Food and Chemical Toxicology 49 (2011) 1970–1978

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Argentinean Andean propolis associated with the medicinal plant *Larrea nitida* Cav. (Zygophyllaceae). HPLC–MS and GC–MS characterization and antifungal activity

María Belén Agüero [a], Laura Svetaz [b], Marianela Sánchez [c], Lorena Luna [a], Beatriz Lima [a], María Liza López [a], Susana Zacchino [b], Jorge Palermo [c], Daniel Wunderlin [d], Gabriela Egly Feresin [a], Alejandro Tapia [a,*]

[a] *Instituto de Biotecnología – Instituto de Ciencias Básicas, Universidad Nacional de San Juan, Av. Libertador General San Martín 1109 (O), 5400 San Juan, Argentina*
[b] *Farmacognosia, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional del Rosario, Suipacha 531, CP 2000, Rosario, Argentina*
[c] *UMYMFOR, Departamento de Química Orgánica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria, Piso 3, Pabellón 2, (1428), Buenos Aires, Argentina*
[d] *Universidad Nacional de Córdoba-CONICET, Facultad Ciencias Químicas-Dpto. Química Orgánica-ISIDSA-CIBICI. Medina Allende esq. Haya de La Torre, Ciudad Universitaria, 5000 Córdoba, Argentina*

ARTICLE INFO

Article history:
Received 7 April 2011
Accepted 6 May 2011
Available online 12 May 2011

Keywords:
Argentinian Andean propolis
Lignans
HPLC–ESI–MS/MS
GC–MS
VOCs
Antifungal activity

ABSTRACT

The chemical profile and botanical origin of Andean Argentinian propolis were studied by HPLC–ESI–MS/MS and GC–MS techniques as well as the antifungal activity according to CLSI protocols. Dermatophytes and yeasts tested were strongly inhibited by propolis extracts (MICs between 31.25 and 125 μg/mL). The main antifungal compounds were: 3′methyl-nordihydroguaiaretic acid (MNDGA) **1**, nordihydroguaiaretic acid (NDGA) **2** and a NDGA derivative **3**, showing strong activity against *Trichophyton mentagrophytes*, *T. rubrum* and *Microsporum gypseum* (MICs between 15.6 and 31.25 μg/mL). The lignans **1** and **2** showed activities against clinical isolates of *Candidas* spp., *Cryptococcus* spp., *T. rubrum* and *T. mentagrophytes* (MICs and MFCs between 31.25 and 62.5 μg/mL). The lignan and volatile organic compounds (VOCs) profiles from propolis matched with those of exudates of *Larrea nitida* providing strong evidences on its botanical origin. These results support that Argentinian Andean propolis are a valuable natural product with potential to improve human health. Six compounds (**1**–**6**) were isolated from propolis for the first time, while compounds **1** and **3**–**6** were reported for first time as constituents of *L. nitida* Cav.

1. Introduction

Natural products play an important role in the development of drugs for the treatment of human diseases. Thus, many medicines are naturally-derived metabolites and/or by-products from microorganisms, plants or animals, which have been used by humans for thousands of years (Strobel et al., 2004). The last decades have witnessed a growing interest by consumers, the food industry, and researchers, in the ways food may help to maintain human health. In this aspect is where functional foods, also known as nutraceuticals, designed foods, therapeutic foods, superfoods, or medicinal foods, play an important role (Nagai and Inoue, 2004). Among these foods, we may include all those that are originated in beehives, such as honey, propolis, and royal jelly. All of them are important complementary foods not only for their nutritional values but also for their functional and biological properties (Viuda-Marto et al., 2008). Propolis is a resinous substance that bees collect from the exudates of plants and which they use to seal holes in the beehive and as protective barrier against intruders. Propolis is considered responsible for the low incidence of bacteria and moulds within the hive. The antimicrobial activity of propolis is one of the properties which have been appreciated by humans for several centuries and even nowadays (Bankova et al., 2000; Ghisalberti, 1978). Besides its antibacterial, antifungal and antiviral properties, propolis presents many other beneficial biological activities such as antioxidant, anti-inflammatory, antitumor, hepatoprotective, immunostimulatory, antimutagenic, etc. (Kalogeropoulos et al., 2009). For these reasons propolis has gained wide acceptance (Ishida et al., 2011), and is currently used as a constituent of biocosmetics, health foods and for numerous other applications (Bankova et al., 2000; Banskota et al., 2001; Ghisalberti, 1978).

The chemical composition of propolis differs significantly according to its geographical and botanical origin, mainly because bees gather the resins and plant exudates from available sources, which vary from region to region according to climate, terrain,

* Corresponding author. Address: Instituto de Biotecnología, Facultad de Ingeniería, Universidad Nacional de San Juan, Av. Libertador General San Martín 1109 oeste, 5400 San Juan, Argentina. Tel.: +54 264 4211700x294; fax: +54 264 4213672.
E-mail address: atapia@unsj.edu.ar (A. Tapia).

0278-6915/$ - see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.fct.2011.05.008

water availability and other environmental factors (Ahn et al., 2007; Isla et al., 2005; Lima et al., 2009; Park et al., 2002). Several studies on Argentinian propolis have reported antioxidant, free radical scavenger, antimicrobial and anti-tumoral activities in samples of propolis from different regions (Agüero et al., 2010; Chaillou and Nazareno, 2009; Isla et al., 2001, 2005; Nieva Moreno et al., 1999; Nieva Moreno et al., 2000; Quiroga et al., 2006; Zampini et al., 2005, 2007).

In the mountain valleys of Iglesia and Calingasta, located in the province of San Juan, a western area close to the Andes Mountains (between 2000 and 3000 m.a.s.l.), there are a number of small rural settlements (Bauchaceta, Chita, and Espota). In these settlements, since the 18th century, there are many people living in the so called "goat posts", which are minimal units of organized rural population dedicated to the exploitation of rural resources (Gambier, 1988). Their activities are based on subsistence farming, goat breeding for their own use and the production of goat-dairy products such as cheese. They also possess small-scale apiaries for the production of honey and propolis, which are of high demand. These products are generally associated to the native flora of the Andes, obtained through a sustainable production system over the time, and claimed to have medicinal properties. So far, there are no reports on the chemical composition of Andean propolis from Argentina associated with *Larrea* genera.

The main goal of this work was to characterize propolis samples collected from apiaries located in Bauchaceta settlements (2700 m.a.s.l.), province of San Juan, Argentina, by assay-guided isolation of their most bioactive compounds. Furthermore, we look for evaluating their antifungal efficiency and characterizing their botanical origin by analysis of chemical profiles from both, the propolis and the endemic species *Larrea nitida*. Thus, we look to verify the correspondence between bioactive compounds present in plants native from the sampling area with those present in propolis produced in the same area as well as the correspondence between their volatiles profile.

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 (Darmstadt, Germany). Ultra pure water (<5 μg/L) was obtained from an Arium 61316-RO plus Arium 611 UV (Sartorius, Germany) purification system. TLC was carried out on Merck Sílicagel 60 F_{254} plates.

2.2. Equipments

^{1}H and ^{13}C NMR spectra were performed on Bruker Avance 2 (500 MHz) and AC-200 (200 MHz) spectrometers, using $CDCl_3$, CD_3OD and DMSO-d_6 as solvent. ^{1}H and ^{13}C NMR chemical shifts were referenced to the solvent residual signals, in the case of $CDCl_3$ at δ_H 7.26 and the central peak at δ_{13C} 77.0. Homonuclear ^{1}H connectivities were determined by COSY experiments. The edited reverse-detected single quantum heteronuclear correlation (DEPT-HSQC) experiment allowed the determination of carbon multiplicities as well as one-bond proton-carbon connectivities, and the heteronuclear multiple bond correlation (HMBC) experiment allowed the determination of long-range proton–carbon connectivities. The relative stereochemistry was determined by gradient-enhanced NOESY experiments. All 2D NMR experiments were performed using standard pulse sequences. HRESI mass spectra were recorded using a Micro TOF QII Bruker mass spectrometer. HPLC separations were performed using HPLC-grade solvents, a Thermo Separations Spectra Series P100 pump, a Thermo Separations Refractomonitor IV RI detector, a Thermo Separations Spectra Series UV 100 UV detector and an YMC RP-18 (5 μm, 20 mm × 250 mm) column. UV detection was performed at 220 nm.

