natural resinous product collected by honeybees from buds and exudates of various plant sources, has been used empirically as a traditional remedy in folk medicine for centuries [14]. It is well known for its potential benefits to human health and for presenting valuable biological activities such as antioxidant [10,15], antibacterial [16], anticariogenic [17], anti-inflammatory [18], and anticancer [19] properties. It has recently gained popularity as a healthy food supplement and has been extensively used in foods and beverages in different parts of the world, where it is claimed to improve health and prevent ailments such as inflammation, heart diseases, diabetes, and even cancer [14].

The present study aimed at evaluating the antioxidant and antimicrobial activities of Brazilian red propolis extracts

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and fractions and, subsequently, isolating isoflavonoids through bioassay-guided fractionation techniques.

2. Materials and methods

2.1. Materials

Silica gel 60 and Brain Heart Infusion Agar (BHI) were purchased form Merck (Darmstadt, Germany); 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH), (±) α-tocopherol (VE), trans-β-carotene, Tween 40, and chlorhexidine from Sigma Co. (St. Louis, MO); linoleic acid from Agros Organics (Geel, Belgium); sodium carbonate, butylated hydroxytoluene (BHT), and butylated hydroxyanisole (BHA) standards from Synth (Diadema, SP, Brazil); resazurin from Sigma Co. (St. Louis, MO); and Sephadex LH-20 from Amersham Pharmacia (Uppsala, Sweden). All the solvents used for chromatography were of high performance liquid chromatography (HPLC) grade and all the other chemicals were of analytical-reagent grade.

A total of 11 crude propolis samples were obtained from colonies of *Apis mellifera* collected from March, 2007 to January, 2008, in an apiary located in the municipality of Marechal Deodoro, state of Alagoas, in the Northern Region of Brazil. The samples with the same chemical profile by HPLC were cleaned, ground after addition of liquid nitrogen, weighed, mixed, and stored at −18 °C until analysis.

2.2. Extraction and isolation of bioactive compounds

The representative propolis sample (100 g) was extracted with ethanol 80% (450 mL) in water bath at 70 °C for 30 min and after filtration yielded the ethanolic extract of propolis (EEP). The EEP was further fractioned by liquid–liquid extraction with hexane and chloroform yielding 11.4 g of hexanic fraction (Hex-fr) and 30 g of chloroform fraction (Chlo-fr), respectively. The active Chlo-fr (30 g) was subjected to open dry column chromatography on normal phase silica gel (particle size: 0.0063–0.2 mm; pore size: 60 Å; pore volume: ~0.8 cm^3/g; and specific surface area: 500 m^2/g) and eluted with a solvent mixture of chloroform/ethyl acetate (70:30, v/v) to afford seven major subfractions. The subfractions 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 ultra violet (UV) light at the wavelengths of 254 nm and 366 nm [10]. Subfractions 1, 6, and 7 showed no activity and negligible activities were found in subfractions 2, 4, and 5. The most bioactive subfraction (3) was chromatographed over a Sephadex LH-20 column (5 cm × 30 cm) using methanol to yield three bioactive subfractions. Active subfractions 3.2 and 3.3 were purified by semi-preparative reverse-phase HPLC [Shimadzu PREP-ODS (H) 250 × 20 mm column eluted with a gradient starting with CH_3OH:H_2O (65:35) to CH_3OH:H_2O (95:5) in 35 min, flow rate 3 mL/min] and yielded three active compounds.

2.3. Chromatography with mass spectrometry (GC–MS)

Aliquots of 400 μL (10 mg/mL) of each compound were transferred to vials, received 1 mL of CH_2N_2 solution for methylation, and were maintained in ice bath for 4 h to complete the methylation reaction. Samples of the methylated solutions were analyzed by GC–MS using a capillary column CBP5 (30 m × 0.25 mm × 0.25 μm) installed in a Hewlett-Packard 5890 Series II instrument interfaced with a HP-5971 mass selective detector operated in scanning mode (*m*/*z* 40–400). GC–MS analysis was previously programmed from 50 °C (0.3 min hold) to 285 °C (15 min hold) at a rate of 6 °C/min. Samples were injected with an auto injector using a splitless injection technique (0.6 μL injection volume) and the 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 [12].

