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Anti-inflammatory activity and phenolic profile of propolis from two locations in Región Metropolitana de Santiago, Chile

Gabriela Valenzuela-Barra [a], Consuelo Castro [a], Catalina Figueroa [a], Andrés Barriga [b], Ximena Silva [c], Beatriz de las Heras [d], Sonsoles Hortelano [e], Carla Delporte [a,*]

[a] Laboratorio de Productos Naturales, Facultad de Ciencias Químicas y Farmacéuticas, Universidad de Chile, Casilla 233, Santiago 1, Chile
[b] Unidad de espectrometría de masas, Facultad de Ciencias Químicas y Farmacéuticas, Universidad de Chile, Casilla 233, Santiago 1, Chile
[c] Unidad de Pruebas Biológicas, Instituto de Salud Pública de Chile, Marathon 1000, Santiago, Chile
[d] Departamento de Farmacología, Facultad de Farmacia, Universidad Complutense de Madrid, Plaza Ramón y Cajal s/n, 28040, Madrid, Spain
[e] Unidad de Inflamación y Cáncer, Área de Biología Celular y del Desarrollo, Centro Nacional de Microbiología, Instituto de Salud Carlos III, Majadahonda-Pozuelo, Km 2.200, 28220 Majadahonda, Madrid, Spain

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ABSTRACT

Ethnopharmacological relevance: Propolis has long been used as a popular folk medicine due to its wide spectrum of alleged biological and pharmaceutical properties. In Chile, propolis is widely used by folklore medicine as an anti-inflammatory agent; however, this property has not been demonstrated by scientific methods.

Aim of the study: The objective of this study was to determine the anti-inflammatory activity *in vivo* and *in vitro* and to establish the phenolic profile of propolis collected in two localities in Región Metropolitana de Santiago (RM), Chile.

Materials and methods: Propolis was collected in the areas of Caleu and Buin, RM Chile. Following that, the samples were unwaxed to obtain the global ethanolic extracts of propolis (EEPs) and, from these, the serial extracts of dichloromethane (EEP-DCMs) and ethanol (EEP-EtOHs). The topic anti-inflammatory effect was evaluated through mice ear edema induced by arachidonic acid (AA) and 12-O-tetradecanoylphorbol-13-acetate (TPA) at a dose of 3 mg/ear. Nitric oxide (NO) measurements were determined spectrophotometrically (Greiss reagent) by the accumulation of nitrite in the medium of macrophages RAW 264.7 stimulated with the lipopolysaccharide (LPS, 1 μg/mL) for 20 h at different concentrations of the EEPs, EEP-DCMs and EEP-EtOHs (6.25–50.00 μg/mL). The content of total phenols and flavonoids were determined through the methods of Folin–Ciocalteau and $AlCl_3$, respectively. The profile of phenolic compounds was determined by HPLC–UV–ESI-MS/MS.

Results: The EEP-EtOH (64%) and EEP (59%) of Buin were the most active in the inflammation induced by TPA and AA respectively, being the anti-inflammatory effect stronger than the same Caleu extracts. Regarding the release of NO, all the extracts from the Buin propolis inhibited significantly its release in a concentration-dependent manner, this inhibition was stronger than the extracts from Caleu propolis.

Conclusions: Our research shows for the first time a comparative study of the topical *in vivo* activity of two Chilean propolis. Both propolis showed *in vivo* topical anti-inflammatory activity against AA and TPA, the most active was Buin propolis and this difference is due in part to the variations in total phenols and flavonoids content and the phenolic profile. The phenols and flavonoids content of Buin propolis was higher than Caleu propolis. The extracts from Buin propolis result in a lower release of NO.

1. Introduction

Propolis is a resinous material elaborated by the bee *Apis mellifera, n.v.*, through the recollection of the exudates from different plant species. The bees use propolis to repair combs, to strengthen the thin borders of the comb, and to make the entrance of the hive watertight or easier to defend. Propolis is also used as an embalming substance to cover the carcass of a hive invader which the bees have killed but cannot transport out of the hive. The bees cover the invader with propolis and wax, and the remains are left at the bottom or on one of the walls of the hive (Toreti et al., 2013). Propolis is constituted by resinous, sticky and balsamic substances; such as waxes, essential oils and pollen, among others (Tosi et al., 2006).

* Corresponding author. Tel.: +56 2 29781697.
E-mail address: cdelpor@uchile.cl (C. Delporte).

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Folk medicine recommends the use of propolis, due to its antibacterial, anti-fungal, and anti-viral effects and its hepatoprotective and anti-inflammatory properties, to increase the resistance against infections and to treat gastroduodenal ulcers (Castaldo and Capasso, 2002). Propolis has attracted researchers' interest in the last decades because of several biological and pharmacological properties, such as immunomodulatory, anti-tumor, anti-inflammatory, anti-oxidant, anti-bacterial, anti-viral, anti-fungal and anti-parasite, among others (Sforcin and Bankova, 2011). Besides, products containing propolis have been intensely marketed by the pharmaceutical industry and health-food stores (Sforcin, 2007). The ethnopharmacological approach, combined with chemical and biological methods, may provide useful pharmacological leads about the medical effects of propolis.

In Chilean propolis, only some of these properties have been evaluated, such as anti-oxidant (Astudillo et al., 2000; Castro et al., 2014; Russo et al., 2004), anti-bacterial (Saavedra et al., 2011), anti-fungal (Herrera et al., 2010) and anti-tumoral (Russo et al., 2004), among others. However, the anti-inflammatory activity through *in vivo* and *in vitro* methods has not been investigated.

The chemical composition of propolis is complex and varied, which is why a great amount of compounds have been identified, such as: alcohols, aldehydes, phenolic acids, amino acids, chalcones, flavonoids, lignans, triterpenes, steroids and sugars, among others. However, phenolic compounds are the most abundant (Righi et al., 2013).

