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

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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

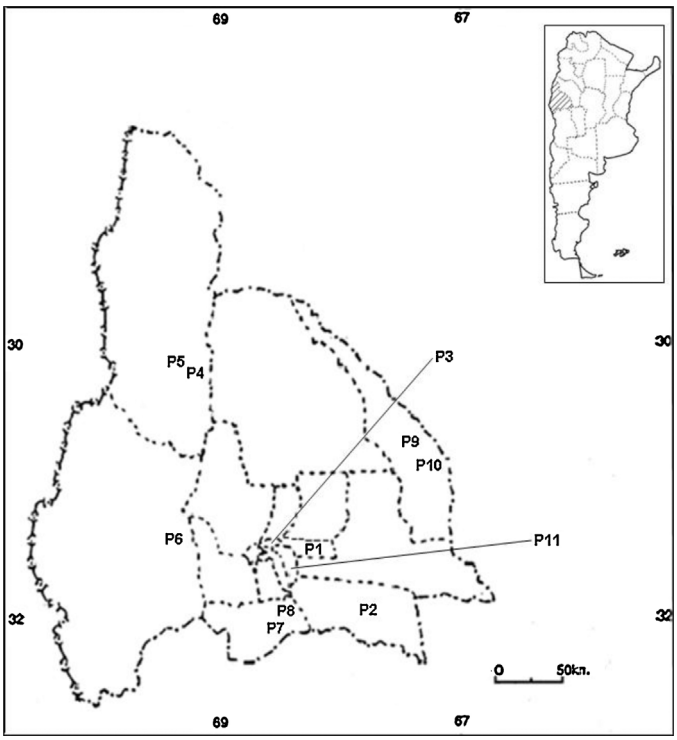


Fig. 1. Map of San Juan province, Argentina. Collection sites of propolis: P1, San Martin; P2, 25 de Mayo; P3, Chimbas; P4, Rodeo, Iglesia; P5, Tudcum, Iglesia; P6, Calingasta; P7, Sarmiento, Sarmiento; P8, Cochagual, Sarmiento; P9, Tumanas, Valle Fertil; P10, Astica, Valle Fertil; P11, 9 de Julio.

districts: San Martín (P1); 25 de Mayo (P2); Chimbas (P3); Iglesia (Rodeo, P4; Tudcum P5); Calingasta (P6); Sarmiento (Sarmiento, P7; Cochagual, P8); Valle Fértil (Tumanas, P9; Astica, P10); and 9 de Julio (P11). Propolises were collected with propolis traps to minimize their contamination with foreign substances and were first frozen and then stored at −20 °C until analysis. Sampling areas are showed in Fig. 1. Propolis samples references are deposited at the Instituto de Biotecnología, National University of San Juan, identified as P1, P2, P3, P4, P5, P6, P7, P8, P9, P10 and P11.

2.4. Preparation of propolis ethanolic extracts

A representative amount (100 g) from each propolis sample was extracted at room temperature with 500 mL ethanol during 24 h. Combined extracts were filtered and concentrated under reduced pressure to afford the corresponding propolis ethanolic extracts (PEE). They were stored at −20 °C in the dark until analysis. The extraction yields (% w/w) ranged between 41% and 76% of raw propolis samples (Table 1).

2.5. Extraction and isolation of the antifungal compounds

A representative sample of the antifungal PEE4 (32 g) was applied to a Sephadex LH-20 column (length 42 cm, diameter 4 cm, equilibrated with MeOH). Some 20 fractions, of 75 mL each, were obtained. After TLC comparison (silica gel, by using petroleum ether (PE):ethyl acetate (EtOAc) 7.5:2.5 as the mobile phase), fractions with similar TLC patterns were combined. The following groups of fractions were obtained: I (1745 mg; fractions 1–3), II (2040 mg, fraction 4), III (1449 mg, fractions 5 and 6), IV (784 mg, fractions 7–9), V (604 mg, fractions 10), VI (389 mg, fractions 11–13), VII (251 mg, fractions 14–16), VIII (308 mg, fractions 17–20).