2.4. Nuclear magnetic resonance (NMR)

The NMR spectra, obtained in CD_3OD using TMS as internal standard, were recorded in a Brucker DPX 500 MHz spectrometer, operating at 500 MHz for ^1H. The chemical shifts are expressed in δ (parts per million) and the coupling constants (*J*) in Hz.

All the compounds, already described in the literature, were identified by comparison of their spectral data (UV, nuclear magnetic resonance – NMR, and mass spectrometry) with reported values.

Vestitol (**1**): UV_{max} (EtOH): 208, 280 (sh); EIMS (70 eV) *m*/*z* (rel. int.): 300 [M^+], 272 (38), 150 (100), 137 (32), 123 (11). ^1H- and ^{13}C-NMR data were consistent with those previously reported [20].

Neovestitol (**2**): UVmax (EtOH): 211, (sh); EIMS (70 eV) *m*/*z* (rel. int.): 300 [M^+], 272 (38), 150 (16,7), 137 (100), 121 (15). ^1H- and ^{13}C-NMR data were consistent with those previously reported [21].

Isoliquiritigenin (**3**): ^1H- and ^{13}C-NMR data were in agreement with the reported literature values [22].

2.5. Antioxidant activity on linoleic acid oxidation

This analysis was carried out using the method of [23] with some modifications. We dissolved 10 mg of β-carotene in 100 mL of chloroform, an aliquot of 3 mL was added to 40 mg of linoleic acid and 400 mg of Tween 40, and the chloroform was removed under a stream of nitrogen gas. Oxygenated distilled water (100 mL) was added and thoroughly mixed. Aliquots of 3 mL of the β-carotene/linoleic acid emulsion were mixed with 50 μL of test samples and incubated in water bath at 50 °C. The antioxidant activity was expressed as percent inhibition relative to the control after 120 min incubation using the equation:

$$AA = \frac{[(DR_{control} - DR_{sample})]}{DR_{control}} \times 100$$

AA = antioxidant activity; $DR_{control}$ = degradation rate of β-carotene without addition of antioxidant; DR_{sample} = degradation rate of β-carotene with addition of antioxidant.

The degradation rates were calculated according to first order kinetics:

$$DR = \text{natural log}\left(\frac{A_t}{A_x}\right) \times \left(\frac{1}{t}\right)$$

A_t = initial absorbance at 470 nm and $t = 0$; A_x = absorbance at 470 nm and $t = 120$ min.

Test samples were evaluated at the final concentration of 90 μg/mL and VE and BHT were used as the reference samples. All analyses were carried out in triplicate.

2.6. Antimicrobial activity

The antimicrobial activity was determined by minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) in accordance with the Clinical and Laboratory Standards Institute [24], using the following bacterial strains: *Staphylococcus aureus ATCC 25923, Streptococcus mutans* Ingbritt 1600, and *Actinomyces naeslundii ATCC 12104*. To determine MIC, the starting inoculum was 5×10^5 CFU/mL. The technique was developed in 96-well microplates, which received 190 μL of BHI broth previously inoculated and 10 μL of the samples in concentrations ranging from 500 to 3.9 μg/mL (serial dilution of reason 2). We

