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Urban propolis from San Juan province (Argentina): Ethnopharmacological uses and antifungal activity against *Candida* and dermatophytes

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Dedicated to the memory of Dr. Luis Astudillo Saavedra (University of Talca, Chile).

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ABSTRACT

Propolis is widely used in the folk medicine of San Juan province (Argentina) to treat several diseases, including cold, cough, muscle aches and superficial mycoses. We report the in vitro antifungal activity of urban propolis, evaluated with CLSI protocols in addition to the evaluation of their chemical profile by HPLC-ESI-MS/MS techniques.

The dermatophytes *Microsporum gypseum*, *Trichophyton mentagrophytes* and *Trichophyton rubrum* were the most susceptible species and guided the fractionation of urban propolis, which was performed with Sephadex LH-20 leading to eight fractions (I–VIII). These fractions showed high antifungal activities against dermatophytes (MICs = 16.0–62.5 μg/mL) and yeasts (MICs = 31.2–125 μg/mL) being III, V and VI the most active ones (MIC_{100} = 16–31.2 μg/mL). They also, showed fungicidal capacity (a condition highly appreciated in antifungal drugs to avoid recurrence) with MFC values between 31.2 and 62.5 μg/mL. From the most active fractions, two lignans: 3′-methyl-nordihydroguaiaretic acid (MNDGA) (**1**), and nordihydroguaiaretic acid (NDGA) (**2**), in addition to three flavonoids: chrysin (**3**), pinocembrin (**4**) and galangin (**5**), were isolated and quantified by HPLC–PDA-MS/MS as the main antifungal compounds. Lignans **1** and **2** showed strong activities against *T. mentagrophytes*, *T. rubrum* and *Microsporum gypseum* (MICs between 31.2 and 62.5 μg/mL), and 1 showed strong activity against *Candida albicans*, *Candida tropicalis* and *Cryptococcus neoformans* (MICs between 31.2 and 62.5 μg/mL). Regarding flavonoids, all yeasts were sensitive to **5** (MIC = 31.2 μg/mL), whereas the dermatophytes *T. mentagrophytes* and *T. rubrum* and all yeasts were moderately inhibited by **4** (MIC = 31.2–250 μg/mL). Finally, chrysin (**3**) showed low activity against yeasts and dermatophytes (MIC = 250 μg/mL). These results support that Argentinean urban propolis, which are frequently used by beekeepers for the preparation of syrups, tinctures and creams, are valuable natural product for the improving of human health, particularly fungal infections. It is also worthy to take into account that its chemical composition contains mainly two antifungal lignans, associated with the medicinal *Larrea* genus.

1. Introduction

Propolis is a resinous substance prepared by bees as a result of mixing the resin collected from plants with their salivary secretions and beeswax. It is used for sealing unwanted open spaces in the hive, and to protect the colony against different pathogens (Marcucci et al., 2001). Its use in traditional medicine dates back to 300 years BC (Ghisalberti, 1979) and, at present, it continues to be used worldwide. In the last years, propolis has been the subject of intensive biological and pharmacological studies, including antiviral (Schnitzler et al., 2010), anticancer (Valente et al., 2011), antioxidant (Moreira et al., 2008), hepatoprotective (Banskota et al., 2001), cariostatic (Libério et al., 2009), anti-inflammatory (Silva

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et al., 2012), as well as antibacterial and antifungal ones (Sforcin et al., 2001; Santos et al., 2008; Ngatu et al., 2011). Due to its different activities, propolis have gained wide acceptance to promote health, preventing diseases (Ishida et al., 2011) and it is widely used in cosmetology and food industries (Banskota et al., 2001).

However, since honey bees are opportunists gathering the resins from available sources, the chemical composition of propolis varies considerably from region to region, accompanying the vegetation of the area (Daugsch et al., 2008; Lima et al., 2009; Agüero et al., 2010, 2011). As a consequence, the biological properties of propolis show variations that depend on its chemical composition.

San Juan province is located in the central-western part of Argentina, in the intersection of 31°S latitude and 69°W longitude to the western Andean slopes. The province has a rich tradition in folk medicine including the use of medicinal plants, honey, pollen, and propolis, which may be associated with native or introduced plant species depending on the location (environment) of the hives. The native flora comprises the species *Larrea divaricata* Cav., *Larrea cuneifolia* Cav., *Tessaria absinthioides* Hook. et Arn. DC. and *Zuccagnia punctata* Cav. and a large number of species including *Prosopis*, *Acacia* and *Baccharis* genera, distributed in different ecosystems of particular edaphic and climatic conditions. On the other hand, there are a high number of exotic plants including species such as *Populus* spp., *Eucalyptus* spp., *Tamarix gallica* L., *Pinus* spp., *Medicago sativa* L., *Vitis vinifera* L., *Olea europaea* L., plum, and peaches in urban zones from the province. The economic activity of the province is mainly focused on agriculture, standing out the production of grapes, olives, peaches, plums, onions, garlics, melons, and tomatoes. In the last decade, in Argentina the so-called craft fairs in which small farmers sell their products like jams, crafts, textiles, medicinal plants, honey, pollen, and propolis have greatly increased in the last years. Among the products sold at these fairs, propolis has increased its importance in San Juan province as a therapeutic product, being widely used in folk medicine to treat several diseases, including cold, cough, muscle aches and superficial mycoses.

