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Brazilian red propolis improves cutaneous wound healing suppressing inflammation-associated transcription factor NFκB

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ABSTRACT

The use of natural products in wound healing has been extensively studied in the context of complementary and alternative medicine. Propolis, a natural product, is a polyphenol-rich resin used for this purpose. This study aimed to investigate the effect of Brazilian Red Propolis Extract (BRPE) on inflammation and wound healing in mice, using a tissue repair model. The BRPE polyphenol content was analyzed by liquid chromatography coupled to mass spectrometry (LC/MS). A full-thickness excision lesion was created, and mice were treated orally with daily doses of vehicle solution (water-alcohol solution containing 2% of ethanol, control group) or 100 mg/kg of BRPE (P100 group) during nine consecutive days. BRPE chemical composition analysis showed that this complex matrix contains several phenolic compounds such as phenolic acids, phenolic terpenes and flavonoids (especially catechins, flavonols, chalcones, isoflavones, isoflavans, pterocarpans and bioflavonoids). After BRPE administration, it was observed that, when compared to the control group, P100 group presented faster wound closure ($p < 0.001$); less neutrophils per mm^2 ($p < 0.05$) and macrophages ($p < 0.01$) in tissue analyses, down regulation of the inflammatory transcription factor pNF-κB protein expression, and reduced production of inflammatory cytokine, such as TGF-β, TNF-α ($p < 0.0001$), and IL-6 ($p < 0.001$). These findings suggest a positive role of BRPE oral administration in the wound healing process via suppressing the inflammatory response during tissue repair.

1. Introduction

Wound healing can be defined as a dynamic and complex process of skin repair that occurs in response to an injury. This process comprises the steps of inflammation, reepithelialization (proliferation, migration, and differentiation of epithelial cells) and remodeling, which overlaps in time, but are coordinated. It also involves immune and inflammatory cellular and biochemical events, and requires the participation of several intracellular and extracellular metabolic regulation pathways. The purpose of these activities is to regain tissue integrity and restore the local homeostasis [1,2].

The use of herbal products and medicinal plants in the treatment of skin lesions consists of a very ancient practice in human history. In the last decades, it was replaced by the application of chemicals and synthetic drugs, which however, have been shown to present shortcomings, limitations and side effects. Currently, natural products have been investigated as an alternative source of drugs which modulate the inflammatory process in a shorter time period and with minimal complications. The interest in this area of complementary and alternative medicine has increased, resulting in multiple studies on the use of natural products for tissue repair [3–6].

Propolis is a natural polyphenol-rich resinous substance produced by honey bees with the purpose of protecting their beehives against insects and infectious agents [7,8]. Thirteen different types of propolis have been identified and classified according to their physicochemical characteristics. Red propolis is

Abbreviations: BRPE, Brazilian red propolis extract; HPLC–MS, high performance liquid chromatography–mass spectrometry; DAB, diaminobenzidine; NF-, κBNF-kappaB; pNF-κB, phospho-NF-kappaB; IL-6, interleukin-6; TNF-α, tumor necrosis factor α; TNF, tumor necrosis factor; TGF-β, transforming growth factor-β; IL-1, interleukin-1; PF-4, platelet factor 4; MCP-1, monocyte chemotactic protein-1; IFN-γ, interferon gamma; CD36, thrombospondin receptor; MMP-9, matrix metallopeptidase-9; IL-1β, interleukin-1β; VEGF, vascular endothelial growth factor; TGF-β_1, transforming growth factor-β_1; α-SMA, smooth muscle α-actin.

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a type of propolis found in the northern and northeastern areas of Brazil [9]. Its color is due to the red exudates found on the surface of *Dalbergia ecastophyllum* (L.) Taub which is collected by bees living in these specific regions [10].

Among the chemical components of red propolis, the bioactive compounds or phytochemicals are noteworthy and are mainly represented by polyphenols. Compared with the green and brown types, red propolis presents the highest content of polyphenols [11]. More than 30 phenolic compounds have been identified so far and they belong to the groups of phenolic acids, flavonoids, polyisoprenylated guttiferones, terpens, tannins and xanthones [9–14]. These compounds are considered bioactive markers of Brazilian red propolis and are indicated as the potential compounds responsible for the pharmaceutical and nutraceutical effects of this matrix [14].

Several biological properties have already been attributed to Brazilian red propolis, such as antibacterial [15], immunomodulatory [16], antifungal [17], antitumoral [14], and anti-oxidative [18]. Data on the anti-inflammatory effects of Brazilian propolis are abundant [19–24] and the effects of this natural product on wound healing have also been reported [25]. However, there are few studies on the anti-inflammatory effects [26–28] and healing properties [3,29] of Brazilian red propolis. In addition, the knowledge about these biological properties after red propolis oral administration in animals is scarce [30] and these issues have not been studied in mice so far.

Given that wound healing is a complex event which involves inflammation and intercellular interactions and considering that red propolis has been suggested to play a role in inflammation and wound closure, the present study set to evaluate the effect of oral administration of BRPE in mice using a tissue repair model.

