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Anti-Inflammatory and Antimicrobial Evaluation of Neovestitol and Vestitol Isolated from Brazilian Red Propolis

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ABSTRACT: The objective of this study was to evaluate anti-inflammatory and antimicrobial activities of neovestitol and vestitol isolated from Brazilian red propolis (BRP). BRP ethanolic extract (EEP), neovestitol, and vestitol were evaluated by anti-inflammatory properties using a neutrophil migration assay. The antimicrobial activity was evaluated by minimal inhibitory and bactericidal concentrations (MIC and MBC) against *Streptococcus mutans*, *Streptococcus sobrinus*, *Staphylococcus aureus*, and *Actinomyces naeslundii*. Neovestitol, vestitol, and EEP inhibited neutrophil migration at a dose of 10 mg/kg. Regarding antimicrobial activity, neovestitol showed MICs ranging from <6.25 to 25−50 μg/mL and MBCs ranging from 25−50 to 50−100 μg/mL, while vestitol showed MICs ranging from 25−50 to 50−100 μg/mL and MBCs ranging from 25−50 to 50−100 μg/mL. Both isoflavonoids neovestitol and vestitol are consistent bioactive compounds displaying anti-inflammatory and antimicrobial activities that can strongly act in a low dose and concentration and have a promising potential to be applied in the pharmaceutical and food industries.

KEYWORDS: *anti-inflammatory, antimicrobial, isoflavonoids, neovestitol, vestitol, red propolis*

INTRODUCTION

Brazilian propolis has attracted scientific interest due to the discovery of new drugs, such as CAPE (caffeic acid phenyl ester),[1] apigenin, *tt*-farnesol,[2,3] and artepillin C (3,5-diprenyl-4-hydroxycinnamic acid), as well as more than 250 patents related to its applications and therapeutic and nutrition uses.[4] Since the chemical composition of propolis is dependent on vegetation around the beehive, Brazilian propolis was classified into 13 different types according to its physical-chemical properties and geographical location.[5,6] The most recent one, red propolis, was classified as type 13 on the basis of its unique chemical composition, particularly rich in isoflavonoids. Recently, its botanical origin was determined as *Dalbergia ecatosphyllum*, from the Leguminosae family,[6] which is known for its high content of isoflavonoids, with particular interest in neovestitol and vestitol for antioxidant activity.[7]

Recent studies have shown that red propolis contains elevated amounts of a certain type of isoflavonoids, i.e., isoflavones. This particular group of polyphenols exhibits a wide range of biological properties, including antiviral, antimicrobial, anti-inflammatory, and even anticancer activities. Furthermore, isoflavonoids have been used as food additives due to low toxicity and also as a health food supplement.[8,9]

Although a previous study has shown the biological activity of crude extracts of red propolis,[10] these authors did not find antimicrobial activity for neovestitol because the yield was insufficient, so this research activity was neglected at that time. Whether the isoflavonoids are associated with bioactivity remains to be elucidated because other studies concerning red propolis, such as those by Piccinelli et al.[11] and Righi et al.,[12] did not correlate the isolated isoflavonoids with biological activity. Moreover, it is important to note that there are no reports in the literature about anti-inflammatory properties of Brazilian red propolis or its bioactive compounds neovestitol and vestitol. As isoflavonoids have recognized pharmacological activities,[13−18] we hypothesize that the specific isoflavonoids neovestitol and vestitol present in this distinctive propolis are associated with antimicrobial and anti-inflammatory effects.

Therefore, the aim of this study was to evaluate the anti-inflammatory and antimicrobial activities of neovestitol and vestitol isolated from Brazilian red propolis.

