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Argentinean Propolis from *Zuccagnia punctata* Cav. (Caesalpinieae) Exudates: Phytochemical Characterization and Antifungal Activity

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This paper reports the in vitro antifungal activity of propolis extracts from the province of Tucumán (Argentina) as well as the identification of their main antifungal compounds and botanical origin. The antifungal activity was determined by the microdilution technique, using reference microorganisms and clinical isolates. All dermatophytes and yeasts tested were strongly inhibited by different propolis extracts (MICs between 16 and 125 μg mL^{-1}). The most susceptible species were *Microsporum gypseum*, *Trichophyton mentagrophytes*, and *Trichophyton rubrum*. The main bioactive compounds were 2′,4′-dihydroxy-3′-methoxychalcone **2** and 2′,4′-dihydroxychalcone **3**. Both displayed strong activity against clinical isolates of *T. rubrum* and *T. mentagrophytes* (MICs and MFCs between 1.9 and 2.9 μg mL^{-1}). Additionally, galangin **5**, pinocembrin **6**, and 7-hydroxy-8-methoxyflavanone **9** were isolated from propolis samples and *Zuccagnia punctata* exudates, showing moderate antifungal activity. This is the first study matching the chemical profile of *Z. punctata* Cav. exudates with their corresponding propolis, giving strong evidence on the botanical origin of the studied propolis.

KEYWORDS: Argentinean propolis; antifungal activity; chalcones; HPLC-ESI-MS/MS

INTRODUCTION

Propolis is a resinous product that bees collect from botanical sources to seal their hives. The chemical composition of propolis differs significantly according to its geographical and botanical origins, mainly because bees gather the resins and plant exudates from available sources, which vary from region to region depending on the climate, soil, and other factors (*1−4*). Propolis is worldwide recommended for external topical use, relieving various types of bacterial and fungal dermatitis (*5*, *6*). The high rate of fungal infections, along with the limited efficacy and high toxicity of available antifungal drugs, renders imperative the need of new fungicides against the most clinically important fungi (*7*). Natural fungicides could present advantages over synthetic ones because of good efficacy and low toxicity. However, both efficacy and toxicity must be tested before such natural products are used in humans.

The different propolises from northwestern Argentina and, particularly, from the province of Tucumán are recommended for treating bacterial, fungal, and viral infections and inflammatory conditions as well as immunostimulants. Studies undertaken in the past 10 years have reported antioxidant, free radical scavenger, antimicrobial, and anticarcinogen activities for propolis from northern Argentina (*2*, *8−14*). There are few reports on the chemical composition of propolis from Argentina. Propolis extract from the province of Tucumán and its isolated compounds pinocembrin and galangin have antifungal effect against filamentous fungi of agricultural relevance (*13*). The chemical characterization as well as the assessment of the geographical origin of propolis from several areas of the province of San Juan has been recently reported by our group, including the isolation, identification, and quantitation of six main flavonoids,

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comprising the flavanones 7-hydroxy-8-methoxyflavanone, pinocembrin, and pinobanksin, the flavones chrysin and tectochrysin, and the flavonol galangin (*4*).

However, the activity of propolis from the province of Tucumán against human opportunistic and pathogenic fungi has not been yet reported and, therefore, there is no support for its traditional use as an antifungal. Nevertheless, the antifungal behavior of propolis from Tucumán (as well as of other propolises of different geographical regions) will have significance only if reported together with its chemical composition and possible botanical origin.

Our main goal was to characterize different propolis samples, collected in the province of Tucumán (Argentina) by assay-guided isolation of their most bioactive compounds, evaluating their antifungal efficiency, and characterizing their botanical origin by analysis of the chemical profile isolated from both propolis and endemic species *Zuccagnia punctata* Cav. Therefore, we looked for the correspondence between bioactive compounds present in plants from the sampling area with those present in propolis samples from these plants.

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 the equipment Arium 611 UV (Sartorius, Germany). TLC analysis was carried out on aluminum-coated silica gel plates (Sigma Aldrich, Inc., USA).

2.2. Equipment. Nuclear magnetic resonance (NMR) spectra were obtained using 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. ESI-HRMS spectra were recorded on a Bruker micrOTOFQ2 mass spectrometer.