The pooled fraction III–V (2.5 g) was treated with MeOH to afford a MeOH-soluble fraction (A, 2 g) and a MeOH-insoluble yellow precipitate (B, 0.4 g) from which 150 mg of chrysin (**3**) were obtained as yellow needles.

Repeated column chromatographies (Sephadex LH-20, elution solvent PE:MeOH:$CHCl_3$ (2:1:1) of MeOH-soluble fraction A (1.5 g), led to the isolation of 80 mg of 3′-methyl-nordihydroguaiaretic acid (**1**), 100 mg of nordihydroguaiaretic acid (**2**) and 150 mg of pinocembrin (**4**). A representative sample of fraction VI (260 mg) was applied to a Sephadex LH-20 column (column length 46 cm, diameter 2 cm, equilibrated with PE:Cl_3CH:MeOH, 2:1:1) affording galangin (**5**, 56 mg) as yellow needles. The structures of isolated compounds **1–5** (Fig. 2) were elucidated by spectroscopic data (^1H, ^{13}C NMR, and MS) which were in agreement with those reported in the literature (Mabry et al., 1977; Agrawal, 1989; Gnabre et al., 1995; Torres et al., 2003; Abou-Gazar et al., 2004; Vargas-Arispuro et al., 2005; Agüero et al., 2011).

2.6. Antifungal activity

2.6.1. Microorganisms and media

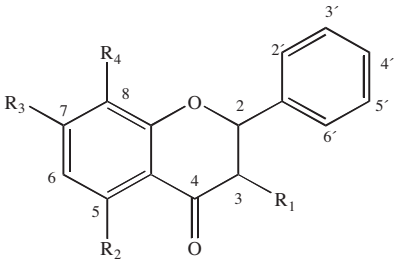
For the antifungal evaluation, strains from the American Type Culture Collection (ATCC), Rockville, MD, USA and CEREMIC (CCC), Centro de Referencia en Micología, Facultad de Ciencias Bioquímicas y Farmacéuticas, Suipacha 531, 2000-Rosario, Argentina were used: *Candida albicans* ATCC 10231, *Candida tropicalis* CCC 131-2000, *Saccharomyces cerevisiae* ATCC 9763, *Cryptococcus neoformans* ATCC 32264, *Aspergillus flavus* ATCC 9170, *Aspergillus fumigatus* ATTC 26934, *Aspergillus niger* ATCC 9029, *Trichophyton rubrum* CCC 110, *Trichophyton mentagrophytes* ATCC 9972 and *M. gypseum* CCC 115. Inocula of cell or spore suspensions were obtained according to reported procedures and adjusted to 1–5 × 10^3 cells/spores with colony forming units (CFU)/mL according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2002, 2008).

2.6.2. Antifungal susceptibility testing

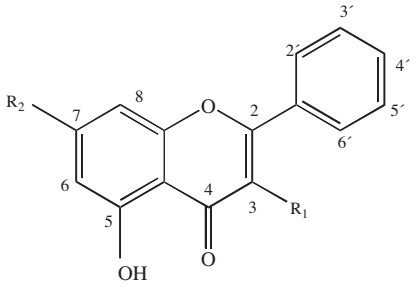
Minimum inhibitory concentration (MIC) of each extract, fractions or pure compound was determined by broth microdilution techniques (CLSI, 2008) by using RPMI-1640 medium

Table 1
Propolis collection sites, yields (%) and ethnopharmacological uses of propolis ethanolic extracts (PEE).

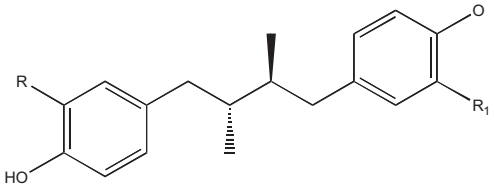
Propolis extracts	Collection site	Disctrict	Yield extract (% w/w)	Ethnopharmacological uses
PEE1	San Martin	San Martin	52	"The ethanolic extracts of the San Juan propolis are prepared in several presentations such as syrup, tincture and creams which are sold as regional products in local market and are highly demanded by people seeking a natural choice for the treatment of fungal infections"
PEE2	25 de Mayo	25 de Mayo	55	
PEE3	Chimbas	Chimbas	50	
PEE4	Rodeo	Iglesia	59	
PEE5	Tudcum	Iglesia	69	
PEE6	Calingasta	Calingasta	63	
PEE7	Sarmiento	Sarmiento	70	
PEE8	Cochagual	Sarmiento	41	
PEE9	Tumanas	Valle Fertil	49	
PEE10	Astica	Valle Fertil	76	
PEE11	9 de Julio	9 de Julio	63	