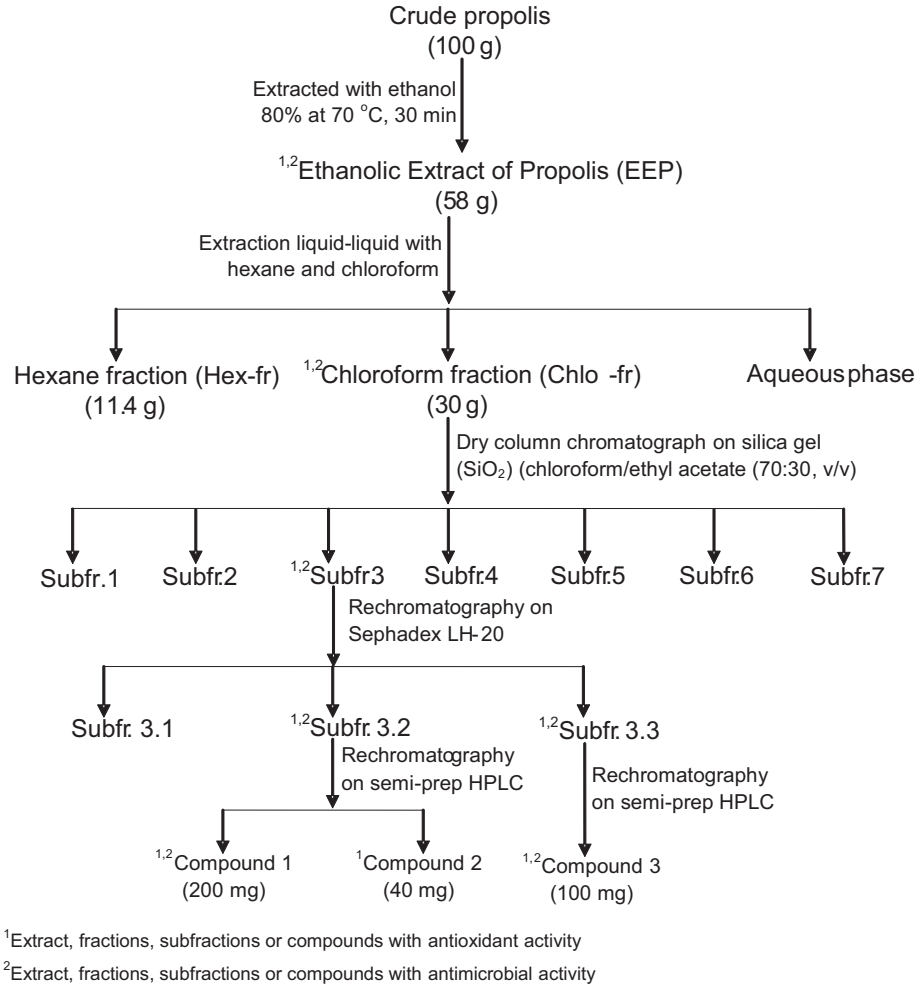

Fig. 1. Procedure for bioguided isolation of compounds from Brazilian red propolis.

used chlorhexidine 0.12% (w/v) as positive control and 80% ethanol (v/v), employed to solubilize the samples, as negative control. The microplates were incubated at 37 °C for 24 h and, after incubation, 30 μL of resazurin (0.01%, w/v) was added to identify the wells presenting bacterial growth. The wells presenting no changes in color were considered free of viable bacteria and those presenting changes in color were considered positive for bacterial growth.

To determine MBC, aliquots of 10 μL of all incubated wells presenting concentrations higher than the MIC were subcultured on BHI agar. The MBC was defined as the lowest concentration that allowed no visible growth on the agar. All the analyses were performed in six replicates.

3. Results and discussion

In this study, the antimicrobial and antioxidant activities of the EEP, Hex-fr, Chlo-fr, and isolated compounds from Brazilian red propolis were investigated. Bioguided purification of the EEP using a series of chromatographic separations (Fig. 1) resulted in the isolation of three compounds: 200 mg of vestitol (**1**), 40 mg of neovestitol (**2**), and 100 mg of isoliquiritigenin (**3**) (Fig. 2). The structures of the compounds were identified by their spectroscopic data (^{1}H NMR, ^{13}C NMR, UV, and mass spectrometry) measurement and by comparison with published values. The structure of these compounds has never been reported in other 12 types of Brazilian propolis [25].

3.1. Antioxidant activity

The first goal of this work was to detect and isolate compounds from Brazilian red propolis with potential antioxidant capacity. We performed analyses for the detection of antioxidant activity at every step of isolation of compounds from the EEP. The analyses using the β-carotene–linoleic acid model system led us to the three purified compounds (vestitol, neovestitol, and isoliquiritigenin) that were obtained from subfractions presenting antioxidant activity (Fig. 2).