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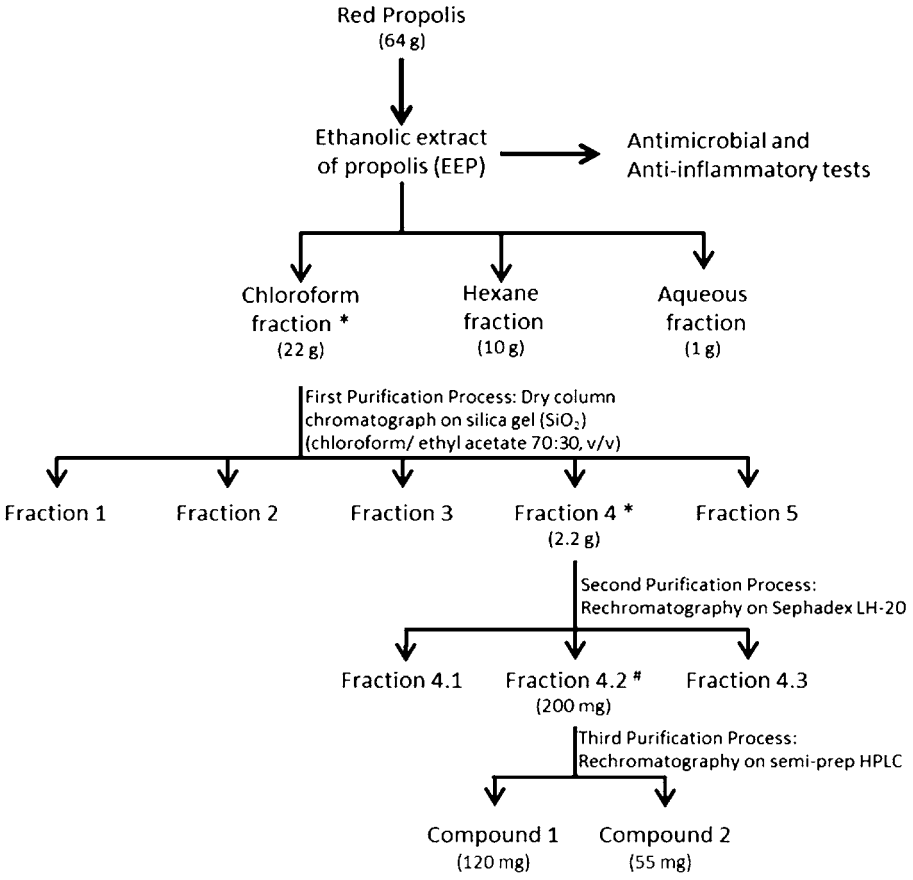

Figure 1. Bioassay-guided fractionation focused on identification of antimicrobial and anti-inflammatory compounds. Antimicrobial tests: MIC and MBC against *Strep. mutans, Strep. sobrinus, Staph. aureus,* and *A. naeslundii*. Anti-inflammatory test: evaluation of neutrophil migration. Key: *, best antimicrobial and anti-inflammatory activities when compared with others from the same line; #, easier to dissolve when compared with others from the same line.

MATERIALS AND METHODS

Brazilian red propolis samples were collected from Maceio, Alagoas State, northeast of Brazil, 9°40′ S, 35°41′ W, at the end of summer, during the month of March. Three samples from three different boxes (beehives) were collected to be used in this study.

Extraction and Isolation of Bioactive Compounds. Propolis was ground to a fine powder, and 2 g (dry weight) was mixed with 25 mL of 80% (v/v) ethanol and shaken at 70 °C for 30 min. After extraction, the mixture was centrifuged, and the supernatant was evaporated under low pressure to produce the ethanolic extract of propolis (EEP).[6]

A bioassay-guided fractionation was designed to focus on the isolation and identification of compounds with anti-inflammatory and antimicrobial activities[19] as shown in Figure 1.

On the basis of our previous results, reported by Oldoni et al.,[10] we modified the extraction method to obtain a larger amount of neovestitol and vestitol. These results include the biological inactivity of fractions from the beginning of the open dry column which led us to work only with the second half of the column (the last 10 cm length). Furthermore, the division criteria to obtain the fractions were not only by color but also per size, again different from method of Oldoni et al.[10] The maximum length of one fraction was 2 cm. If the fraction (with the same color) was longer than 2 cm, we divided it into two different fractions. Therefore, the maximum volume of each fraction was 3.5 cm^3.

The fractions obtained were monitored by thin-layer chromatography (TLC) using the anisaldehyde reagent (4-methoxybenzaldehyde, acetic acid, sulfuric acid, 1.0:48.5:0.5), followed by heating at 100 °C for 5 min. Fluorescent substances were visualized under ultraviolet (UV) light at wavelengths of 254 and 366 nm.

In order to select the bioactive subfraction and, consequently, the bioactive compounds neovestitol and vestitol, we focused on isolating the red-orange band (in TLC). Subfractions 1 and 5 showed no biological activity, and negligible biological activities were found in fractions 2 and 3. The most bioactive fraction (4) was chromatographed over a Sephadex LH-20 column (5 × 30 cm) using methanol to yield three bioactive fractions. Fractions 4.2 and 4.3 were active; however, fraction 4.3 was rejected because it was impossible to dissolve in buffer solution (AB buffer and phosphate buffer) routinely used to perform anti-inflammatory and antimicrobial tests. Thus, fraction 4.2 (200 mg) was purified by semipreparative reversed-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 two active compounds.