Compound	R_1	R_2	R_3	R_4
4. pinocembrin	H	OH	OH	H



Compound	R_1	R_2
3. chrysin	H	OH
5. galangin	OH	OH



Compound	R	R_1
1: 3´methyl-nordihydroguaiaretic acid	OCH_3	OH
2: nordihydroguaiaretic acid	OH	OH

Fig. 2. Isolated compounds of urban propolis samples from San Juan province, Argentine.

(Sigma–Aldrich, St. Louis, MO, USA), buffered to pH 7.0 with MOPS. Microtiter trays were incubated at 35 °C for yeasts and *Aspergillus* spp. and at 28–30 °C for dermatophytes in a moist, dark chamber. MICs were visually recorded at 48 h for yeasts, and at a time according to the control fungus growth, for the rest of fungi. For the assay, stock solutions of pure compounds were two-fold diluted with RPMI medium from 250 to 0.98 μg/mL (final volume 100 μL) to reach a final dimethyl sulfoxide (DMSO) concentration ≤1%. A volume of 100 μL of inoculum suspension was added to each well with the exception of the sterility control, where sterile water was added to the well instead. Ketoconazole, terbinafine, and amphotericin B were used as positive controls. Endpoints were defined as the lowest concentration of drug resulting in total inhibition (MIC_{100}) of visual growth compared to the growth in the control wells containing no antifungal. MIC_{80} and MIC_{50} were defined as the lowest concentration of a fraction that induced 80% or 50% reduction of the growth control respectively (culture media with the microorganism but without the addition of any compound) and was determined spectrophotometrically with the aid of a VERSA Max microplate reader (Molecular Devices, USA). The minimum fungicidal concentration (MFC) of each fraction or compound was determined as follows. After determining the MIC, an aliquot of 5 μL was withdrawn from each clear well of the microtiter tray and plated onto a 150 mm RPMI-1640 agar inoculated plate buffered with MOPS (Remel, Lenexa, KS). After 48 h incubation at 30 °C, MFCs were recorded. The MFC was defined as the lowest concentration of each compound that resulted in total inhibition of visible fungal growth in the plates (Rodero and Córdoba, 2007).

3. Results and discussion

3.1. Antifungal activity of urban propolis ethanolic extracts, fractions and isolated compounds

PEEs of P1–P11 urban propolis from San Juan province (Argentina) were tested against a panel of standardized strains of yeasts, filamentous fungi and dermatophytes. Results showed (Table 2), that the species of *Aspergillus* genus were not sensitive to urban propolis extracts (MICs > 250 μg/mL) (data no shown). Instead, most dermatophytes and yeasts were inhibited by urban propolis, with MIC values between 31.2 and 250 μg/mL, being the dermatophytes *M. gypseum*, *T. mentagrophytes* and *T. rubrum* the most susceptible species (MICs range from 31.2 to 125 μg/mL). Regarding the yeasts, these were moderately inhibited by the different propolis extracts with MICs between 62.5 and 250 μg/mL. Based on these antifungal results (Table 2), sample availability, and preliminary HPLC/MS studies, the urban PEE from Iglesia district (Rodeo, P4), was submitted to chromatographic fractionation and the fractions obtained PEE4.I–VIII were tested against the same panel of yeasts and dermatophytes by using three endpoints: MIC_{100}, MIC_{80} and MIC_{50} (the minimum concentration of compounds that inhibit 100, 80 and 50% of growth respectively) and also the minimum fungicide concentration (MFC) (Table 3).