HPLC–ESI–MS/MS analysis were carried out using a MicroTOFQ II apparatus (Bruker Daltonics, MA, USA), equipped with an ESI ion source operated at either positive or negative mode with nitrogen as nebulizing gas (4 psi) and drying gas (8 L/min, 200 °C); capillary 4500 V and end plate offset at 500 V. Mass accuracy was verified by infusing Na-formiate (10 mM, Sigma–Aldrich, Argentina) dissolved in isopropyl alcohol:water (50:50). Pure compounds were first characterized by direct infusion to ESI using a syringe pump (Harvard Apparatus 11 Plus) recording both MS and MS/MS spectra. Because of better performance obtained during direct infusion to ESI, here we only report MS and MS/MS spectra obtained in the negative mode.

HPLC was used to separate and characterize extract profiles coupling a PDA detector (Agilent DAD SL G1315C, monitoring at 280 nm) before the ESI source. A C18-RP column (Agilent ZORBAX XDB-C18, 1.8 μm × 50 mm × 3.0 mm i.d.) was used. Samples were introduced in the HPLC (5 μL for standards and 20 μL for samples) using an autosampler (Agilent HiP-ALSSL+) and a column oven (Agilent 1200 series, TCC-SL, G1316B) operated at 40 °C. Solvent delivery was performed at 0.4 mL/min by a binary pump (Agilent 1200 SL), using ultra-pure water supplemented with 0.1% formic acid (A) and HPLC-grade methanol with 0.1% formic acid (B) and a program, starting with 30% B, changing to 70% B within 10 min, held by 4.5 min, returning to 30% B in 0.5 min and keeping this condition for five additional minutes to achieve column stabilization before next run (total run time 20 min). Eluted compounds were recorded using PDA and auto MS–MS/MS mode with Argon in the collision cell; resulting ions were analyzed by TOF, recording the total ionic current (TIC) spectra.

For the analysis of volatile organic compounds (VOCs), a Perkin Elmer Series Clarus 600 gas chromatograph, with splitless injection was directly coupled to a Perkin Elmer Clarus 600 mass spectrometer. VOCs were separated on a fused silica DB-5 MS capillary column (60 m, 0.25 mm i.d., and 0.25 μm film thickness). Samples were analyzed using the following chromatographic and MS detection conditions: oven temperature was programmed from 50 °C (2 min), increased at 5 °C/min to 250 °C, and held for 10 min, using Helium (5.0 grade) as the carrier gas (49.6 psi). A column head pressure of 15 psi and an injector temperature and FID detector of 250 °C were used. The GC transfer line was maintained at 200 °C. Ionization was carried out in the mass spectrometer under vacuum by electron impact at 70 eV ionization energy. Chromatograms were acquired in "scan" mode scanning the quadrupole from m/z 50 to m/z 300 (scan time: 0.2 s, inter-scan time: 0.1 s).

2.3. Plant material

Aerial parts of *L. nitida* Cav. (Zygophyllaceae) were collected in December 2008, on Bauchaceta locality, Iglesia district, province of San Juan (Argentina). The plant was identified by Dra. Gloria Barboza, IMBIV (Instituto Multidisciplinario de Biología Vegetal), Facultad de Ciencias Exactas, Físicas y Naturales, Universidad Nacional de Córdoba, Argentina. A voucher specimen was deposited at the herbarium of the Botanic Museum of Córdoba (CORD 1335).

2.4. Propolis samples

Three representative raw propolis samples were kindly provided by beekeepers from the Bauchaceta district. Hives were located in the same geographical area where *L. nitida* grows between 2500 and 2700 m.a.s.l. at pre-mountain area close to the Andes range. Propolis samples were collected during December 2008, using propolis traps to minimize contamination and stored at −20 °C until analysis. Reference propolis samples were deposited at the Instituto de Biotecnología, Universidad Nacional de San Juan, identified as LnP1, LnP2 and LnP3.

2.5. Preparation of propolis extracts

One hundred grams of propolis were successively extracted at room temperature (3 × 24 h) with petroleum ether (PE), dichloromethane (DCM), ethyl acetate (EtOAc) and ethanol (EtOH). Then, solvents were evaporated under vacuum to give semisolid extracts: PPEE, PDCME, PEtOAcE and PEtOHE, affording 12.6%, 37.6%, 37.2% and 5% yield (w/w), respectively. Additionally, PMeOHE extract from a propolis sample (100 g) was obtained using methanol (MeOH) (3 × 24 h) (52.9% w/w yield).

2.6. Isolation of antifungal compounds from propolis extracts

A representative sample (4.5 g) of the PDCME extract was permeated on a Sephadex LH-20 column (43 cm length, 4 cm i.d.), using PE:MeOH:$CHCl_3$ (2:1:1) as eluant, collecting 32 fractions (50 mL each). Each fraction was compared by TLC, using silica gel, PE:EtOAc 70:30 as mobile phase, UV detection and further reaction with diphenylboric acid–ethanolamine complex in MeOH. Fractions showing similar TLC patterns were combined and evaluated for antifungal activity as described in the Section 2.10. In this way, 19 combined fractions were grouped: I (350 mg, fractions 1–3); II (130 mg, fraction 4); III (303 mg, fractions 5 and 6); IV (145 mg, fraction 7); V (124 mg, fraction 8); VI (405 mg, fractions 9–11); VII (304 mg, fractions 12 and 13); VIII (152 mg, fraction 14); IX (330 mg, fraction 15); X (474 mg, fraction 16); XI (500 mg, fraction 17); XII (330 mg, fractions 18 and 19); XIII (200 mg, fractions 20–24); XIV (32 mg, fractions 25 and 26); XV (90 mg, fractions 27 and 28); XVI (85 mg, fraction 29); XVII (150 mg, fraction 30); XVIII (370 mg, fraction 31) and XIX (22 mg, fraction 32).The pooled antifungal fractions IX–XI (1100 mg), were applied to a Sephadex LH-20 column (43 cm length, 4 cm i.d.), eluting with MeOH, yielding 15 sub-fractions (25 mL each) after TLC. The strongest antifungal sub-fraction 2 (270 mg) was successively chromato-

Table 1
Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of propolis extracts, *Larrea nitida* exudates and compounds isolated against yeasts and dermatophytes (MIC and MFC values in μg/mL).

Extract or compound	*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
PPEE	125	125	125	125	125	125	125	125	125	125	62.5	62.5	1000	>1000
PDCME	62.5	125	125	125	31.25	31.25	31.25	62.5	62.5	62.5	62.5	62.5	62.5	62.5
PEtOAcE	125	125	250	250	62.5	62.5	250	250	62.5	62.5	62.5	62.5	125	125
PEtOHE	500	500	1000	>1000	1000	1000	500	1000	250	250	250	250	250	500
PMeoHE	500	500	500	500	125	125	62.5	62.5	250	250	250	250	250	250
*Ln*DCME	62.5	125	125	125	125	125	50	125	125	125	62.5	625	62.5	625
C 1	62.5	125	62.5	125	31.25	31.25	31.25	31.25	62.5	62.5	62.5	62.5	62.5	62.5
C 2	250	250	250	250	250	250	250	250	62.5	62.5	62.5	62.5	62.5	62.5
C 3	62.5	125	125	250	62.5	62.5	62.5	62.5	31.25	31.25	15.6	31.25	15.6	31.25
C 4	>250		>250		>250		>250		250	250	250	250	250	250
C 5	>250		>250		>250		>250		>250		>250		>250	
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 or compound that inhibit 100% of the growth control. *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 Reference; PPEE, propolis petroleum ether extract; PDCME, propolis dichloromethane extract; PEtOAcE, propolis ethyl acetate extract; PEtOHE, propolis ethanol extract; PMeOHE propolis methanol extract; *Ln*DCME, *L.nitida* dichloromethane exudates; C 1, 3 -methyl-nordihydroguaiaretic acid; C 2, nordihydroguaiaretic acid; C 3, 4-[4-(4-hydroxy-phenyl)-2,3-dimethyl-butyl]-benzene-1,2-diol; C 4, meso-(rel 7S,8S,7'R,8'R)-3,4,3',4' -tetrahydroxy-7,7'-epoxylignan; C 5, (7S, 8S,7'S, 8'S)-3,3',4'-trihydroxy-4-methoxy-7,7'-epoxylignan; C 6, isomer of C 1; amp B, amphotericin B; keto, ketoconazole; terb, terbinafine; **–** : Not tested.

graphed on silica gel (column length 40 cm, internal diameter 2 cm; 50 g silica gel (0.063-0.2 mesh, Merck 60); eluant: PE–PE:EtOAc–EtOAc gradient to afford 247 mg of pure compound **1**.