2. Materials and methods

2.1. Brazilian red propolis extract (BRPE)

For BRPE preparation, a crude red propolis sample was obtained from Barra de Santo Antônio (Alagoas, Brazil) in a mangrove area (09°11′19.67″S/35°27′47.03″W), where *Dalbergiaecastophyllum* (L.) Taub was the main botanical source. Crude Brazilian red propolis was mixed in a 1:3 (w/v) proportion with ethanol:water (80:20, v/v) in an amber-colored bottle and incubated for 30 min at 60 °C under constant agitation. The mixture was then filtered through a 0.22 μm filter (Millipore®, São Paulo, Brazil) to separate the ethanol extract from the solid propolis residue. The extract was left at 4 °C overnight to promote precipitation of waxes and other poorly soluble waste compounds, and was then centrifuged at 3000 × *g* for 10 min at 4 °C to obtain a clear supernatant. Subsequently, the BRPE was evaporated using a rotary evaporator (Fisatom 801, Fisatom Equipment Scientific Ltda. São Paulo, Brazil) and lyophilized (HetoPowerDry LL3000, Thermo Scientific).

2.2. Chemical characterization of BRPE

BRPE total phenolic content was estimated using the Folin–Ciocalteu reagent [31]. Results were expressed as gallic acid equivalents (GAE) (mg GAE/g dry extract) through a calibration curve using known solutions of gallic acid standard in the same conditions of the samples (λ = 765 ou 750 nm).

The content and the profile of phenolic compounds were investigated using HPLC–MS. Samples were reconstituted in an ethanol:water (80:20, v/v) solution, and filtered through a 0.45 μm cellulose ester membrane (Millipore®, São Paulo, Brazil), prior to HPLC injection. HPLC analysis was performed in a Shimadzu system (Kyoto, Japan) equipped with a quaternary pump (LC-20AT, Shimadzu®, Kyoto, Japan), a degasser (DGU-20A5), an automatic sample injector (SIL-20AHT), and a diode-array detector (DAD, SPD-M20A). Chromatographic separation was achieved using a Luna™ C18 column (250 × 3 mm, 5 μm). The mobile phase consisted of a gradient of 0.3% aqueous formic acid (solvent A), methanol (solvent B) and acetonitrile (solvent C), at a flow rate of 1.0 mL/min. During analysis, the concentration of solvent C (1%) was kept constant. Prior to injection, the column was equilibrated with 18% solvent B. After sample injection, the mobile phase composition changed to 20% B (0–1 min), 43% B (2–19 min), and 85% B (20–24 min), and was then kept constant (25–30 min). Compounds were also identified by mass spectra analysis using a mass spectrometer (MS), equipped with an electrospray ionization source (Esquire HCT, BrukerDaltonics, Billerica, MA, USA), which operated in negative mode. Helium was used as collision gas. The compounds identification was based on the analysis of the UV spectrum and MS/MS fragmentation pattern.

2.3. Animals and experimental protocol

We used 12 two-month old *Swiss* male mice, with an average weight of 22 ± 2 g. The animals were maintained in a temperature-controlled room (22 °C) under a 12- hour light, 12-h dark cycle, with *ad libitum* access to water and commercial food (NUVILAB CR-1). The animals were divided into two groups (n = 10 animals per group). Using oral gavage, the control group (control) received a vehicle solution (ethanol:water 2:98, v/v), while the propolis group (P100) received a daily dose of 100 mg/kg of BRPE. The dose used in this study was based on previous reports [32,33] demonstrating the biological activity and safety dose of propolis polyphenols. The treatment started the day before the lesion was created (d-1), as a single daily dose of 0.3 mL (Fig. 1). At d0, a lesion was created on the back of the animals. BRPE was administrated until d9 (9 days after the lesion), and the animals were euthanized on d10 (10 days after the lesion). This study was approved by the Ethics Committee for Use of Animals of the State University of Rio de Janeiro (CEUA/028/2014).

2.4. Wounding model and macroscopic analysis

Before wounding, all animals were anesthetized with intraperitoneal injection of ketamine (150 mg/kg) and xylazine (15 mg/kg). After trichotomy, a full-thickness excisional wound (8 mm diameter) was created with punch biopsy in the back of the animal and was not sutured or covered with a dressing, but was left to heal by secondary intention. The area of the lesions was measured on the day of the lesion (d0) and three, eight, and ten days afterward [34]. The areas were scanned and analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, version 1.43). The contraction data were expressed as a percentage of initial lesion area.

2.5. Tissue processing and microscopic analysis

One part of the lesion (with the surrounding normal skin) was formalin-fixed (pH 7.2), paraffin-embedded, and used for histological analysis; the other part (without adjacent healthy skin) was frozen at −80 °C and then used for molecular analysis. Histological analyses were performed by staining the sections with hematoxylin and eosin, in order to estimate the density of inflammatory cells and count blood vessels in the granulation tissue. Slides were digitalized using a Pannoramic scanner MIDI (3DHistech Ltd., Budapest, Hungary), and analyzed with the Pannoramic Viewer (3DhistechKft).The density of inflammatory cells present in the lesion was examined using a previously described stereological method [35]. Five fields per animal were analyzed.