Fractions II–VI from PEE4 showed high antifungal activity against dermatophytes (MICs = 16–62.5 μg/mL) whereas the yeasts were inhibited at higher MICs (31.2–125). Fractions I–VI from PEE4 showed the strongest activities against dermatophytes (MICs = 16–31.2 μg/mL). In addition, these fractions were fungicidal with MFC values between 31.2 and 62.5 μg/mL; a condition highly appreciated in antifungal drugs to avoid recurrence. Fraction PEE4.VI showed the best antifungal activity against *M. gypseum*, *T. mentagrophytes* and *T. rubrum* with similar MIC values of 16 μg/mL. The yeasts *C. albicans*, *C. tropicalis*, *S. cerevisieae* and *C. neoformans* were moderately inhibited by the fractions PEE4.III–VI with MIC values between 62.5 and 125 μg/mL.

Pure compounds **1**–**5** isolated and identified during the bioassay-guided fractionation of urban propolis PEE4 were also assessed for antifungal effect (Table 3).

For pure compounds, we determined not only MIC_{100} (the concentration that completely inhibits the fungal growth, but MIC_{80} and MIC_{50}, concentrations that inhibit 80 and 50% of fungal growth respectively. The application of less stringent endpoints

Table 2
Minimum inhibitory concentrations (MIC) and minimum fungicidal concentration (MFC) of urban propolis extracts of the districts from San Juan province, Argentine against yeast and dermatophytes fungus (MIC and MFC values in μg/mL).

Extract	*Ca*		*Ct*		*Sc*		*Cn*		*Mg*		*Tr*		*Tm*	
	MIC_{100}	MFC	MIC_{100}	MFC	MIC_{100}	MFC	MIC_{100}	MFC	MIC_{100}	MFC	MIC_{100}	MFC	MIC_{100}	MFC
PEE1	250		250		125		250		62.5		125		62.5	
PEE2	250		250		125		250		62.5		125		62.5	
PEE3	250		250		125		250		62.5		125		62.5	
PEE4	125		125		125		125		62.5		125		62.5	
PEE5	250	250	250	250	250	250	250	250	62.5	62.5	62.5	62.5	62.5	125
PEE6	250		250		125		250		62.5		125		62.5	
PEE7	125		125		125		125		62.5		62.5		62.5	
PEE8	125	250	125	250	62.5	125	125	125	62.5	62.5	31.25	31.25	31.25	31.25
PEE9	1000	1000	1000	1000	1000	1000	1000	1000	250	250	125	250	125	250
PEE10	125	250	125	250	62.5	250	125	250	62.5	62.5	31.25	31.25	31.25	31.25
PEE11	250	250	250	250	125	250	250	250	125	125	62.5	62.5	62.5	125
amp B	1	1	0.5	0.5	0.5	0.5	0.25	0.25	–	–	–	–	–	–
keto	0.5	–	0.125	–	0.5	–	0.25	–	–	–	–	–	–	–
terb									0.04	–	0.025	–	0.04	–

MIC_{100}, concentration of extract that inhibit 100% of the growth control; MFC, minimum fungicidal concentration; *Ca*, *Candida albicans* ATCC 10231; *Ct*, *Candida tropicalis* CCC 131-2000; *Sc*, *Saccharomyces cerevisiae* ATCC 9763; *Cn*, *Cryptococcus neoformans* ATCC 32264; *Mg*, *Microsporum gypseum* CCC 115; *Tr*, *Trichophyton rubrum* CCC 110; *Tm*, *Trichophyton mentagrophytes* ATCC 9972; ATCC, American Type Culture Collection; CCC, Center of Mycological; amp B, amphotericin B; keto, ketoconazole; terb, terbinafine; –: not tested.

such as MIC_{80} and MIC_{50} has been showed to consistently represent the in vitro activity of compounds (Frost et al., 1995) and many times provides a better correlation with other measurements of antifungal activity such as the MFC (Singh, 2003). The main isolated compounds were: 3′-methyl-nordihydroguaiaretic acid (MNDGA) (**1**) and nordihydroguaiaretic acid (NDGA) (**2**), chrysin (**3**), pinocembrin (**4**) and galangin (**5**). Lignans **1** and **2** showed activity against *T. mentagrophytes*, *T. rubrum* and *M. gypseum* (MICs = 31.25–62.5 μg/mL), **5** inhibits all yeasts at 31.2 μg/mL and **4** inhibited *T. mentagrophytes* and *T. rubrum* with MIC = 31.2 μg/mL and yeasts in the range 62.5–250 μg/mL. Instead, **3** showed marginal activity against yeasts and dermatophytes (MIC = 250 μg/mL).