Invasive fungal infections are of great concern for human beings because they are associated with unacceptable high mortality rates. More than 90% of all reported fungal-related deaths result from species that belong to one of three genera: *Cryptococcus*, *Candida* and *Aspergillus*. In turn, superficial infections of the skin and nails are the most common fungal diseases in humans, affecting ca. 25% of the population worldwide (Karan et al., 2009). These infections are primarily caused by dermatophytes, which give rise to well-known conditions such as athlete's foot (occurs in one out of five adults), ringworm of the scalp (common in young children) and infection of the nails (affects ca. 10% of the population worldwide (Brown et al., 2012). Although antifungal drugs designed to cope with invasive and superficial fungal infections have increased substantially in the past decade, they are not completely effective and usually show severe toxicity. Thus, there is a general consensus that the search of new antifungal compounds is necessary to improve both preventive and therapeutic issues of fungal infections.

Propolis of San Juan province is prepared in several forms, including syrups, tinctures and creams, constituting a natural alternative for the treatment of fungal infections. Contrasting with this, there are few reports on the chemical composition and antifungal activity of the many propolis varieties used by beekeepers for the preparation of the propolis containing products.

The main goals of this study were: (a) to evaluate the antifungal activity of urban propolis from several areas of San Juan province (Argentina); (b) to find their bioactive compounds by assay-guided fractionation; (c) to evaluate the contribution of native flora to the chemical profile of studied propolis.

2. Materials and methods

2.1. Chemicals

All solvents used were of analytical grade. Chloroform was purchased from Fisher (USA), methanol (MeOH) from J.T. Baker (USA), acetonitrile from Caledon Lab. Ltd. (Canada), and formic acid from Merck (Germany). Ultrapure water was obtained from an Arium 611 UV (Sartorius, Germany) equipment. TLC analyses were carried out on aluminum-coated silica gel plates (Sigma–Aldrich, St. Louis, MO, USA). Pinocembrin, galangin, chrysin and nordihydroguaiaretic acid were purchased from Sigma–Aldrich, St. Louis, MO, USA).

2.2. Equipments

2.2.1. NMR studies

Nuclear magnetic resonance (NMR) spectra were obtained with a Bruker Avance (400 MHz) or Avance II (500 MHz) spectrometer operating at 400 or 500 MHz for ^{1}H and at 100 and 125 MHz for ^{13}C, respectively. CD_3OD, DMSO-d_6, and $CDCl_3$ were used as solvents.

2.2.2. Identification and quantification of active compounds by HPLC–ESI-MS/MS

An Agilent Series 1200 LC System (Agilent, USA) coupled to a PDA detector and a MicrOTOF Q II (Bruker Daltonics, USA) in tandem was used for HPLC–PDA-ESI-MS/MS analysis. The HPLC system consisted in a micro-vacuum degasser, binary pumps, an autosampler (40 μL sample loop) and a thermostated column compartment. The mass spectrometer was used in both MS and MS/MS modes for the structural analysis of phenolic compounds.

HPLC analyses were performed on a thermostatic oven (40 °C) (Agilent 1200L series), using a Phenomenex Luna C18 250 × 4.6 mm (5 μm) column operated at 0.4 mL/min flow rate, using 0.5% (v/v) formic acid (solvent A) and MeOH (solvent B). Gradient runs were performed using the following sequence: starting with 20% B and changing to 50% B along 3 min, kept for 5 min, followed by a second ramp to 80% B during 5 min, maintained for 17 min, and a third ramp to 20% B in 1 min, remaining at this last condition for 10 min before the next run. The injection volume was 40 μL.

ESI-MS detection was performed in negative ion mode with mass acquisition between 100 and 1500 Da. Nitrogen was used as drying and nebulizer gas (3.5 bar). For MS/MS experiments, fragmentation was achieved by using the Auto MS^2 option from the software. PDA analyses were carried out in the range between 200 and 700 nm, monitoring at 280 nm.

The identification of propolis constituents was carried out by comparison of the retention time and spectral characteristics (UV, ESI-MS and MS/MS) of compounds eluting from the HPLC column with those from pure compounds, when available, or with data from the literature. The standards pinocembrin, galangin, chrysin and nordihydroguaiaretic acid were prepared at a stock concentration of 1000 mg/L. Calibration standard were prepared by appropriate dilutions with MeOH from the stock solutions. Both standard and samples were filtered on Millipore paper (0.45) before use. MS chromatograms (extracted ions) were used for the quantification of reported compounds. Quantification was performed using linear regression plots, constructed from pure compound or from structurally similar compounds when pure compounds were not available. All analyses were performed in triplicate, reporting means and standard deviations (SD).

2.3. Propolis samples

Eleven raw propolis samples were kindly provided by beekeepers from San Juan province (Argentine) belonging to the following