The β-carotene–linoleic acid method has been considered an efficient technique to detect and evaluate antioxidants from propolis and other natural sources by several investigative groups [23,26,27]. The consumption of β-carotene is related to the thermally induced formation of linoleic acid hydroperoxides. After the isolation and identification of the three flavonoids compounds, a comparison of their activities in the β-carotene–linoleic acid system was performed. Their antioxidant activity was compared with that of the EEP, fractions, BHT, and vitamin E, a major natural lipid-soluble antioxidant [28].

The highest antioxidant effect was observed for vitamin E and BHT (Fig. 3), and Chlo-fr presented 57% activity, which was higher than that found for the EEP (26%). This results demonstrate that the liquid–liquid extraction technique yielded a higher concentration of compounds presenting antioxidant activity in the Chlo-fr than in the Hex-fr. Vestitol presented higher antioxidant activity (39.5%) than the EEP (26%), neovestitol (21.4%), and isoliquiritigenin (8.7%),

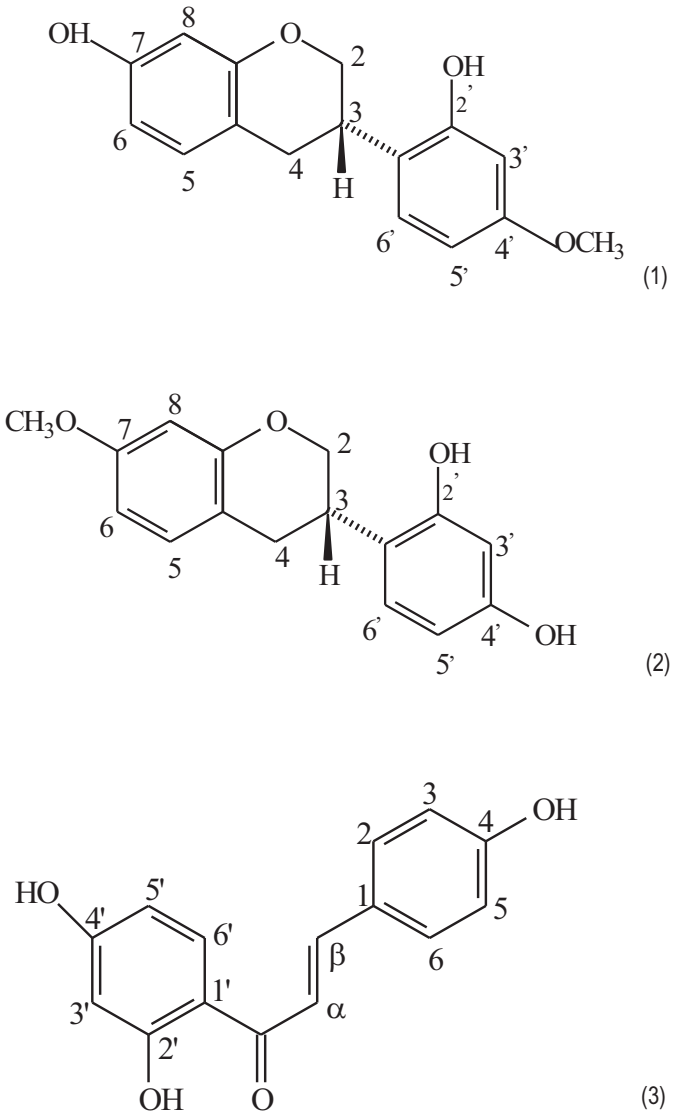

Fig. 2. Structures of the bioactive compounds isolated from Brazilian red propolis: (1) vestitol, (2) neovestitol, and (3) isoliquiritigenin.