Several studies on the antimicrobial activity and chemical composition of other Argentinean propolis have also found some of these antimicrobial compounds, Isla et al. (2005) and Chaillou and Nazareno (2009) found pinocembrin (**4**) in propolis samples from Northern Argentine which possessed antibacterial activity against *Staphylococcus aureus*. Furthermore, this flavonoid along with **3**, were found in honeys from Northwest Argentina, and displayed antimicrobial activity too (Isla et al., 2011). Regarding antifungal activity, Quiroga et al. (2006) proved the effects of **4** and **5** on yeasts, xylophagous and phytopathogenic fungi. Recently, we reported on the antifungal effect of isolated compounds from propolis associated to *Z. punctata* Cav. from the Tucumán province against pathogenic fungi (Agüero et al., 2010), and the antifungal activity of Andean Argentinean propolis associated with *Larrea nitida* Cav. against dermatophytes and clinical isolates of *Candida*, *Cryptococcus*, and *Trichophyton* species (Agüero et al., 2011) including its chemical profile.

These results give a scientific support to the traditional use of PEE of San Juan province (Argentine) for fungal infections, because it is worth to consider that the signs or symptoms that are related to a traditional antifungal use are mainly related to skin or mucosal conditions which are easy to detect and to follow when they are under treatment. These ailments are produced by dermatophytes (*Trichophyton*, *Microsporum* and *Epidermophyton* spp.), which are the ethiological agent of tinea unguium (producer of invasive nail infections), tinea manuum (palmar and interdigital areas of the hand infections) and tinea pedis (Athlete's foot), the latter being the most prevalent fungal infections in developed countries, and the first one accounting for 50% and 90% of all fingernail and toenail infections respectively (Weitzman and Summerbell, 1995).

3.2. Characterization and quantification of antifungal compounds from urban propolis ethanolic extracts

Taking into account the antifungal activity of bioactive compounds isolated from fractions of PEE4, we were interested in identifying and quantifying these compounds in the rest of propolis extracts (PPE1–3 and PEE5–11) under study. The antifungal compounds **1**–**5** were identified and quantified using HPLC ESI-MS/MS in all ethanolic propolis extracts. A HPLC chemical profile of each extract is showed in Fig. 3. Under our experimental conditions, **1**, **2**, **3**, **4** and **5** eluted at Rt = 23.4, 19.6, 22.2, 22.7 and 22.9 min, respectively.

Qualitative and quantitative results are shown in Table 4. Compounds **3**–**5**, were identified in all PEE. Propolis ethanolic extracts PEE1, PEE4, PEE5 and PEE8, showed the highest concentration of compound **4** and **5**: 91, 109, 127, and 114 mg/g of PEE respectively. Particularly, PEE1 and PEE8 showed the largest amount of **3**, having 71.2 and 66 mg/g, respectively. On the other hand, the lignans **1** and **2**, that are recognized constituents of *Larrea* genus growing in Argentina, were identified in all analyzed extracts. PEE1 had the highest amount of **2** (106 mg/g of PEE) followed by PEE4 and PEE5 which have a 65 and 64 mg/g, respectively. PEE4, PEE5 and PEE11 showed a significant amount of **1** (49–120 mg/g).

Our present results agree with those reported by Kumazawa et al. (2004) who found propolis ethanolic extracts containing a large amount of **3** (68.5 mg/g), **4** (68.7 mg/g), **5** (32.5 mg/g), cinnamylidene acetic acid (30.4 mg/g), pinobanksin (22.5 mg/g), pinobanksin 3-acetate (56.3 mg/g), and tectochrysin (31.4 mg/g).