The PEtOAcE extract (5.2 g) was permeated on a Sephadex LH-20 column (43 cm length, 4 cm i.d., eluant: MeOH). After TLC comparison (silica gel, PE:EtOAc 70:30), 15 combined fractions (A1-A15) were obtained. Separation of A5 (294 mg) by preparative HPLC with MeOH:H_2O (70:30) as eluant afforded **2** (25 mg) and **5** (38 mg) and one impure fraction (12 mg), which was purified by preparative TLC using DCM:MeOH 9:1 as mobile phase to yield **4** (3.2 mg). The strongest antifungal fraction A8 (410 mg) was successively permeated through a Sephadex LH-20 column (43 cm length, 4 cm i.d., eluant: MeOH) and by preparative HPLC (eluant: MeOH:H_2O 70:30) to yield 99.2 mg of compound **2** and 5.6 mg of compound **3**.

2.7. L. nitida exudate

The resinous exudate from the aerial parts of *L. nitida* Cav. was obtained by dipping the fresh plant (100 g) in cold CH_2Cl_2 (DCM, 1000 mL) at room temperature (26 °C) for 40 s. The DCM solution was filtered and evaporated under vacuum to give a semisolid yellow residue (*Ln*DCME; 9.35 g, 9.35% w/w yield).

2.8. ESI–MS–MS/MS analysis of main identified compounds

High resolution MS and MS/MS spectra of pure compounds were determined by direct infusion as described in Section 2. Parent ions as well as fragment ions are reported in the Table 6, using both positive and negative ionization modes. Then, PDCME, PEtOAcE, PMeOHE and *Ln*DCME were evaluated by HPLC–PDA–ESI–MS–MS/MS (auto, negative mode), confirming the identity of eluted pure compounds.

2.9. VOCs from aerial part of L. nitida and propolis

Volatile organic compounds (VOCs) were sampled from the headspace using a manual PMDS/DVB (Supelco Inc.) fibre. The fibre was activated according to the manufacturer's instructions. For the extractions, either plant material (5 g) or propolis (10 g) were placed in 40 mL amber glass vials, sealed with PTFE/silicone septa (volume ratio headspace/solid matrix was 1:3), equilibrated during 30 min at 42 °C and inserted into the injector port (250 °C) of the GC–MS system for 15 min. Identification of VOCs was performed by comparison of mass spectra with commercial libraries (ADAMS) using the AMDIS software (version 2.69), matching with MS literature as well as evaluation of the retention time of target compound relative to the series of *n*-alkanes (Table 7).

Table 2
Minimum inhibitory concentration (MIC) and Minimum fungicidal concentration (MFC) of PDCME; PEtOAcE, *Ln*DCME, **1** and **2** against clinical isolates *Trichophyton rubrum* and *T. mentagrophytes* (MIC and MFC in μg/mL).

Strain		PDCME		PEtOAcE		*Ln*DCME		MNDGA (**1**)		NDGA (**2**)		terb
		MIC_{100}	CFM	MIC_{100}	CFM	MIC_{100}	CFM	MIC_{100}	CFM	MIC_{100}	CFM	
T. rubrum	CCC 113	62.5	62.5	62.5	62.5	62.5	62.5	31.25	31.25	62.5	62.5	0.06
T. rubrum	CCC 110	31.25	31.25	31.25	31.25	31.25	31.25	31.25	31.25	62.5	62.5	0.06
T. rubrum	CCC 135	31.25	31.25	31.25	62.5	31.25	31.25	31.25	31.25	62.5	62.5	0.06
T. rubrum	CCC 136	62.5	62.5	62.5	62.5	31.25	31.25	31.25	62.5	62.5	62.5	0.06
T. rubrum	CCC 137	31.25	31.25	31.25	31.25	31.25	62.5	31.25	31.25	31.25	62.5	0.06
T. rubrum	CCC 139	62.5	62.5	62.5	62.5	31.25	31.25	62.5	62.5	62.5	62.5	0.12
T. rubrum	CCC 140	31.25	31.25	125	125	31.25	31.25	31.25	31.25	62.5	62.5	0.03
T mentagrophytes	ATCC 9972	62.5	62.5	125	125	62.5	62.5	62.5	62.5	62.5	62.5	0.06
T mentagrophytes	CCC 108	31.25	31.25	62.5	62.5	31.25	31.25	31.25	31.25	62.5	62.5	0.06
T mentagrophytes	CCC 364	62.5	62.5	125	125	15.6	31.25	125	125	62.5	62.5	0.06
T mentagrophytes	CCC 539	31.25	31.25	62.5	62.5	31.25	31.25	31.25	31.25	62.5	62.5	0.06
T mentagrophytes	CCC 738	62.5	125	125	125	31.25	31.25	62.5	62.5	62.5	62.5	0.06
T mentagrophytes	CCC 943	31.25	31.25	125	125	31.25	31.25	31.25	31.25	31.25	31.25	0.06
T mentagrophytes	CCC 726	62.5	62.5	62.5	62.5	31.25	31.25	62.5	62.5	62.5	62.5	0.12
T mentagrophytes	CCC 189	31.25	31.25	125	125	15.6	31.25	31.25	31.25	62.5	62.5	0.03

MIC_{100}, concentration of extract or compound that inhibit 100% of the growth control; PDCME, propolis dichloromethane extract; PEtOAcE, propolis ethyl acetate extract; *Ln*DCME, *L.nitida* dichloromethane exudate; MNDGA (**1**), 3́methyl-nordihydroguaiaretic acid; NDGA (**2**), nordihydroguayaretic acid; *T. rubrum*, *Trichophyton rubrum*; *T. mentagrophytes*, *Trichophyton mentagrophytes*; ATCC, American Type culture Collection; CCC, Center of Micological Reference (Rosario, Argentina); terb, terbinafine.

Table 3
Minimum inhibitory concentration (MIC) and Minimum fungicidal concentrations (MFC) of PDCME, PEtOAcE and *Ln*DCME against clinical isolates of *Candida* genus (MIC and MFC in μg/mL).

Strain		PDCME		PEtOAcE		*Ln*DCME		amp B	ket
		MIC_{100}	MFC	MIC_{100}	MFC	MIC_{100}	**MFC**	MIC_{100}	MIC_{100}
C. albicans	ATCC 10231	62.5	125	125	125	125	125	1	0.5
C. albicans	CCC 125-2000	125	250	125	250	125	125	0.78	6.25
C. albicans	CCC 126-2000	62.5	62.5	62.5	125	125	125	1.56	1.56
C. albicans	CCC 127-2000	125	125	125	125	62.5	62.5	0.78	6.25
C. albicans	CCC 128-2000	62.5	62.5	62.5	125	62.5	62.5	1.56	6.25
C. albicans	CCC 129-2000	62.5	62.5	62.5	125	125	125	0.78	12.5
C. albicans	CCC 130-2000	125	250	125	250	125	125	0.39	6.25
C. glabrata	CCC 115-2000	125	250	125	125	125	250	0.39	1.56
C. parapsilopsis	CCC 124-2000	125	250	125	250	62.5	125	0.78	0.78
C. lusitaniae	CCC 131-2000	62.5	125	125	125	125	250	0.39	25
C. colliculosa	CCC 122-2000	125	125	125	125	62.5	62.5	0.36	0.78
C. krusei	CCC 117-2000	250	>250	250	>250	125	125	0.39	50
C. kefyr	CCC 123-2000	62.5	125	62.5	125	62.5	62.5	0.78	0.78
C. tropicalis	CCC 131-2000	62.5	125	62.5	125	125	250	0.5	0.125

MIC_{100}, concentration of extract that induced 100% reduction of the growth control; *C. albicans, Candida albicans*; *C. glabrata*, *Candida glabrata*; *C. parapsilopsis, Candida parapsilopsis*; *C. lusitaniae*, *Candida lusitaniae*; *C. colliculosa*, *Candida colliculosa*; *C. krusei; Candida krusei*; *C. kefyr*, *Candida kefyr*; *C. tropicalis*, *Candida tropicalis;* ATCC, American Type culture Collection; CCC, Center of Micological Reference; PDCME, propolis dichloromethane extract; PEtOAcE, propolis ethyl acetate extract, *Ln*DCME, *L. nitida* dichloromethane exudates; amp B, amphotericin B; ket, ketoconazole.

Table 4
Minimal inhibitory concentrations (MIC) and Minimal fungicidal concentrations (MFC) of PDCME, PEtOAcE and *Ln*DCME against clinical isolates *Cryptococcus neoformans* (MIC and MFC in μg/mL).