The UV spectra were measured using a Heλios α V-3.06 UV−vis spectrophotometer; MeOH was used as solvent. HPLC-DAD was used for the identification and quantification of the main components of crude extract fractions and as a purity criterion for the isolated compounds. The HPLC-DAD equipment used was a Merck-Hitachi (La Chrom, USA) consisting of an L-7100 pump, an L-7455 UV diode array detector, and a D-7000 chromatointegrator. A C18-RP column (Phenomenex, 5 μm, 250 mm × 4.60 mm. i.d.) was used. UV−vis spectra were recorded from 200 to 400 nm, with detection at 254 nm. Gradient elution was carried out with water/0.1% formic acid (solvent A) and 20% solvent A in 80% acetonitrile (solvent B) at a constant flow rate of 0.75 mL min^{-1}. The linear gradient elution program was as follows: 0−15 min, 40−60% B; 15−30 min, 60% B; 30−40 min, 60−80% B; 40−50 min, 80−100% B; 50−60 min, back to 40% B.

HPLC-ESI-MS/MS analyses were carried out using a Varian 1200 triple quadrupole, equipped with an ESI ion source operated at either positive or negative mode with nitrogen as main gas (75.5 psi), drying gas (21 psi, 300 °C), and nebulizing gas (50 psi); needle, 5000 V' and shield, 600 V. Ultrapure synthetic air (99.9% pure; Indura, Argentina) was used as nebulizing gas during negative mode analysis. Pure compounds were first characterized by direct infusion to ESI using a syringe pump (Harvard Apparatus 11 Plus), recording MS and MS/MS spectra. HPLC was then used to separate and characterize extract profiles (qualitative analysis only). A C18-RP column (Waters Symmetry 3.5 μm × 100 mm × 2.1 mm. i.d.) was used. Samples were introduced in the HPLC using a Varian ProStar 410 autosampler equipped with a 20 μL loop and column oven operated at 40 °C. Solvent delivery was performed at 0.3 mL min^{-1} by two pumps, Varian Prostar 210 Dynamax, using ultrapure water supplemented with 0.1% formic acid (A) and methanol with 0.1% formic acid (B), and a program starting with 40% B, changing to 100% B within 20 min, heldfor 5 min, returning to 40% B in 1 min, and keeping this condition for an additional 5 min to achieve column stabilization before the next run (total run time = 30 min). Eluted compounds were recorded using MRM mode by selecting the corresponding parent ion ($M + H^+$ or $M - H^+$) at the first quadrupole (Q1), fragmented in Q2 using Ar with variable collision energy, and analyzing resulting ions at Q3.

2.3. Propolis Samples. Three representative raw propolis samples were kindly provided by beekeepers from the Amaicha del Valle district, province of Tucumán, Argentina. Hives were placed in the same geographical area where *Z. punctata* grew abundantly. Propolis were collected with propolis traps, minimizing its contamination with foreign substances, and stored frozen at −20 °C. References are deposited at the Instituto de Biotecnología, National University of San Juan, identified as P1, P2, and P3.

2.4. Extracts. Twenty grams of each propolis (P1, P2, and P3) was extracted at room temperature using MeOH (200 mL × 3 times × 24 h each), filtered, and concentrated under reduced pressure to afford the corresponding crude MeOH extract (MEP). Extraction yields (% w/w) were as follows: MEP1, 72.7%; MEP2, 60.8%; MEP3, 66.1%. These extracts were submitted to antifungal evaluation according to section 2.8.

2.5. Isolation of Antifungal Compounds. The MEPs of the three propolis samples were compared by TLC, HPLC-DAD, and HPLC-ESI-MS/MS. Extracts were pooled because all of them showed similar chromatographic profiles.

A representative sample (2.2 g) of the pooled MP extract was fractionated by column chromatography (CC) (column length, 70 cm; internal diameter (i.d.), 3.5 cm) on silica gel (200 g, 0.063−0.2 mesh, Merck 60). The column was eluted with a solvent gradient starting with petroleum ether (PE) (300 mL), followed by PE/EtOAc mixtures to reach 100% EtOAc. About 50 fractions of 100 mL each were obtained. After TLC comparison (silica gel, PE/EtOAc 80:20 as the mobile phase; detection under UV light followed by spraying with diphenylboric acid/ethanolamine complex in MeOH), fractions with similar TLC patterns were combined and evaluated for antifungal activity according to section 2.8. Thirty-two groups of fractions were obtained: 1 (10 mg), 2 (7.7 mg), 3 (12 mg), 4 (10 mg), 5 (3 mg), 6 (5.6 mg,), 7 (7.4 mg), 8 (8.5 mg), 9 (14 mg); 10 (15 mg); 11 (74 mg); 12 (90 mg); 13 (200 mg); 14 (81 mg); 15 (80 mg); 16 (65 mg); 17 (45 mg); 18 (28.5 mg); 19 (27.8 mg), 20 (40 mg); 21 (30.8 mg), 22 (41 mg), 23 (30 mg), 24 (41 mg), 25 (38 mg), 26 (50 mg), 27 (56 mg), 28 (73 mg), 29 (93 mg), 30 (76 mg), 31 (105 mg), and 32 (19 mg).