Identification of Bioactive Compounds. Neovestitol and vestitol were identified using high-resolution mass spectrometry (Q-TOF-MS/MS) and nuclear magnetic resonance (NMR) based on the findings of Oldoni et al.[10] The isolated neovestitol and vestitol were highly purified (<99.8% pure). The chemical structures are shown in Figure 2.

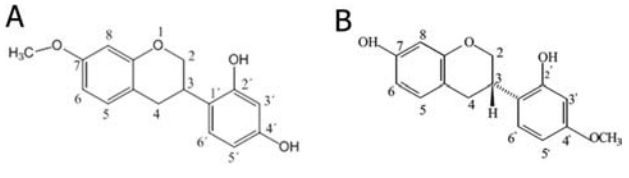

Figure 2. Chemical structures of the bioactive compounds (A) neovestitol and (B) vestitol.

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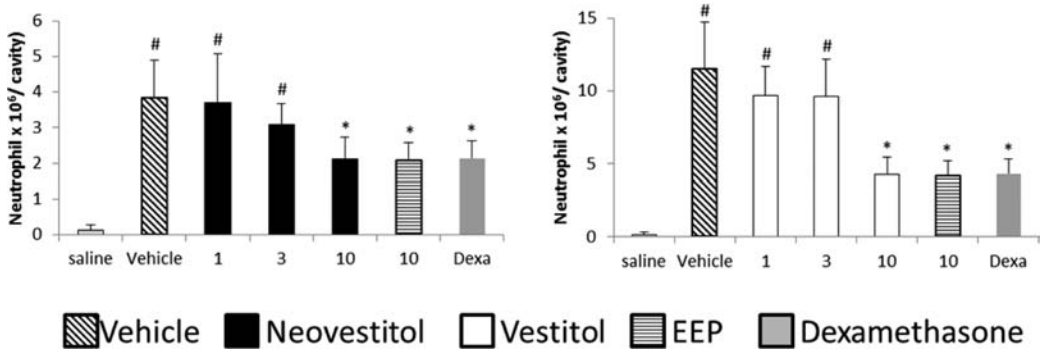

Figure 3. Recruitment of leukocytes into the peritoneal cavity induced by carrageenan. The neutrophil migration was determined 4 h after injection of carrageenan (500 μg/cavity). Mice were previously treated with vehicle (saline), EEP, neovestitol, and vestitol, followed by carrageenan injection. The results are expressed as the mean ± SEM, n = 5−6. Key: #, statistical difference compared to the saline group; *, statistical difference compared to the carrageenan group (one-way ANOVA followed by the Bonferroni test, $p < 0.05$).

Anti-Inflammatory Test. The anti-inflammatory test consisted of evaluating neutrophil migration to peritoneal cavity after treatment with EEP, neovestitol, and vestitol. Male Balb/c mice (20−25 g) were housed in temperature-controlled rooms (22−25 °C) with ad libitum access to water and food. All experiments were conducted in accordance with National Institutes of Health guidelines for the welfare of experimental animals and with the approval of the institutional Committee for Ethics in Animal Research (protocol number 1484-1). The animals were used only in a single experimental group. For the determination of neutrophil migration to peritoneal cavity, EEP, neovestitol, and vestitol were administered by subcutaneous injection (1, 3, or 10 mg/kg) 15 min before the administration of inflammatory stimuli by intraperitoneal injection of carrageenan at 500 μg/cavity in naive mice. Mice were killed 4 h after the challenge (carrageenan) administration, and the peritoneal cavity cells were harvested by washing the cavity with 3 mL of phosphate-buffered saline (PBS) containing 1 mM EDTA. The volumes recovered were similar in all experimental groups and equated to approximately 95% of the injected volume. Total counts were performed in a cell counter (COULTER A CT; Coulter, Miami, FL), and differential cell counts (100 cells total) were carried out on cytocentrifuge (Cytospin 3; Shandon Lipshaw, Pittsburgh, PA) slides stained with Rosenfeld. The results are presented as the number of neutrophils per cavity.[20]