Strain		*Ln*DCME		PDCME		PEtOAcE		amp B	ket	itz	5FC	vcz
		MIC_{100}	MFC	MIC_{100}	MFC	MIC_{100}	MFC	MIC_{100}	MIC_{100}	MIC_{100}	MIC_{100}	MIC_{100}
C. neoformans	ATCC 32264	62.5	62.5	125	125	62.5	62.5	0.25	0.25	0.15		
C. neoformans	IM 983040	62.5	62.5	125	125	62.5	62.5	0.13	–	<0.015	7.8	<0.015
C. neoformans	IM 972724	125	125	250	250	62.5	62.5	0.06	–	0.25	3.9	<0.015
C. neoformans	IM 042074	62.5	62.5	125	125	62.5	62.5	0.25	–	<0.015	3.9	<0.015
C. neoformans	IM 983036	62.5	62.5	62.5	62.5	62.5	62.5	0.13	–	<0.015	7.8	<0.015
C. neoformans	IM 00319	125	125	125	125	62.5	62.5	0.25	–	<0.015	n.t.	<0.015
C. neoformans	IM 972751	125	125	62.5	62.5	62.5	62.5	0.25	–	<0.015	15.6	0.030
C. neoformans	IM 031631	62.5	125	125	250	62.5	62.5	0.13	–	0.25	7.8	0.25
C. neoformans	IM 031706	62.5	62.5	125	250	62.5	125	0.25	–	0.50	7.8	0.50
C. neoformans	IM 961951	125	250	125	125	62.5	62.5	0.06	–	<0.015	3.9	0.015
C. neoformans	IM 052470	125	125	125	250	62.5	62.5	0.50	–	<0.015	7.8	0.030

MIC_{100}, concentration of extract that inhibit 100% of the growth control; *C. neoformans*, *Cryptococcus neoformans*; ATCC, American Type culture Collection; IM, Malbran Institute; PDCME, propolis dichloromethane extract; PEtOAcE, propolis ethyl acetate extract, *Ln*DCME, *L. nitida* dichloromethane exudate; amp B, amphotericin B; ket, ketoconazole; itz, itraconazole; 5FC, 5-fluorocitosine; vcz, voriconazole; -: Not tested.

2.10. Antifungal activity

2.10.1. Microorganisms and media

For the antifungal evaluation, we used 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. The first panel was integrated by the following microorganisms: *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 *Microsporum gypseum* CCC 115. The second panel of clinical isolates included 13 *Candida* spp., 10 *C. neoformans*, 6 *T. rubrum* and 7 *T. mentagrophytes* strains (voucher numbers reported in Tables 2–5). Inocula of cell or spore suspensions were obtained according to reported procedures and adjusted to 1–5 $\times$ 10^3 cells/spores with colony forming units (CFU)/mL (CLSI, 2002, 2008).

2.10.2. Antifungal susceptibility testing

Minimum inhibitory concentration (MIC) of each extract or pure compound was determined by broth microdilution techniques, in according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2002, 2008). MIC values were determined in RPMI-1640 medium (Sigma, St. Louis, MO, USA), buffered to pH 7.0 with MOPS. Microtiter trays were incubated at 35 °C for yeasts and hialohyphomycetes and at 28–30 °C for dermatophytes strains 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 256 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 instead added to the well. Ketoconazole, terbinafine, itraconazole, 5-fluorocitosine, voriconazole 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 compound 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 compound for each isolate 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 plate buffered with MOPS (Remel, Lenexa, Kans). Inoculated plates were incubated at 30 °C, and MFCs were recorded after 48 h. The MFC was defined as the lowest concentration of each compound that resulted in total inhibition of visible growth in the plates (Rodero and Córdoba, 2007).

3. Results and discussion

3.1. Antifungal activity of propolis extracts and L. nitida exudate

The PPEE, PDCME, PEtOAcE, PEtOHE and PMeOHE extracts of the three collected raw propolis samples and *Ln*DCME were assayed for antifungal activity against the first panel of fungi (see Section 2.10.1).

Table 5
Minimum inhibitory concentrations (MIC) and Minimum fungicidal concentrations (MFC) of MNDGA and NDGA against clinical isolates of *Candida* genus and *Cryptococcus neoformans* (MIC and MCF in μg/mL).

Strain		MNDGA (**1**)				NDGA (**2**)				amp	ket.	itz	5FC	vcz
		MIC_{100}	MIC_{80}	MIC_{50}	CFM	MIC_{100}	MIC_{80}	MIC_{50}	CFM	MIC_{100}	MIC_{100}	MIC_{100}	MIC_{100}	MIC_{100}
C. albicans	ATCC 10231	62.5	62.5	31.25	125	250	250	125	250	1.0	0,5	–	–	–
C. albicans	CCC 125-2000	125	62.5	62.5	125	250	250	250	500	0.78	6.25	–	–	–
C. albicans	CCC 126-2000	62.5	31.25	31.25	62.5	250	250	125	250	1.56	1.56	–	–	–
C. albicans	CCC 127-2000	62.5	31.25	31.25	125	250	250	125	250	0.78	6.25	–	–	–
C. albicans	CCC 128-2000	62.5	31.25	15.6	62.5	250	250	125	250	1.56	6.25	–	–	–
C. albicans	CCC 129-2000	62.5	31.25	31.25	62.5	250	250	125	250	0.78	12.5	–	–	–
C. albicans	CCC 130-2000	125	125	62.5	250	250	250	125	250	0.39	6.25	–	–	–
C. glabrata	CCC 115-2000	125	62.5	62.5	125	250	250	250	500	0.39	1.56	–	–	–
C. parapsilopsis	CCC 124-2000	125	62.5	31.25	250	250	250	250	250	0.78	0.78	–	–	–
C. lusitaniae	CCC 131-2000	125	62.5	62.5	125	500	500	250	1000	0.39	25	–	–	–
C. colliculosa	CCC 122-2000	62.5	31.25	31.25	62.5	250	250	125	500	0.36	0.78	–	–	–
C. krusei	CCC 117-2000	125	125	62.5	250	500	500	250	1000	0.39	50	–	–	–
C. kefyr	CCC 123-2000	31.25	31.5	15.6	62.5	250	250	125	500	0.78	0.78	–	–	–
C. tropicalis	CCC 131-1997	62.5	62.5	31.25	125	250	250	125	500	0.5	0.125	–	–	–
C. neoformans	ATCC 32264	31.25	15.6	7.8	31.25	250	250	62.5	250	0.25	0.25	0.15	–	–
C. neoformans	IM 983040	31.25	31.25	15.6	31.25	250	250	125	250	0.13	–	<0.015	7.8	<0.015
C. neoformans	IM 972724	15.6	15.6	7.8	15.6	250	250	62.5	250	0.06	–	0.25	3.9	<0.015
C. neoformans	IM 042074	31.25	31.25	15.6	31.25	250	250	62.5	250	0.25	–	<0.015	3.9	<0.015
C. neoformans	IM 983036	31.25	31.25	15.6	31.25	–	–	–	–	0.13	–	<0.015	7.8	<0.015
C. neoformans	IM 00319	31.25	15.6	15.6	31.25	125	125	62.5	125	0.25	–	<0.015	n.t.	<0.015
C. neoformans	IM 972751	31.25	15.6	15.6	31.25	250	250	125	250	0.25	–	<0.015	15.6	0.030
C. neoformans	IM 031631	31.25	31.25	15.6	31.25	250	250	125	250	0.13	–	0.25	7.8	0.25
C. neoformans	IM 031706	31.25	31.25	31.25	31.25	250	250	125	250	0.25	–	0.50	7.8	0.50
C. neoformans	IM 961951	31.25	31.25	15.6	31.25	250	250	125	250	0.06	–	<0.015	3.9	0.015
C. neoformans	IM 052470	31.25	31.25	15.6	31.25	250	250	125	250	0.50	–	<0.015	7.8	0.030

MIC_{100}, MIC_{80} and MIC_{50}: concentrations of a compound that induced 100%, 80% and 50% reduction of the growth control respectively. *C. albicans, Candida albicans*; *C. glabrata, Candida glabrata*; *C. parapsilopsis, Candida parapsilopsis*; *C. lusitaniae, Candida lusitaniae; C. colliculosa, Candida colliculosa; C. krusei, Candida krusei; C. kefyr, Candida kefyr; C. tropicalis, Candida tropicalis; C. neoformans, Cryptococcus neoformans*; ATCC, American Type culture Collection; CCC, Center of Micological Reference; IM, Malbrán Institute. MNDGA (**1**), 3́methyl-nordihydroguaiaretic acid; NDGA (**2**), nordihydroguayaretic acid; amp B, amphotericin B; ket, ketoconazole; itz, itraconazole; 5FC, 5-fluorocitosine; vcz, voriconazole; –: Not tested.