After purification on Sephadex LH-20 (column length, 30 cm; i.d., 1.5 cm; PE/MeOH/$CHCl_3$ 2:1:1), the combined fractions 6−9 (25 mg) afforded 10 mg of galangin-7-methyl ether (izalpinin) **1** and 6 mg of 7-hydroxy-8-methoxyflavanone **9**.

The antifungal fractions 10−13 (450 mg) afforded after permeation on a Sephadex LH-20 column (column length, 40 cm; i.d., 5 cm; PE/MeOH/$CHCl_3$ 2:1:1) 20 mg of 2′,4′-dihydroxy-3′-methoxychalcone **2**, 15 mg of 2′,4′-dihydroxychalcone **3**, 5 mg of galangin **5**, and 5 mg of pinocembrin **6**.

The fraction pool 14−16 (200 mg) was repeatedly chromatographed on silica gel (column length, 40 cm; i.d., 2 cm; 50 g of silica gel (0.063−0.2 mesh, Merck 60); eluant PE/EtOAc gradient to afford 60 and 12 mg of pure chalcone **3** and pinostrobin **10**, respectively.

The pooled MeOH-soluble fractions 20−25 (200 mg) were permeated successively on a Sephadex LH-20 column (column length, 30 cm; i.d., 1.5 cm; MeOH) to afford 8 mg of 7-hydroxyflavanone **4** and 10 mg of 7-hydroxy-8-methoxyflavanone **9**.

The pooled MeOH-soluble fractions 27−30 (298 mg) were permeated successively on a Sephadex LH-20 column (column length, 30 cm; i.d., 1.5 cm; MeOH) to afford 12 mg of pure kaempferol 7-methyl ether (rhamnocitin) **7** and **5** mg of 7-hydroxyflavanone **4**. The compounds were characterized by NMR and UV spectra with a purity ≥97%.

2.6. *Z. punctata* Exudate. The resinous exudate from the aerial parts of *Z. punctata* Cav. collected in Amaicha del Valle district (province of Tucumán, Argentina) was obtained by dipping the fresh plant (300 g) in cold CH_2Cl_2 (1000 mL) at room temperature (26 °C) for 40 s. The CH_2Cl_2 solution was filtered and evaporated under reduced pressure to give a semisolid yellow orange residue (*Zp*DCME; 45 g, 15% w/w yield). The plant material was identified by Dr. A. Slanis at the Faculty of Natural Sciences, Institute Miguel Lillo, National University of Tucumán, Argentina. A voucher herbarium specimen has been kept at the Instituto Lillo (LIL 609629).

2.6.1. Isolation of the Main Exudate Constituents from Tucumán-Grown Z. punctata. *Zp*DCME (25 g) was applied onto a column (column length, 70 cm; i.d. 5 cm) containing 500 g of silica gel

Table 1. Compounds Identified and Quantified from Different Propolis (Methanolic Extracts MEP1−3) and *Zuccagnia punctata* Exudates (*Zp*DCME), Indicating Their RT, HPLC-DAD, UV, MS, and MS/MS Characteristics