Antimicrobial Tests. Antimicrobial tests consisted of the determination of the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of EEP, neovestitol, and vestitol. The tested microorganisms were *Streptococcus mutans* UA159, *Streptococcus sobrinus* 6715, *Staphylococcus aureus* ATCC25923, and *Actinomyces naeslundii* ATCC12104. The methodology described by Koo et al.[21] was modified into a microtechnique, in which 190 μL of BHI broth with inoculum ($(1-2) \times 10^5$ UFC/mL) and 10 μL of EEP, neovestitol, vestitol, and control solution (ethanol, 4%, v/v) were dispensed onto a microplate. Concentrations of the tested extracts for MIC ranged from 12.5 to 800 μg/mL, and bacterial growth was assessed by adding 0.01% resazurin stain (Aldrich). MIC values were defined as the lowest concentration of a given extract that could inhibit bacterial growth. An aliquot (30 μL) of a concentration higher than MIC was cultured on BHI agar supplemented with 5% defibrinated sheep blood for 18−24 h, at 37 °C, with 10% CO_2 to determine MBC. MBC was the lowest concentration that allowed no visible bacterial growth on agar.[21]

RESULTS AND DISCUSSION

The inflammation process is a human body response to any damage or infection, such as those associated with oral inflammation such as periodontal complications (gingivitis and periodontitis), and the anti-inflammatory agent could act locally. This process involves a cascade of successive events that will result in neutrophil migration to the inflammatory focus.[22,23] The maintenance of the inflammatory process (chronic inflammation) in the tissues may promote their destruction and bone loss; thus, the modulation of the host response, reducing the severity of inflammation, is somehow necessary to avoid the deleterious effects.[20,24−27]

Thus, we used bioassay-guided fractionation, which is a proven method for the discovery of active principles from natural products, to isolate bioactive compounds from Brazilian red propolis ethanolic extract. On the basis of the study by Oldoni et al., we used different chromatographic methods combined with anti-inflammatory and antimicrobial assays to obtain neovestitol and vestitol. It is important to note that the main achievement of our research at this step (bioguided fractionation) was the modification of the method of extraction to obtain neovestitol and vestitol, which improved the yield of neovestitol from 0.04%[10] to 0.2% (present data); the vestitol yield was not changed.[10]

In this way, animals treated with EEP, neovestitol, and vestitol showed inhibitory activity against neutrophil migration at a 10 mg/kg dose (Figure 3). It is important to note that the three substances were as effective as the gold standard dexamethasone at 10 mg/kg (Figure 3) in preventing neutrophil migration to the peritoneal cavity, demonstrating that the compounds have a noteworthy anti-inflammatory potential that should be explored in the future.

Thus, both isolated compounds presented anti-inflammatory effects, and to the best of our knowledge, this is the first report of anti-inflammatory properties of Brazilian red propolis extract and its isolated compounds, such as neovestitol and vestitol. Also they may act as a synergistic association.

Natural products are recognized as an important source of new therapeutic agents. Over 70% of all new drugs approved between 1981 and 2012 were obtained from natural products, which demonstrates their value as sources for the discovery of novel bioactive agents.[28] *Canavalia grandiflora* seed lectin, a literature-recognized natural product with anti-inflammatory properties,[7,20] inhibited neutrophil migration at a dose of 10 mg/kg[29] as determined using the exact same anti-inflammatory model used in this study. It is apparent that the bioactive compounds of Brazilian red propolis are as effective as a well-known naturally occurring anti-inflammatory agent (Figure 3). The isoflavones, particularly neovestitol and vestitol, may have potential therapeutic application to modulate inflammation processes, such as those involved with periodontal diseases.

Table 1. Results of Antimicrobial Tests (MIC and MBC) of Isolated Compounds

	MIC (μg/mL)				MBC (μg/mL)			
compd	*Strep. mutans*	*Strep. sobrinus*	*Staph. aureus*	*A. naeslundii*	*Strep. mutans*	*Strep. sobrinus*	*Staph. aureus*	*A. naeslundii*
EEP	100−200	<6.25	100−200	25−50	100−200	50−100	200−400	50−100
neovestitol	25−50	<6.25	25−50	25−50	50−100	25−50	50−100	50−100
vestitol	50−100	25−50	50−100	50−100	100−200	200−400	100−200	>1600