Table 6
Compounds identified and quantified from propolis extracts (PMeOHE, PDCME, PEtOAcE) and *L. nitida* exudate (*Ln*DCME), indicating their Rt, HPLC-QTOF, UV, MS characteristics.

Peak number	RT (min)	$[M - H]^-$	QTOF MS/MS (fragments)	Molecular formula	Compounds	*Ln*DCME[a]	PMeOHE[a]	PDCME[a]	PEtOAcE[a]
1	6.3	315.1594	300.1355, 241.0473, 149.0593, 122.0360	$C_{19}H_{24}O_4$	**1**	20.97 ± 0,12	7.13 ± 1.05	14.10 ± 0.01	9.17 ± 0.79
2	4.6	301.1457	273.1496, 122.0373	$C_{18}H_{22}O_4$	**2**	30.41 ± 0.12	17.98 ± 0.47	10.07 ± 0.10	32.22 ± 5.49
3	5.9	285.1511	122.0376	$C_{18}H_{22}O_3$	**3**	7.38 ± 0.06	5.91 ± 0.32	6.97 ± 0.06	7.17 ± 0.69
4	3.9	329.1415	177.0924, 151.0402, 137.0239	$C_{19}H_{22}O_5$	**4**	2.68 ± 0.03	2.57 ± 0.01	2.58 ± 0.50	2.39 ± 0.09
5	2.0	315.1253	137.0246	$C_{18}H_{20}O_5$	**5**	2.91 ± 0.22	2.90 ± 0.04	2.29 ± 0.01	5.41 ± 0.51
6	6.4	315.1584	300.1353, 241.0471, 149.0590, 122.0365	$C_{19}H_{24}O_4$	**6 (Isomer of 1)**	5.60 ± 0.16	1.59 ± ± 0.20	6.97 ± 0.26	4.81 ± 0.29

[a] Data determinated by HPLC-QTOF-MS method, are reported in g compounds/100 g propolis and correspond to the mean standard deviation (SD) calculated from at least three replicates.

Results (Table 1) showed that all tested dermatophytes and yeasts were inhibited by the propolis extracts with MIC values between 62.5 and 1000 μg/mL. The dermatophytes *M. gypseum*, *T. mentagrophytes* and *T. rubrum* were moderately inhibited with MIC values between 62.5 and 250 μg/mL. Among different extracts, PDCME and PEtOAcE showed the highest activities, MICs values from 62.5 to 125 μg/mL. The yeasts *C. albicans*, *S. cerevisiae* and *C. neoformans* were the most susceptible species (MICs values between 31.2 and 125 μg/mL), displaying PDCME the highest activity. It is worth to mention that the extracts were not only fungistatic but fungicide, with PDCME having the lowest MFC values (31.2 to 125 μg/mL). Regarding PMeOHE inhibit the growth of *S. cerevisiae* and *C. neoformans* with MICs of 62.5 and 125 μg/mL. The species of *Aspergillus* genus were less sensitive to propolis extracts (MICs between 250 and 1000 μg/mL, data not shown).

Similarly, *Ln*DCME exhibited antifungal activity against dermatophytes and yeasts with MIC values ranging from 50 to 125 μg/mL, while it was slightly active against species of *Aspergillus* genus (250–1000 μg/mL, data not shown) in according to previous report on *L. tridentata* (Vargas-Arispuro et al., 2005).

In order to gain insight to their activity spectrum , extracts displaying MIC values <125 μg/mL (PDCME, PEtOAcE and *Ln*DCME) against dermatophytes, *Candida* spp. and *C. neoformans* of the first panel were tested against a second panel of clinical isolates of *T. mentagrophytes*, *T. rubrum*, *Candida* spp. and *C. neoformans* (Tables 2–4).

Three extracts (PDCME, PEtOAcE and *Ln*DCME) displayed strong activities against *T. rubrum* second panel (Table 2), with MICs values of 31.25 μg/mL. These extracts were also fungicide against all strains (MFC range from 31.25 to 62.5 μg/mL), which is highly appreciated in antifungal drugs to avoid recurrence. Regarding to *T. mentagrophytes* clinical isolates, extracts showed a similar spectrum.

Over 90% of invasive *Candida* infections can be attributed to five species: *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis* and *C. krusei*. *C. albicans* remains the most common species, but in recent

Table 7
Chemical composition for the volatile profile of *L. nitida* and propolis.

Compound	RI[a]	*L. nitida*	Propolis	Identification[b]
2-Heptanone	889	+	+	GC–MS; RI
Tricyclene	935	+	+	GC–MS; RI
Camphene	952	+	+	GC–MS; RI
(E)-hexenol acetate	978	+		GC–MS; RI
N-octanal	1003	+		GC–MS; RI
Terpinene	1019	+	+	GC–MS; RI
o-Cymene	1027	+	+	GC–MS; RI
Limonene	1032	+	+	GC–MS; RI
Phellandrene	1034	+		GC–MS; RI
m-Cresol	1036		+	GC–MS; RI
Terpinene	1061	+	+	GC–MS; RI
Acetophenone	1070	+	+	GC–MS; RI
Terpinolene	1088		+	GC–MS; RI
2-Nonanone	1091	+	+	GC–MS; RI
p-Cymenene	1092	+	+	GC–MS; RI
Linanool	1100	+	+	GC–MS; RI
N-nonanal	1105		+	GC–MS; RI
Benzyl acetate	1164		+	GC–MS; RI
Borneol	1186	+	+	GC–MS; RI
4-Terpineol	1197	+	+	GC–MS; RI
Methyl salicylate	1199	+		GC–MS
Terpineol	1206	+	+	GC–MS; RI
N-decanal	1433		+	GC–MS; RI
(E)-Caryophyllene	1433	+	+	GC–MS; RI
cis-Bergamotene	1440		+	GC–MS; RI
Humulene	1469		+	GC–MS; RI
AR-curcumene	1487		+	GC–MS; RI
Hexadecane	1524		+	GC–MS; RI
cis-Guaiene	1638	+	+	GC–MS; RI
Eudesmol	1670	+	+	GC–MS; RI
Nonadecane	1777		+	GC–MS; RI
Eicosane	1791		+	GC–MS; RI

[a] Experimental RI determined on the DB-5 column relative to homologous series of *n*-alkanes.
[b] Compounds were identified by comparison of their retention indices (RI) and mass spectra with literature data and reference compound.

years has a considerable increase in the proportion of non-albicans *Candida* species (Infante-López and Rojo-Conejo, 2009). PDCME, PEtOAcE and *Ln*DCME showed similar and good activities against this second panel *Candida* spp. with MICs between 62.5 and 125 μg/mL.

Table 4 shows results of antifungal activities of PDCME, PEtOAcE and *Ln*DCME against clinical isolates of *C. neoformans*. PEtOAcE was the most active as fungistatic and fungicide against all clinical isolates with equal MICs and MFC (62.5 μg/mL). The activity of this extract was comparable to that shown by the *Ln*DCME (MICs between 62.5 and 125 μg/mL). PDCME showed a slightly lower but significant antifungal activity against all clinical isolates of *C. neoformans* (MICs values from 62.5 to 125 μg/mL).

These results undoubtedly give a scientific support to the traditional use of propolis from the studied area for fungal related infections. It is worthy to consider that the signs or symptoms that are related to a traditional antifungal use are easy to see such as skin or mucosal conditions (tineas, athlete's foot, dandruff, etc.), which are usually produced by dermatophytes (*Trichophyton* and *Microsporum* spp.) (Weitzman and Summerbell, 1995).

3.2. Antifungal activity of main isolated compounds

Pure compounds **1**-**5,** identified during the bioassay-guided fractionation of propolis, were also assessed for antifungal effect. Regarding their activity against dermatophytes, compounds **1**-**2** were fungistatic and fungicidal against all the strains of the first panel, with equal MICs and MFC (62.5 μg/mL) (Table 1). Compound **3** was the most active metabolite, showing a selective and strong activity against dermatophytes species of the first panel with MICs values between 15.6 and 31.25 μg/mL. *T. mentagrophytes* and *T. rubrum* presented the lowest MICs values (15.6 μg/mL). Compound **4** was slightly active against dermatophytes with MICs of 250 μg/mL, while compound **5** was inactive (MIC > 250 μg/mL) (Table 1).