Volpi and Bergonzini (2006) described that ethanolic extracts of propolis from Argentina, Italy and Spain had a great amount of pinocembrin (approximately 49%, 48% and 39% of the total identified flavonoids, respectively). Gardana et al. (2007) suggest that European, Chinese and Argentinean propolis are characterized by the presence of phenolic acids and flavonoids, being chrysin (2–4%), pinocembrin (2–4%), pinobanksin acetate (1.6–3%) and galangin (1–2%) the most abundant.

In this study, the urban propolis collected in eleven localities from San Juan province (Argentine) are characterized by lignans **1** and **2** as major constituents in the analyzed propolis, it could indicate that the bees obtained resins from species of the genus Larrea, since large patches of native flora mainly *L. divaricate* are around hives are. These lignans were recognized as major constituents in extracts of *Larrea* spp. (Mabry et al., 1977; Gnabre et al., 1995;

Table 3
Minimum inhibitory concentration (MIC) and Minimum fungicidal concentration (MFC) of fractions from urban propolis (PEE4) and isolated compounds against yeast and dermatophytes.

Fractions of PEE4	*Ca*				*Ct*				*Sc*				*Cn*				*Mg*		*Tr*		*Tm*	
	MIC			MFC	MIC			MFC	MIC			MFC	MIC			MFC	MIC	MFC	MIC	MFC	MIC	MFC
	100 μg/mL	80 μg/mL	50 μg/mL		100 μg/mL	80 μg/mL	50 μg/mL		100 μg/mL	80 μg/mL	50 μg/mL		100 μg/mL	80 μg/mL	50 μg/mL		100 μg/mL		100 μg/mL		100 μg/mL	
PEE4	125	–	–	–	125	–	–	–	125	–	–	–	125	–	–	–	62.5	–	125	–	62.5	–
PEE4.I	>250	–	–	–	>250	–	–	–	>250	–	–	–	>250	–	–	–	>250	–	>250	–	>250	–
PEE4.II	125	62.5	31.2	>250	125	62.5	31.2	>250	125	62.5	62.5	>250	125	62.5	62.5	>250	62.5	62.5	62.5	125	62.5	125
PEE4.III	125	62.5	31.2	>250	125	62.5	31.2	>250	62.5	62.5	62.5	>250	125	62.5	31.2	>250	31.2	62.5	31.2	62.5	31.2	62.5
PEE4.IV	125	62.5	31.2	>250	125	62.5	31.2	>250	62.5	62.5	62.5	>250	125	62.5	31.2	>250	31.2	62.5	31.2	62.5	31.2	62.5
PEE4.V	125	62.5	31.2	250	125	62.5	62.5	250	125	62.5	62.5	>250	250	62.5	31.2	>250	31.2	31.2	31.2	62.5	16	31.2
PEE4.VI	62.5	62.5	31.2	125	62.5	62.5	31.2	125	62.5	62.5	62.5	250	125	62.5	31.2	>250	16	31.2	16	62.5	16	62.5
PEE4.VII	250	125	62.5	>250	250	125	62.5	>250	250	250	125	>250	250	62.5	31.2	>250	62.5	62.5	62.5	125	62.5	62.5
PEE4.VIII	>250	–	–	–	>250	–	–	–	>250	–	–	–	125	–	–	–	>250	–	>250	–	>250	–
1	62.5	62.5	31.2	125	62.5	62.5	31.25	125	31.25	–	–	31.25	31.25	15.6	7.8	31.25	62.5	62.5	31.2	31.2	62.5	62.5
2	250	250	125	250	250	250	125	500	250	–	–	250	250	250	62.5	250	62.5	62.5	62.5	62.5	62.5	62.5
3	250	–	–	–	250	–	–	–	250	–	–	–	250	–	–	–	250	–	250	–	250	–
4	62.5	–	–	–	125	–	–	–	125	–	–	–	250	–	–	–	125	–	31.2	–	31.2	–
5	31.2	–	–	–	31.2	–	–	–	31.2	–	–	–	31.2	–	–	–	–	–	–	–	–	–
amp B	1				0.5	–	–		0.5				0.25									
keto	0.5				0.125	–			0.5				0.25									
terb																	0.04		0.025		0.04	