peak	compd	RT	UV_{max} (MeOH)	M_w	$(m/z)^-$ MS^2	$(m/z)^+$ MS^2	MEP1−3[a]	*Zp*DCME[a]
1	izalpinin	40.00	260, 302sh, 354	284	283, 268, 239, 211, 267	285, 270, 242	1133 ± 83	3106 ± 104
2	2′,4′-dihydroxy-3′-methoxychalcone	27.97	228, 338	270	269, 254, 150, 106	271, 167, 152, 131, 103	10400 ± 104	10000 ± 100
3	2′,4′-dihydroxychalcone	26.40	266, 319, 345	240	239, 197, 135	241, 195, 131, 103	4500 ± 104	4900 ± 100
4	7-hydroxyflavanone	17.20	233, 275, 310	240	239, 197, 135	241, 163, 137, 131, 103	100 ± 25	148 ± 20
5	galangin	21.15	267, 305sh, 359	270	269, 151	271, 215, 165, 153	2132 ± 363	2614 ± 190
6	pinocembrin	19.60	289, 325sh	256	255, 213, 151, 107	257, 153, 131, 153	1194 ± 58	1248 ± 166
7	rhamnocitrin	17.00	364, 267, 238	300	299, 284, 271, 240, 165	301, 258, 183, 133 121	2697 ± 250	3842 ± 140
8	3-hydroxy-7,8-dimethoxyflavone	22.15	227sh, 312, 353	298	297, 282, 239, 166, 155	299, 284, 255, 238, 165	1093 ± 90	1388 ± 56
9	7-hydroxy-8-methoxyflavanone	38.50	233, 283	270	269, 254, 150, 106	271, 167, 152, 131, 103	105 ± 15	148 ± 20
10	pinostrobin	24.90	288	270	nd	nd	505 ± 25	650 ± 30

[a] Data analyzed by HPLC-DAD are reported in mg of compound 100 g^{-1} and correspond to the mean ± one standard deviation (SD), calculated from at least three replicates. nd, not determined.

(0.063−0.2 mesh, Merck 60) and eluted with a PE−PE/EtOAc−EtOAc gradient. About 28 fractions of 1 L each were obtained. After TLC comparison (silica gel, PE/EtOAc 8:2 as the mobile phase; detection under UV light and after spraying with diphenylboric acid, ethanolamine complex in methanol), fractions with similar TLC patterns were combined. The following groups of fractions were obtained: 1 (6 mg), 2 (236 mg), 3 (713 mg), 4 (988 mg), 5 (105 mg), 6 (746 mg), 7 (208 mg), 8 (1822 mg), 9 (3680 mg); 10 (2472 mg); 11 (870 mg); 12 (376 mg); 13 (620 mg); 14 (665 mg); 15 (657 mg); 16 (2362 mg); 17 (1376 mg); 18 (602 mg); 19 (752 mg), 20 (1648 mg); 21−28 (533 mg). Fraction 6 (240 mg) was permeated on Sephadex LH-20 (column length, 46 cm; i.d., 2 cm; PE/MeOH/$CHCl_3$ 2:1:1). Some 17 fractions of 10 mL each were obtained. After TLC comparison (silica gel, PE/EtOAc 8:2), fractions with similar TLC patterns were combined as follows: 1 (21 mg; fraction 1); 2 (24 mg, fraction 2); 3 (64 mg, fractions 3−5); 4 (47 mg, fractions 6−9), 5 (27 mg, fractions 10−11); 6 (31 mg, fractions 12−17). Fraction 2 yielded 24 mg of izalpinin **1**.

The MeOH-soluble fraction 8 (1.5 g) was applied to a Sephadex LH-20 column (column length, 40 cm; i.d., 5 cm), eluant PE/MeOH/$CHCl_3$ 2:1:1). Some 21 fractions of 10 mL each were obtained. After TLC comparison (silica gel, PE/EtOAc 8:2 as the mobile phase, detection under UV light, and after spraying with diphenyl boric acid, ethanolamine complex in methanol), fractions with similar TLC patterns were combined as follows: 1 (80 mg; fraction 1); 2 (20 mg, fraction 2); 3 (420 mg, fractions 3−5); 4 (928 mg, fractions 6−9), 5 (24 mg, fractions 10−11); 6 (20 mg, fractions 12−21). Fraction 3 was successively percolated by Sephadex LH-20 column (column length, 46 cm; diameter, 2 cm) equilibrated with PE/MeOH/$CHCl_3$ (2:1:1) to afford 100 mg of pure 2′,4′-dihydroxy-3′-methoxychalcone **2** and 15 mg of pure 7-hydroxy-8-methoxyflavanone **9**. Fraction 4 was successively percolated by Sephadex LH-20 column (column length, 46 cm; diameter, 2 cm) equilibrated with PE/MeOH/$CHCl_3$ (2:1:1), affording 100 mg of pure 2′,4′-dihydroxy-3′-methoxychalcone **2** and 15 mg of pure 7-hydroxy-8-methoxyflavanone **9**. Fraction 6 yielded 20 mg of pure pinocembrin **6**.