On the other hand, compounds **1**, **2** and **3** were active against the tested yeasts (MICs between 31.25 and 250 μg/mL). Compound **1** showed fungistatic and fungicidal effect against *S. cereviceae* and *C. neoformans* (MICs and MFCs values of 31.25 μg/mL) (Table 1).

Compounds **1** and **2** were also tested against the second panel. Lignan **1** was active against 5/6 clinical isolates of *T. rubrum* and four out of seven strains of *T. mentagrophytes* with equal MICs and MFCs (31.25 μg/mL). Compound **2** displayed activity against *T. rubrum* and *T. mentagrophytes* with MICs and MFCs values between 31.25 and 62.5 μg/mL (Table 2). There is a close similarity in the results against dermatophytes between PDCME, PEtOAcE, *Ln*DCME and compounds **1-2**. This suggests that the activity of this propolis could be partially attributed to the presence of **1** and **2** in its composition or an additive action or synergism with other compounds is possible.

In addition, lignans **1** and **2** were tested against clinical isolates, including *Candida* strains (*C. albicans* and non-albicans *Candida* spp.) and strains of *C. neoformans* 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 minimum fungicide concentration (MFC) (Table 5). The application of less stringent endpoints such as MIC_{80} and MIC_{50} has been showed to consistently represent the *in vitro* activity of compounds (CLSI, 2002) and many times provides a better correlation with other measurements of antifungal activity such as the MFC (Ernst et al., 2002).

The selection of *Candida* and *Cryptococcus* strains for the second panel was due to the relevance of these fungi in the epidemiology of fungal infections. *Candida* spp. are among the leading causes of nosocomial blood stream infections worldwide and, although *C. albicans* was in the past associated with invasive mycoses, at present non-albicans *Candida* spp. (*C. tropicalis, C. glabrata, C. parapsilopsis, C. krusei* and others) comprise more than half of the isolates of candidosis in immunocompromised hosts (Almirante et al., 2005). In turn, *C. neoformans* remains an important life-threatening complication for weakened patients, particularly for those who have undergone transplantation of solid organs, and therefore, new compounds acting against this fungus are highly welcome (Singh, 2003).

Results (Table 5) showed that MIC values of compound **1** were particularly low against *C. neoformans,* with values as low as 15.6 μg/mL in one strain and 31.25 μg/mL in 9/10 strains tested. Regarding non-albicans *Candida* spp., the most sensible strains was *C. kefir* (MIC of 31.25 μg/mL). Compound **2** was moderately active against all tested clinical isolates (MIC values between 125 and 250 μg/mL). These results give additional support to the antifungal activity of the medicinal plant *L. nitida*, an endemic species from Argentina.

Regarding *Aspergillus* genus, the three tested species are the cause of infections in the respiratory tract in both immunocompetents and immunocompromised hosts. Among them, *A. fumigatus* is the main pathogen causing invasive aspergillosis, which is usually lethal in immunocompromised hosts. Since the incidence of these highly sporulating fungi has increased sevenfold from 1970 to date, new agents for treating this fungal infection are highly welcome (Denning and Stevens, 1990). The three species of *Aspergillus* genus were less sensitive to propolis extracts and *L. nitida* exudate (MICs values between 250 and 1000 μg/mL, data not shown). The moderate antifungal activity of the extracts may be related to the presence of lignans **1** and **2**, which has been reported as effective in inhibiting mycelial growth of *A. flavus* and *A. parasiticus* (MICs values between 300 and 500 μg/mL) (Vargas-Arispuro et al., 2005).

3.3. Isolation and characterization of antifungal compounds from propolis extracts and LnDCME. Evaluation of the botanical origin of studied propolis

The geographical location and associated flora determine the propolis composition. The genus *Larrea* is almost endemic to Argentina. In the province of San Juan three out of four species reported for Argentina are present: *L. nitida, L. cuneifolia* and *L. divaricata*, which are commonly known as the creosote bush, and are widely used in the folk medicine (Ladio and Lozada, 2009). *L. nitida* is distributed mainly in pre-Andes mountain area from the province of Salta (north Argentina) to the province of Chubut (south Argentina). In the province of San Juan, *L. nitida* is present in the west pre-Andes mountain area (Calingasta and Iglesia) between 1500 and 3000 m.a.s.l. (Kiesling, 2003). Bioassay-guided isolation of the antifungal propolis extracts PDCME, PEtOAcE and *L. nitida* exudate (*Ln*DCME) led to the isolation of 3́methyl-nordihydroguaiaretic acid (MNDGA) **1** and nordihydroguaiaretic acid (NDGA) **2** and compound **3** (4-[4-(4-hydroxy-phenyl)-2,3-dimethyl-butyl]-benzene-1,2-diol) as the compounds mainly responsible for the antifungal activity. Additionally, two epoxylign ans: compound **4** meso-(rel 7S,8S,7'R,8'R)-3,4,3',4'-tetrahydroxy-7,7'-epoxylignan and compound **5** (7S,8S,7'S,8'S)-3,3',4'-trihy droxy-4-methoxy-7,7'-epoxylignan) were isolated. Compounds **1-5** were identified by their spectroscopic data (^{1}H and ^{13}C NMR), which are in agreement with those reported in the literature (Abou-Gazar et al., 2004; Gnabre et al., 1995; Mabry et al., 1977). Further structural confirmation was achieved by HPLC–PDA–ESI–MS–MS/MS analyses on both exudates and propolis extracts from *L. nitida* (Fig. 1). Some of these and other lignans have been previously reported in extracts from *Larrea* spp. (Abou-Gazar et al., 2004; Gnabre et al., 1995; Mabry et al., 1975; Torres et al., 2003; Vargas-Arispuro et al., 2005). Table 7 shows the compounds which were identified and quantified from different propolis extracts and exudates from *L. nitida*, indicating their chromatographic characteristics in addition to high resolution MS and MS/MS spectra**.** Also, Table 6 shows compound **6** which, was identified by means of HPLC–PDA–ESI–MS–MS/MS analyses on both exudates and propolis extracts from *L. nitida*. Compound **6** displayed a molecular ion at m/z 315 (HPLC–ESI–MS: m/z: 315.1584 $[M-H]^{-}$), indicating a molecular formula of $C_{19}H_{24}O_4$. The mass spectra of compounds **1** and **6** are nearly identical. This fact supports that these compounds are isomers. As the structure of compound **1** has been unequivocally assigned to 3′-methyl-nordihydroguaiaretic acid, a tentative structure could be proposed for **6** as 4′-methyl-nordihydroguaiaretic acid. Compound **6** has been previously isolated from *Larrea tridentata* (Gnabre et al., 1995). From the resinous exudate of twigs end leaves of *L. nitida*, two lignans nor isoguaiacine, nor-dihydroguaiaretic acid (**2**) and ferulic acid were reported (Torres et al., 2003). However, to the extent of our knowledge, this is the first report on the isolation of **1–6** from propolis. In addition compound **1** and **3–6** were identified and reported for first time as constituents of *L. nitida* Cav.

Considering that *L. nitida* is the main floral and exudates source within the studied beehives' area, we decided to evaluate the possible relation between propolis samples and *L. nitida* exudates by HPLC–PDA–ESI–MS–MS/MS, by comparison of the chemical profiles of exudates and different propolis extracts (PDCME, PEtOAcE, PMeOHE and *Ln*DCME). In order to obtain exudate samples, fresh aerial parts of *L. nitida* were collected at Bauchaceta (Iglesia district, province of San Juan), and the corresponding extracts were prepared by dipping in dichloromethane (*Ln*DCME). Propolis samples were collected from beehives present in the same area.

Comparative HPLC–PDA–ESI–MS–MS/MS fingerprints of PDCME, PEtOAcE, PMeOHE and *Ln*DCME are shown in Fig. 2**.** Compounds **1–5** were detected and unambiguously identified by high resolution MS and by comparison with pure compounds isolated from extracts which were characterized by ^{1}H and ^{13}C NMR, high resolution MS and MS/MS. Pure compounds were also analyzed and quantified by HPLC–PDA–ESI–MS–MS/MS affording typical retention times (R_t) and MS information. The results of these analyses are summarized in Table 6.

In a previous paper we reported the chemical profile match of *Z. punctata* Cav. exudates with their corresponding propolis from the province of Tucumán (Argentina) using HPLC–ESI–MS/MS using a triple quadrupole instrument (Agüero et al., 2010).