MIC_{100}, MIC_{80} and MIC_{50}, concentrations of a fraction that induced 100, 80 and 50% reduction of the growth control respectively; *Ca*, *Candida albicans* ATCC 10231; *Ct*, *Candida tropicalis* CCC 131 2000; *Sc*, *Saccharomyces cerevisiae* ATCC 9763; *Cn*, *Cryptococcus neoformans* ATCC 32264; *Mg*, *Microsporum gypseum* CCC 115; *Tr*, *Trichophyton rubrum* CCC 113; *Tm*, *Trichophyton mentagrophytes* ATCC 9972; ATCC, American Type Culture Collection (Illinois, USA); CCC, Center of Mycological Reference (Rosario, Argentina); PEE4, Iglesia-Rodeo propolis ethanolic extract; PEE4.I–VIII, fractions from PEE4; amp B, amphotericin B; keto, ketoconazole; terb, terbinafine; compounds **1**, 3′-methyl-nordihydroguaiaretic acid; **2**, nordihydroguaiaretic acid; **3**, chrysin; **4**, pinocembrin; **5**, galangin.

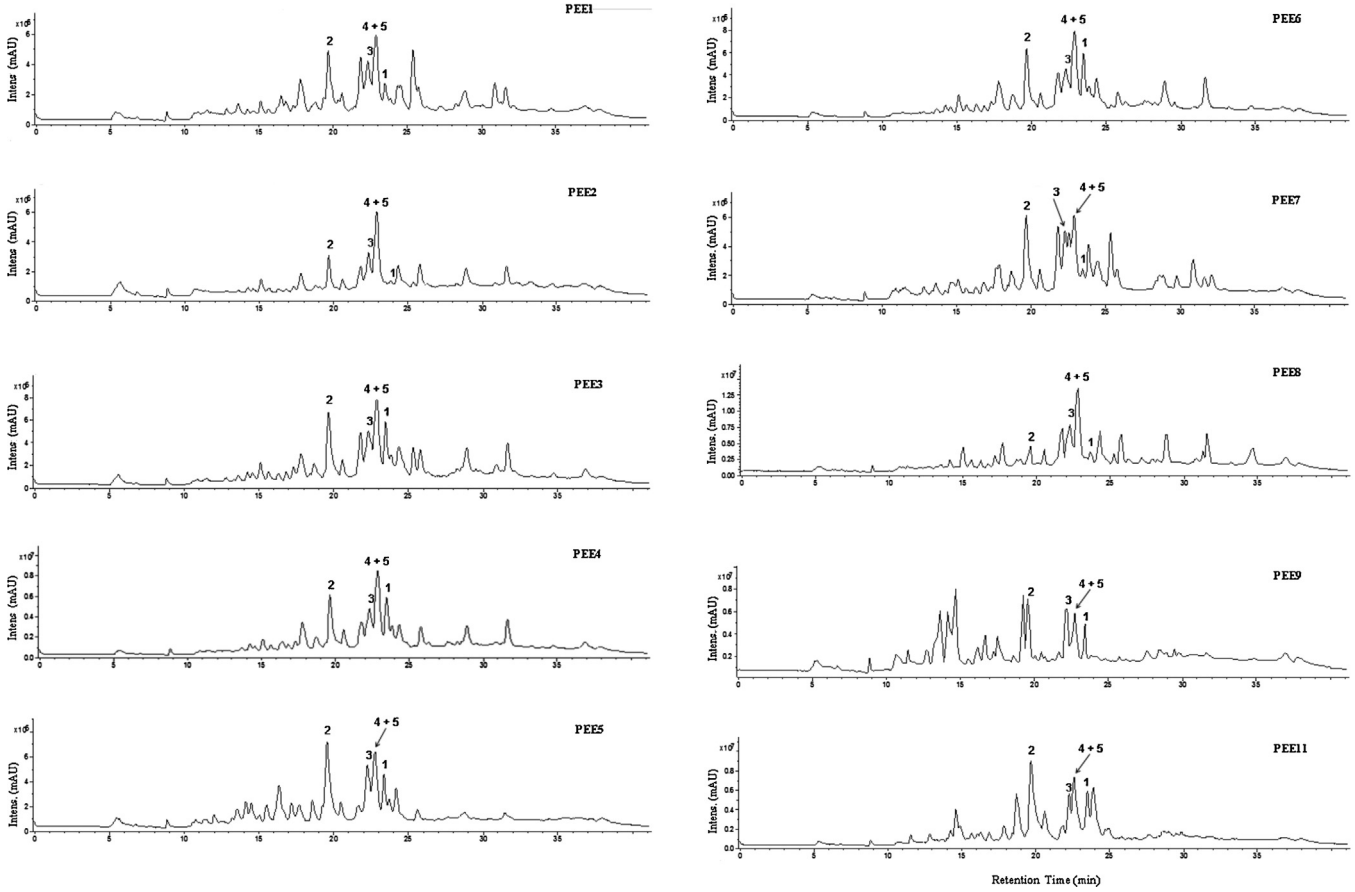


Fig. 3. HPLC-QTOF chromatograms of the San Juan urban propolis ethanolic extracts (PEE). Compounds and retention time (Rt, min). MNDGA (**1**, 23.4 min), NDGA (**2**, 19.6 min), chrysin (**3**, 22.2 min), pinocembrin (**4**, 22.7 min), and galangin (**5**, 22.9 min).

Table 4
Main antifungal compounds identified and quantified in urban propolis extracts from San Juan province.[a]

Compound	[M-H]-(-MS2[M-H])-	PEE1	PEE2	PEE3	PEE4	PEE5	PEE6	PEE7	PEE8	PEE9	PEE11
1 MNDGA	315, 300, 241	22 ± 3	4 ± 2	31 ± 7	49 ± 2	60 ± 5	36 ± 1	11 ± 3	11 ± 2	9 ± 2	120 ± 10
2 NDGA	301, 273, 122	106.7 ± 0.3	5.2 ± 0.6	58 ± 5	65 ± 4	64 ± 16	44 ± 10	53 ± 2	8 ± 3	47 ± 2	44 ± 5
3 Chrysin	253, 209	71.2 ± 0.5	15.2 ± 0.7	44 ± 7	55 ± 2	63 ± 4	40 ± 2	32 ± 4	66 ± 2	42 ± 3	33 ± 8
4 Pinocembrin	255, 213, 151	91 ± 5	44 ± 1	69 ± 7	109 ± 3	127 ± 8	71 ± 3	49 ± 4	114 ± 3	46 ± 4	70 ± 8
5 Galangin	269	91 ± 5	44 ± 1	69 ± 7	109 ± 3	127 ± 8	71 ± 3	49 ± 4	114 ± 3	46 ± 4	70 ± 8