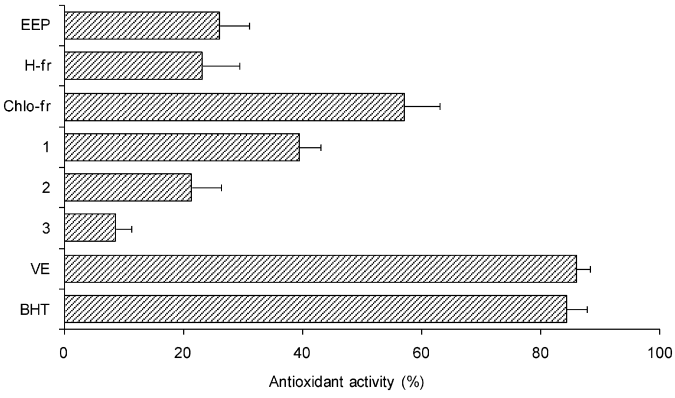

Fig. 3. Antioxidant activity of the ethanolic extract of propolis (EEP), hexanic fraction (Hex-fr), chloroform fraction (Chlo-fr), vestitol (1), neovestitol (2), isoliquiritigenin (3), α-tocopherol (VE), and butylated hydroxytoluene (BHT). Measurements were performed in triplicate and the standard deviations are indicated.

but all of them are highly important antioxidant compounds found in the EEP. In fact, isoflavonoids have been considered the most abundant and effective antioxidant compounds found in soybean [27]. The confirmed occurrence of isoflavonoids in this novel type of Brazilian propolis also suggests the presence of other biological activities in addition to the antioxidant activity detected in this study.

Ahn et al. [30] reported the antioxidant activity of propolis from various areas of Korea. The EEP from Cheongju presented stronger antioxidant activity (~40%) than those from other regions. EEP from Muju also had high antioxidant activity (~28%) evaluated by β-carotene–linoleic acid system. In the study mentioned, the contents of both total polyphenol and flavonoid were high, indicating a correlation between total polyphenol content and antioxidant activity. Flavonoids have been reported to be the most abundant and most effective antioxidant in propolis [31–33]. In our study, two isoflavonoids and one chalcone were important compounds responsible for the antioxidant activity in Brazilian red propolis.

3.2. Antimicrobial activity

The antimicrobial activity of EEP, fractions and compounds isolated were tested against *S. aureus* ATCC 25923, *S. mutans* Ingbritt 1600, and *A. naeslundii* ATCC 12104 (Table 1). Chlo-fr presented MIC values ranging from 31.2 to 62.5 μg/mL for *S. aureus* and from 62.5 to 125 μg/mL for *S. mutans* and *A. naeslundii*, the latter ones lower than those found for the Hex-fr and EEP. As to MBC, Chlo-fr was as effective as the EEP for *S. aureus* and *S. mutans*, and for *A. naeslundii* it proved to be more bioactive than the EEP, presenting values ranging from 62.5 to 125 μg/mL.

These results are similar to those found by [10], when evaluating MIC and MBC of red propolis collected in the state of Alagoas and its Hex-fr and Chlo-fr using *S. aureus* ATCC 25923 and *S. mutans* UA159. The authors attributed the high antimicrobial activity of red propolis and its Chlo-fr to their high content in phenolic compounds, respectively 232 mg/g and 324 mg/g, the highest values ever found in Brazilian propolis samples [10,29]. In this work we were able to isolate and identify the phenolic compounds responsible for antimicrobial activity.

Chlo-fr was found to be the principal source of bioactive compounds during the bioguided fractionation and isolation process since it presented the best antibacterial activity among the fractions. After isolation, we observed that two compounds showed strong activity against all bacterial strains tested (Table 1). Vestitol presented MIC ranging from 31.2 to 62.5 μg/mL, showing no distinction among the microorganisms assessed. Isoliquiritigenin was more potent than vestitol and exhibited MIC values ranging from 15.6 to 31.2 μg/mL for the three bacterial strains used.

Isoliquiritigenin presented the best MBC values, which ranged from 31.2 to 62.5 μg/mL for *S. aureus* and *S. mutans*. No isolated compound showed antibacterial effect against *A. naeslundii*. As reported by [34], the strong antibacterial activity of propolis may be due to the synergistic effect among flavonoids, hydroxy-acids, and sesquiterpens. Therefore, in the present study we also evaluated the synergistic effect among the isolated compounds. The solution was prepared by mixing vestitol and isoliquiritigenin at a concentration of 1:1 and the final concentration in the tests ranged from 125 to 1.8 μg/mL.