In the present study, high-resolution time-of-flight mass spectrometry (TOF-MS) was used instead for the analysis of the chemical profiles. This technique provides a higher order mass identification than that afforded by nominal mass measurement obtained with other types of mass analyzers, such as triple-quadrupole MS and IT-MS (Zhou et al., 2010). Furthermore, ESI sources coupled to tandem quadrupole/collision cell/time-of-flight mass spectrometry (Q-TOF) allow the generation of both MS and MS/MS information with great accuracy, enabling structural information and exact mass, turning on-line HPLC–PDA–ESI–QTOF-MS a highly useful technique for the screening of natural products (Zhou et al., 2009).

Our current results give state of the art HPLC–PDA–ESI–QTOF-MS evidence on the chemical correspondence between native flora

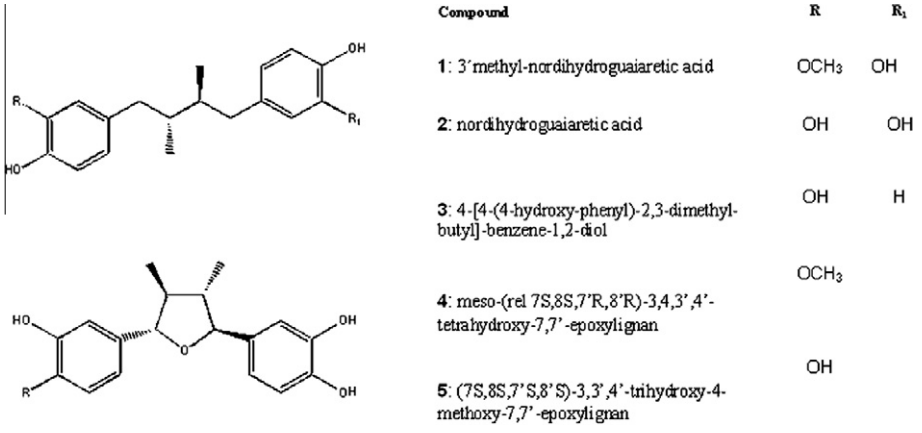

Fig. 1. Main antifungal lignans and epoxylignans from propolis extracts (PDCME, PEtOAcE and PMeOHE) and *Larrea nitida* exudate (*Ln*DCME) from the province of San Juan, Argentina.

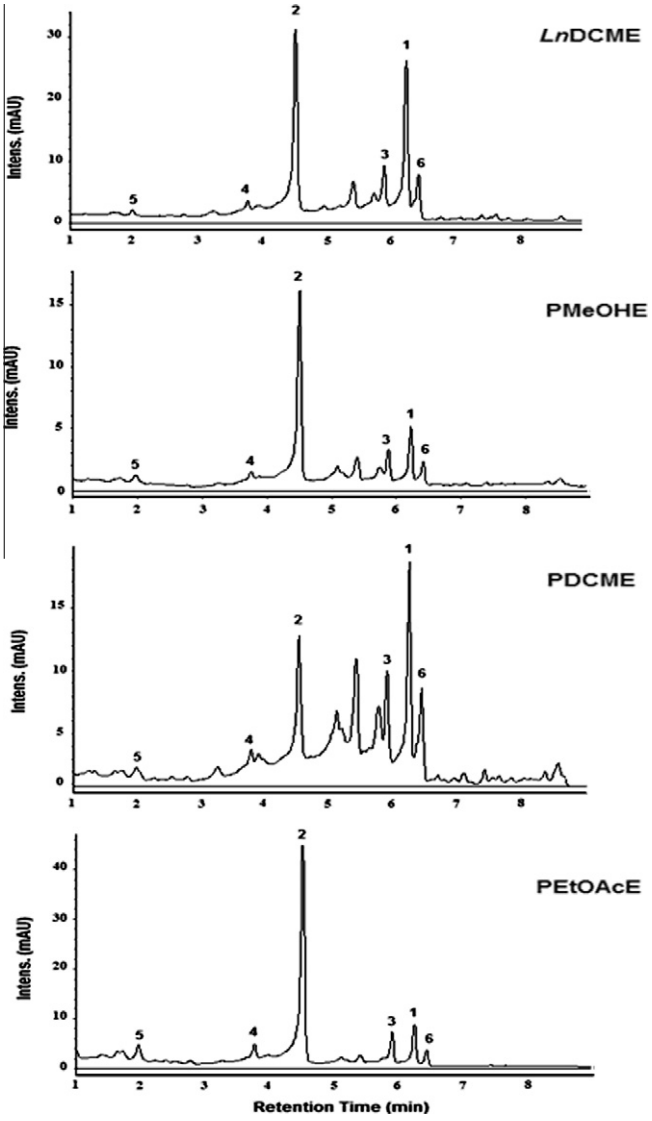

Fig. 2. HPLC-DAD-QTOF trace of *Ln*DCME, PMeOHE, PDCME and PEtOAcE. Peaks: **1**, 3́methyl-nordihydroguaiaretic acid, **2**, nordihydroguaiaretic acid, **3**, 4-[4-(4-hydroxy-phenyl)-2,3-dimethyl-butyl]-benzene-1,2-diol, **4**, meso-(rel 7S,8S,7'R,8'R)-3,4,3',4'-tetrahydroxy-7,7'-epoxylignan and **5**, (7S,8S,7'S,8'S)-3,3',4'-trihydroxy-4-methoxy-7,7'-epoxylignan, **6** isomer of 1.

(*L. nitida*) with propolis exudates. The strong antifungal activity of Andean propolis extracts from the province of San Juan was assessed for first time.

3.4. VOCs correspondence between L. nitida and propolis profiles

The VOCs profile of *L. nitida* reveals the predominance of monoterpenoids in comparison with a much complex profile for propolis (Table 7), including several hydrocarbon sesquiterpenes and C16-20 alkanes. It is noteworthy that 17 common constituents were detected in both samples, while other 12 compounds were only present in the propolis. VOCs from *L. nitida* also detected in the propolis were mainly monoterpenoids (Table 7). Among them, *o*-cymene and limonene were the most abundant monoterpene hydrocarbons present in *L. nitida*, both of them being conserved in the propolis (data not shown). These results show a relative similarity among the VOCs profile of *L. nitida* and that corresponding to the studied propolis, although several constituents, mainly some aldehydes and sesquiterpenes, were exclusively detected in the propolis. Among them, nonanal and α-*cis*-bergamotene, α-humulene and AR-curcumene have been found in Greek propolis (Melliou et al., 2007). Monoterpenes such as limonene, α- and β-terpinene, β-phellandrene and borneol have also been cited as constituents of Greek propolis (Melliou et al., 2007). To the best of our knowledge, this is the first report on the characterization of the VOCs profile for Argentinean propolis.

4. Conclusions

The findings in the present study support the potential of Argentinean Andean propolis to treat fungal diseases, and reinforce their use in Argentina as important complementary food due to their functional and biological properties. On the other hand, considering the close similarity between the propolis composition and those corresponding to *L. nitida* exudates and VOCs profiles, we suggest that *L. nitida* could be considered the botanical origin of the studied propolis. Compounds **1**–**6** were isolated from propolis for the first time, while compounds **1** and **3**–**6** were reported for first time as constituents of *L. nitida* Cav.

Conflict of Interest

None declared.

Acknowledgements

The authors are grateful to ANPCyT Argentina (PICTs 2010-0608, 2008-0554) for financial support; to CICITCA, Universidad Nacional de San Juan, Argentina. S.A.Z. and L.S. are grateful to UNR, ANPCyT and CONICET. Research at the University of Buenos Aires was supported by a Grant from UBA (X-163, Programación 2008-2011). M.B.A., L.S, L.L and B.L. hold fellowships of ANPCyT or CONICET. M.L.L., G.E.F., M.S., J.P. and D.A.W. are researchers from CONICET, Argentina. M.B.A is doctoral student in PROBIOL (UNCuyo).