Compounds **1**, 3′-methyl-nordihydroguaiaretic acid; **2**, nordihydroguaiaretic acid; **3**, chrysin; **4**, pinocembrin; **5**, galangin.
[a] Values (mg compound/g extract) are expressed as mean ± standard deviation (SD) of triplicate analyses for each sample.

Torres et al., 2003; Abou-Gazar et al., 2004; Vargas-Arispuro et al., 2005; Agüero et al., 2011). The genus *Larrea* is characteristic of the ecosystem of the northwest and east central Argentina, including the provinces of San Juan, Mendoza, San Luis, Catamarca, La Rioja, and Tucuman. Therefore it is expected to find resins' compounds as constituents of propolis from these regions of Argentina. Also, this urban propolis possesses characteristic phenolics of poplar origin like **3**–**5** which are present in high concentrations. These results showed that urban propolis extracts from San Juan province (Argentine) collected and used by beekeepers for the preparation of syrups, tinctures and creams display a strong antifungal activity against *Candida* and dermatophytes. Thus, this study could support the traditional use of the analyzed urban propolis, which is mostly used to treat skin fungal infections. Regarding their chemical composition, as these propolises come from beehives located in surrounding urban areas where both the introduced and the native species grow, it could be inferred that the botanical origin of these urban propolis has a contributions from exotic (*Populus*) and native flora (*Larrea*) because they contain mainly antifungal lignans **1** and **2** associated with recognized constituents of resin or exudates of the medicinal plant *L. divaricata*. Additionally, to determine the botanical and geographical origins other complementary studies as palinological analysis should be carried out.

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