Although the mixture of the isolated compounds presented good antibacterial activity, with MIC ranging from 31.2 to 62.5 μg/mL for *S. aureus* and *A. naeslundii* and from 62.5 to 125 μg/mL for *S. mutans*, this effect was not as strong as that shown by the pure isolated compounds (Table 1). Furthermore, when applied in mixture, the compounds annulled the antibacterial activity presented by each of

Table 1
Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the ethanolic extract of propolis (EEP), hexanic fraction (Hex-fr), chloroform fraction (Chlo-fr), and bioactive compounds isolated from Brazilian red propolis.

Treatment	*S. aureus*		*S. mutans*		*A. naeslundii*	
	MIC (μg/mL)	MBC (μg/mL)	MIC (μg/mL)	MBC (μg/mL)	MIC (μg/mL)	MBC (μg/mL)
EEP	62.5–125	250–500	62.5–125	250–500	125–250	125–250
Hex-fr	62.5–125	250–500	125–250	500–1000	250–500	500–1000
Chlo-fr	31.2–62.5	250–500	62.5–125	250–500	62.5–125	62.5–125
Vestitol (1)	31.2–62.5	62.5–125	31.2–62.5	125–250	31.2–62.5	nd
Isoliquiritigenin (3)	15.6–31.2	31.2–62.5	15.6–31.2	31.2–62.5	15.6–31.2	nd
1 + 3 [1:1]	31.2–62.5	nd	62.5–125	nd	31.2–62.5	nd

nd: Activity not detected at the concentration tested. Neovestitiol (2) did not show antimicrobial activity.

them separately. This demonstrates that these compounds are not able to potentialize each other's activity for microbial control, since their antibacterial activity was lower when they were employed together than when they were tested separately.

Koo et al. [16] analyzed MIC and MBC of 30 compounds from Brazilian propolis against *S. mutans* GS-5 and UA159 as well as *Streptococcus sobrinus* 6715. All the flavanones tested inhibited bacterial growth and, among them, pinocembrine proved to be the most efficient compound (MIC 64 μg/mL), although pinobanksin-3-acetate also inhibited the growth of the bacterial strains tested (MIC 157 μg/mL). The concentrations reported by those authors are higher than the ones we found in this study for the flavonoids isolated from Brazilian red propolis that inhibited the growth of *S. mutans* (Table 1). Among all the compounds evaluated by [16], *tt*-farnesol was the most efficient (MIC 28 μg/mL), presenting antibacterial activity similar to that observed for the isoliquiritigenin isolated from Brazilian red propolis reported herein, which exhibited MIC ranging from 15.6 to 31.2 μg/mL.

Pepeljnjak and Kosalec [35] assessed the antibacterial activity of galangin isolated from three samples of propolis collected in Croatia against resistant strains of *S. aureus*, *Enterococcus* spp. and *Pseudomonas aeruginosa* and found MIC values ranging from 1600 to 2400 μg/mL. The high concentration of galangin necessary to inhibit bacterial growth was already expected by the authors since the strains employed showed resistance to several types of antibiotics.

Melliou et al. [36] evaluated the antibacterial effect of α-pinene, a compound found in large amounts in propolis collected in Greece, against *S. aureus* ATCC 25923. Although this compound is widely known for its strong antibacterial activity [29], it presented MIC value of 6500 μg/mL. This concentration was significantly higher than that required to inhibit the growth of *S. aureus* using vestitol and isoliquiritigenin isolated from Brazilian red propolis. In fact, isoflavonoids and chalcones, possess several biological properties such as marked antimicrobial activity [4].

4. Conclusions

The bio-assay guided isolation of active compounds from Brazilian red propolis resulted in the identification of two isoflavonoids (vestitol and neovestitol) and one chalcone (isoliquiritigenin). We assessed the activity of the isolated compounds, by measuring their ability to inhibit β-carotene consumption, and observed that vestitol is a potent antioxidant. Furthermore, isoliquiritigenin presented the most active antimicrobial activity among the compounds tested with no synergistic effect.

The identification of these bioactive isoflavonoids in Brazilian red propolis is important to improve the development and production of this natural product, and in the future, may be used as a mild antioxidant and antimicrobial for food preservation.

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