References

Abou-Gazar, H., Bedir, E., Takamatsu, S., Ferreira, D., Khan, I.A., 2004. Antioxidant lignans from Larrea tridentata. Phytochemistry 65, 2499–2505.
Agüero, M.B., González, M., Lima, B., Svetaz, L., Sánchez, M., Zacchino, S., Feresin, G.E., Schmeda-Hirschmann, G., Palermo, J., Wunderlin, D., Tapia, A., 2010. Argentinean propolis from *Zuccagnia punctata* Cav. (Caesalpinieae) Exudates: phytochemical characterization and antifungal activity. J. Agric. Food Chem. 58, 194–201.
Ahn, M.R., Kumazawa, S., Usui, Y., Nakamura, J., Matsuka, M., Zhu, F., Nakayama, T., 2007. Antioxidant activity and constituents of propolis collected in various areas of China. Food Chem. 101, 1383–1392.
Almirante, B., Rodríguez, D., Park, B.J., Cuenca-Estrella, M., Planes, A.M., Almela, M., 2005. Epidemiology and predictors of mortality in cases of Candida bloodstream infection: Results from population-based surveillance, Barcelona, Spain, from 2002 to 2003. J. Clin. Microbiol. 43, 1829–1835.
Bankova, V.S., De Castro, S.L., Marcucci, M.C., 2000. Propolis: Recent advances in chemistry and plant origin. Apidologie 31, 3–15.
Banskota, A.H., Tezuka, Y., Kadota, S., 2001. Recent progress in pharmacological research of propolis. Phytother. Res. 15, 561–571.
Chaillou, L.L., Nazareno, M.A., 2009. Bioactivity of propolis from Santiago del Estero, Argentina, related to their chemical composition. LWT – Food Sci. Technol. 42, 1422–1427.
CSI, 2002. Reference method for broth dilution antifungal susceptibility testing of yeast. Approved Standard M27, A2.
CSI, 2008. Reference method for broth dilution antifungal susceptibility testing of filamentous fungi. Approved Standard M38, A2.
Denning, D.W., Stevens, D.A., 1990. Antifungal and surgical treatment of invasive aspergillosis: review of 2121 published cases. Rev. Infect. Dis. 12, 1147–1201.
Ernst, E., Roling, E., Petzold, R., Keele, D., Klepser, M., 2002. In vitro activity of micafungin (FK-463) against Candida spp.: microdilution, time-kill, and post-antifungal-effect studies. Antimicrob. Agents Chemother. 46, 3846–3853.
Gambier, M., 1988. La Fase Cultural Punta del Barro. San Juan, Argentina.
Ghisalberti, E.L., 1978. Propolis: A review. Bee World 60, 59–84.
Gnabre, J., Huang, R.C.C., Bates, R.B., Burns, J.J., Caldera, S., Malcomson, M.E., McClure, K.J., 1995. Characterization of Anti-HIV Lignans from Larrea tridentate. Tetrahedron 51, 12203–12210.

Infante-López, M.E., Rojo-Conejo, P., 2009. Utilidad clínica de la micafungina en el tratamiento de las candidiasis invasoras en el neonato. Rev. Iberoam. Micol. 26, 56–61.

Ishida, V.F., Negri, G., Salatino, A., Bandeira, M.F.C.L., 2011. A new type of Brazilian propolis: Prenylated benzophenones in propolis from Amazon and effects against cariogenic bacteria. Food Chem. 125, 966–972.

Isla, M.I., Nieva Moreno, M.I., Sampietro, A.R., Vattuone, M.A., 2001. Antioxidant activity of Argentine propolis extracts. J. Ethnopharmacol. 76, 165–170.

Isla, M.I., Paredes-Guzman, J.F., Nieva-Moreno, M.I., Koo, H., Park, Y.K., 2005. Some chemical composition and biological activity of northern Argentine propolis. J. Agric. Food Chem. 53, 1166–1172.

Kalogeropoulos, N., Konteles, S.J., Troullidou, E., Mourtzinos, I., Karathanos, V.T., 2009. Chemical composition, antioxidant activity and antimicrobial properties of propolis extracts from Greece and Cyprus. Food Chem. 116, 452–461.

Kiesling, R., 2003. Zygophyllaceae. In: Sigma, Estudio. (Ed.), Flora de San Juan. República Argentina. Volumen II, Buenos Aires, pp. 39–46.

Ladio, A.H., Lozada, M., 2009. Human ecology, ethnobotany and traditional practices in rural populations inhabiting the Monte region: Resilience and ecological knowledge. J. Arid. Environ. 73, 222–227.

Lima, B., Tapia, A., Luna, L., Fabani, M.P., Schmeda-Hirschmann, G., Podio, N., Wunderlin, D.A., Feresin, G.E., 2009. Main flavonoids, DPPH activity, and metal content allow determination of the geographical origin of propolis from the Province of San Juan (Argentina). J. Agric. Food Chem. 57, 2691–2698.

Mabry, T.J., Difeo, D.R., Sakakibara, M., Bohnstedt, C.F., Seigler, D., 1977. The natural products chemistry of *Larrea*. In: Mabry, T.J., Hunziker, J.H., Difeo, D.R. (Eds.), Creosote Bush Biology and Chemistry of Larrea in New World Desserts. Dowden HutchinSon and Ross Inc., Stroudsburg Pennsylvania, pp. 115–134.

Melliou, E., Stratis, E., Chinou, I., 2007. Volatile constituents of propolis from various regions of Greece–Antimicrobial activity. Food Chem. 103, 375–380.

Nagai, T., Inoue, R., 2004. Preparation and functional properties of water extract and alkaline extract of royal jelly. Food Chem. 84, 181–186.

Nieva Moreno, M.I., Isla, M.I., Cudmani, N.G., Vattuone, M.A., Sampietro, A.R., 1999. Screening of antibacterial activity of Amaicha del Valle (Tucumán, Argentina) propolis. J. Ethnopharmacol. 68, 97–102.

Nieva Moreno, M.I., Isla, M.I., Sampietro, A.R., Vattuone, M.A., 2000. Comparison of the free radical-scavenging activity of propolis from several regions of Argentine. J. Ethnopharmacol. 71, 109–114.

Park, Y.K., Alencar, S.M., Aguiar, C.L., 2002. Botanical origin and chemical composition of Brazilian propolis. J. Agric. Food Chem. 50, 2502–2506.

Quiroga, E.N., Sampietro, D.A., Soberon, J.R., Sgariglia, M.A., Vattuone, M.A., 2006. Propolis from the northwest of Argentina as a source of antifungal principles. J. Appl. Microbiol. 101, 103–110.

Rodero, L., Córdoba, S., 2007. Método de dilución en medio líquido. In: Zacchino, S., Gupta, M. (Eds.), Manual de Técnicas in vitro para la detección de compuestos antifúngicos. Corpus Ed, Rosario, pp. 85–97.

Singh, N., 2003. Treatment of opportunistic mycoses: how long is long enough? Lancet Infect. Dis. 3, 703–708.

Strobel, G., Daisy, B., Castillo, U., Harper, J., 2004. Natural products from endophytic microorganisms. Nat. Prod. 67, 257–268.

Torres, R., Urbina, F., Morales, C., Modak, B., Delle Monache, F., 2003. Antioxidant properties of lignans and ferulic acid from the resinous exudate of *Larrea nitida*. J. Chil. Chem. Soc. 48, 61–63.

Vargas-Arispuro, I., Reyes-Baeza, R., Rivera-Castañeda, G., Martínez-Téllez, M.A., Rivero-Espejel, I., 2005. Antifungal lignans from the creosotebush (Larrea tridentata). Ind. Crop. Prod. 22, 101–107.

Viuda-Martos, M., Ruiz-Navajas, Y., Fernández-López, J., Pérez-Álvarez, A., 2008. Functional Properties of Honey, Propolis, and Royal Jelly. J. Food Sci. 73, 117–124.

Weitzman, I., Summerbell, R.C., 1995. The dermatophytes. Clin. Microbiol. Rev. 8, 240–259.

Zampini, I.C., Vattuone, M.A., Isla, M.I., 2005. Antibacterial activity of Zuccagnia punctata Cav Ethanolic extracts. J. Ethnopharmacol. 102, 450–456.

Zampini, I.C., Villarini, M., Moretti, M., Dominici, L., Isla, M.I., 2007. Evaluation of genotoxic and antigenotoxic effects of hydroalcoholic extracts of Zuccagnia punctata Cav. J. Ethnopharmacol. 115, 330–335.

Zhou, J.L., Xin, G.Z., Shi, Z.Q., Ren, M.T., Qi, L.W., Li, H.J., Li, P., 2010. Characterization and identification of steroidal alkaloids in Fritillaria species using liquid chromatography coupled with electrospray io nization quadrupole time-of-flight tandem mass spectrometry. J. Chromatogr. A. 1217, 7109–7122.

Zhou, Y., Xu, G., Choi, F.F.K., Ding, L.S., Han, Q.B., Song, J.Z., Qiao, C.F., Zhao, Q.S., Xu, H.X., 2009. Qualitative and quantitative analysis of diterpenoids in Salvia species by liquid chromatography coupled with electrospray ionization quadrupole time-of-flight tandem mass spectrometry. J. Chromatogr. A. 1216, 4847–4858.