Regarding antimicrobial activity, EEP showed MIC values ranging from <6.25 μg/mL (*Strep. sobrinus*) to 100−200 μg/mL (*Strep. mutans* and *Staph. aureus*) and MBCs ranging from 50−100 μg/mL (*Strep. sobrinus*) to 200−400 μg/mL (*Staph. aureus*) (Table 1). In contrast with the anti-inflammatory results, neovestitol displayed a better antimicrobial effect than EEP and vestitol (Table 1). Neovestitol showed potent antibacterial activity with MICs ranging from <6.25 μg/mL (*Strep. sobrinus*) to 25−50 μg/mL. Vestitol was less effective than neovestitol, showing MICs ranging from 25−50 μg/mL (*Strep. sobrinus*) to 50−100 μg/mL (other microorganisms). Regarding MBC, neovestitol showed results from 25−50 μg/mL (*Strep. sobrinus*) to 50−100 μg/mL (*Strep. mutans*, *A. naeslundii*, and *Staph. aureus*), while vestitol showed values in accordance with those of Oldoni et al.[10] (Table 1). Therefore, both neovestitol and vestitol are the main bioactive compounds associated with antimicrobial activity of Brazilian red propolis since they have the same or better MIC and MBC values (antimicrobial parameters used in this study) than EEP.

Other antimicrobial compounds were isolated from Brazilian propolis, such as *tt*-farnesol and pinocembrin.[3,5] The MICs of *tt*-farnesol against *Strep. mutans* and *S. sobrinus*, two microorganisms associated with oral biofilm disorders, were 28 and 14 μg/mL, respectively, while pinocembrim showed 64 μg/mL for both microorganisms.[3] Thus, neovestitol appears to be more effective against *Strep. mutans* than the previously identified compounds.

Although several compounds, including 7-*O*-vestitol, have been identified in red propolis,[30,31] there is no report in the literature on any pharmacological properties or use of these isolated compounds. In addition, to the best of our knowledge, this is the first report in the literature on antimicrobial activity of neovestitol.

Recently, a methanolic fraction of Brazilian red propolis showed antibacterial activity against *Pseudomonas aeruginosa*, *Bacillus subtillis*, *Candida albicans*, *Salmonella typhimurium*, *Klebsiella pnemoniae*, *Enterococcus faecallis*, *Escherichia coli*, *Proteus mirabilis*, and *Streptococcus pyogenes*.[32] However, there were no attempts to precisely isolate and identify the active individual compounds.

The major challenge in the discovery of new natural agents is the isolation and purification of sufficient amounts of active principles (with high purity) from chemically complex crude extracts, such as propolis. The biological effect must be correlated to the effector compound, and preparative separation methods should be refined to yield the necessary quantity of the agent[23] as we did in the present study, attesting that our modification of the Odoni et al.[10] methodology improved the extraction of neovestitol.

Therefore, our study confirms the antimicrobial activity of red propolis and isolated compounds. Clearly, neovestitol and vestitol could be potentially antimicrobial agents against infectious diseases (such as dental caries) pending further investigation using in vitro and in vivo biofilm models and elucidation of their mechanism of action.

Our data support the hypothesis that the antimicrobial and anti-inflammatory activities of Brazilian red propolis are associated with the presence of at least two bioactive isoflavonoids, neovestitol and vestitol. Interestingly, neovestitol (a major component) displayed better antimicrobial activity than vestitol (a minor component), whereas the latter was an equal anti-inflammatory compound. We are currently further characterizing its anti-inflammatory mechanisms of action as well evaluating their effectiveness using in vitro biofilm systems and an in vivo model of dental caries disease. Furthermore, it is plausible that neovestitol and vestitol, as local bucal bioactive agents, can be exploited commercially as they appear to have no cellular toxicity because they have been used as food additives[8,9] or nutraceuticals in preventing oral biofilm disorders. Clearly, neovestitol and vestitol are nutraceuticals as supported by the following definition: "A nutraceutical is a product isolated or purified from foods that is generally sold in medicinal forms not usually associated with food. A nutraceutical is demonstrated to have a physiological benefit or provide protection against chronic disease."[33]

The isoflavonoids neovestitol (2′,4′-dihydroxy-7-methoxyisoflavan) and vestitol (2′,7-dihydroxy- 4′-metoxyisoflavan), both isolated from Brazilian red propolis, showed in vivo anti-inflammatory and in vitro antimicrobial properties. In addition, this is the first literature report on the anti-inflammatory properties of neovestitol and vestitol isolated from Brazilian red propolis and also the antimicrobial activity of neovestitol. Therefore, Brazilian red propolis is a promising natural source of bioactive compounds such as neovestitol and vestitol, which both deserve further investigations to improve the production process, evaluate their effectiveness, elucidate the mechanisms of action, and investigate other possible pharmacological and/or nutraceutical properties.

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Notes

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