In this research, we present a comparative study of the *in vivo* anti-inflammatory activity of two propolis recollected from the localities of Buin and Caleu, which have similar climate and soil conditions but whose main variation was in the surrounding vegetation. Different unwaxed extracts were prepared and their topical anti-inflammatory activities were evaluated in the edema model in mice ear, induced by 12-O-tetradecanoyl-phorbol-13-acetate (TPA) and arachidonic acid (AA). The total phenols and flavonoids content, phenolic profile and inhibitory effect of the release of nitric oxide (NO) were determined for each extract.

2. Materials and methods

2.1. Propolis samples

The samples were recollected during spring in 2011, the hives free of diseases were located in Buin (33°38′43.84″S; 70°39′59.69″O) and Caleu (33°00′12″S; 70°59′37″O), Región Metropolitana de Santiago (RM), Chile. The harvest was done with plastic meshes. Then, the meshes were preserved at −4 °C for 4 h; after this period, the meshes were flexed to release the propolis. Afterwards, the different samples were stored at −20 °C protected from the light.

Buin propolis presented a homogenous aspect, a rigid consistency with brown and green hues. Caleu propolis presented a heterogenous aspect, a rigid consistency, and green and yellow hues.

Buin and Caleu are known for having a Mediterranean weather, long dry season and rainy winter. It is a warm temperate weather in which winter rains are concentrated between May and August. The main difference between both locations is in the vegetation surrounding the hives, identified by the taxonomist Sebastian Teiller. In Buin, there were found: *Agapanthus africanus* (n.v. agapanto), *Argyranthemum frutescens* (n.v. paraquet), *Cyperus alterniflorus* (n.v. paragüita), *Escallonia illinita* (n.v. barraco), *Lavandula angustifolia* (n.v. lavanda), *Medicago sativa* (n.v. alfalfa), *Nerium oleander* (n.v. laurel de flor), *Otholobium glandulosum* (n.v. culén), *Populus* spp. (n.v álamo), *Salvia microphylla* (salvia) *and Verbena officinalis* (n.v. verbena); and in Caleu: *Santolina chamaecyparissus* (n.v. manzanillera), *Acacia caven* (n.v. espino), *Trifolium repens* (n.v. trébol blanco), *Cynodon dactylon* (n.v. chépica), *Lotus corniculatus* (n.v. lotera), *Plantago lanceolata* (n.v. siete venas), *Populus* spp. (n.v. álamo) *and Quillaja saponaria* (n.v. quillay).

2.2. Preparation of propolis extracts

To elaborate the global ethanolic extracts of propolis (EEPs), initially a process of unwaxing was carried out in the crude propolis employing temperature cycles, according to the methodology proposed by Alencar et al. (2007) and Kalogeropoulos et al. (2009). 400 g of raw propolis from each locality was weighed, homogenized in a mortar and added to 750 mL of ethanol. Following this, the ethanolic mixture was introduced in a thermoregulated bath at 70 °C for 30 min; afterwards, it was cooled at room temperature and refrigerated at −20 °C for 12 h. Finally, it was filtered and the supernatant was preserved. This procedure was repeated 3 times with the aim of extracting the wax exhaustively. Once the propolis was unwaxed, the extracts were put in a rotary evaporator at 60 °C until the solvent was completely eliminated, obtaining the EEP.

The serial extracts were elaborated through successive extractions of the EEP with the solvents dichloromethane (DCM) and ethanol (EtOH), resulting in the extracts EEP-DCM y EEP-EtOH, respectively. Each extraction was carried out until the EEPs were completely exhausted; between each extraction the material was dried at room temperature before adding the new dissolvent. The serial extracts were concentrated in a rotary evaporator at reduced pressure.

2.3. In vivo topical anti-inflammatory activity

Two inflammatory agents, AA and TPA, were used to estimate the probable anti-inflammatory action mechanism of the propolis under study. The reference drugs used were indomethacin and nimesulide against TPA and AA, respectively.

All animal experiments were performed according to the ethical guidelines suggested by the "International Norms for the Biomedical Investigation with Animals", elaborated by the Council of International Organizations (1990) and the bio-ethics norms of the Commission of the Instituto de Salud Pública de Chile (ISP) and the Facultad de Ciencias Químicas y Farmacéuticas of the Universidad de Chile (CBE2012-4).

Adult male CF-1 mice (20–25 g), obtained from the stock at the ISP, were used to assess the anti-inflammatory effect. All animals were housed in a climate and light-controlled room with a 12 h light–dark cycle, fasted overnight before the day of the assays, with free access to water. For each of the samples under study, the anti-inflammatory activity was evaluated in two groups. One group of 8 treated mice and the other of 16 control mice. After 5 min of sample treatment (3 mg/ear of the EEP, EEP-DCM and EEP-EtOH), mice received 5 μg of TPA or 2 mg of AA (Sigma, St. Louis, MO, USA) as pro-inflammatory agents, dissolved in 20 μL of acetone (solvent does not interfere with the assay). Control subjects only received TPA or AA at the same concentration. Both, the sample and the TPA or AA, were applied to the inner (10 μL) and outer (10 μL) surfaces of the right ear. The left ear only received acetone. Mice were sacrificed by cervical dislocation (after 6 h of TPA and 1 h of AA), and a 6 mm diameter section of the right and left ears were cut and weighted. The weight differences between both ear sections correspond to the edema value. Topical anti-inflammatory effect (EA) was evaluated according to the following equation: $\%EA = [W_c - W_s/W_c] \times 100$; where W_c and W_s are the difference median values of the weights of the right and the left ear sections of the control (W_c) and the treated animals (W_s) respectively (Delporte et al., 2003).

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