Fraction 9 (2.63 g) was applied to a Sephadex LH-20 column (column length, 40 cm; i.d., 5 cm; PE/MeOH/$CHCl_3$ 2:1:1). Fifteen fractions of 50 mL each were obtained. After TLC comparison (silica gel, PE/EtOAc 8:2), fractions with similar TLC patterns were pooled as follows: 1 (70 mg; fractions 1−4); 2 (39 mg, fraction 5); 3 (113 mg, fraction 6); 4 (645 mg, fractions 7−8), 5 (62 mg, fractions 9−10); 6 (457 mg, fractions 11−13); 7 (328 mg, fractions 14); 8 (11 mg, fraction 15). Pooled fractions 4 (645 mg, fractions 7−8) and 6 (457 mg, fractions 11−13) were purified on Sephadex LH-20 (column length, 46 cm; i.d., 2 cm; PE/MeOH/$CHCl_3$ 2:1:1) to afford 250 mg of 2′,4′-dihydroxy-3′-methoxychalcone **2** and 120 mg of a mixture of the 2′,4′-dihydroxychalcone **3** and flavonoid pinostrobin **10**. The mixture was successively applied onto a column (column length, 40 cm; i.d., 2 cm) containing 50 g of silica gel (0.063−0.2 mesh, Merck 60) and eluted with a PE/EtOAc gradient to afford 60 and 12 mg of pure chalcone **3** and pinostrobin (**10**), respectively.

Fraction 13 (400 mg) was applied to a Sephadex LH-20 column (column length, 42 cm; i.d., 2 cm; MeOH). Some 18 fractions of 20 mL each were obtained. After TLC comparison (silica gel, PE/EtOAc 6:4), fractions with similar TLC patterns were pooled. Fractions 15−17 yielded 18 mg of galangin **5**.

Fraction 14 (600 mg) was applied to a Sephadex LH-20 column (column length, 40 cm; i.d., 2 cm; PE/MeOH/$CHCl_3$, 2:1:1). Twenty fractions of 10 mL each were obtained. After TLC comparison (silica gel, PE/EtOAc, 7.5:2.5), fractions with similar TLC patterns were pooled. Fractions 4−6 (200 mg) were successively percolated by Sephadex LH-20 column (column length, 46 cm; diameter, 2 cm) equilibrated with methanol to afford 20 mg of pure rhamnocitrin **7** and 25 mg of 3-hydroxy-7,8-dimethoxy flavone **8**.

Fraction 16 (1000 mg) was applied to a Sephadex LH-20 column (column length, 40 cm; i.d., 2 cm; MeOH). Twenty-five fractions of 10 mL each were obtained. After TLC comparison (silica gel, PE/EtOAc, 7.5:2.5), fractions with similar TLC patterns were pooled. Fractions 7−9 (200 mg) were successively percolated by Sephadex LH-20 column (column length, 46 cm; i.d., 2 cm) equilibrated with methanol to afford 10 mg of pure 7-hydroxyflavanone **4**. The compounds were characterized by NMR and UV spectra with a purity of ≥97%.

2.7. HPLC-DAD and Quantitative Analysis of the Main Identified Compounds from Propolis and *Z. punctata*. The quantitative analysis of flavonoids and chalcones was performed according to the method of Sánchez-Rabaneda et al. (*15*) with a few modifications (*4*). Retention times (RT) of the 10 identified compounds are shown in **Table 1**.

Calibration curves of pure compounds **1−10** of different concentrations (0.05− 2 mg mL^{-1}) were performed to estimate the content of the main compounds at each sample. The correlation between concentration and peak areas was assessed by the least-squares regression model. Correlation coefficients (R^2) for **1−10** were between 0.989 and 0.998.

The retention times of eluting compounds was checked by co-injection of *Zp*DCME, MEP1, MEP2, and MEP3 with compounds **1−10** isolated from *Z. punctata*. Samples were injected at a final concentration of 5 mg mL^{-1}. Data (mg of compound 100 g^{-1} of extract) are reported in **Table 1** as mean ± standard deviation (SD) from at least three replicates.

2.7.1. LC/ESI-MS/MS Analysis of Main Identified Compounds. MS and MS/MS spectra of pure compounds were determined by direct infusion using a syringe pump as described under Material and Methods. Parent ions as well as breakdown ions (transitions) produced in Q2 (MS^2) are reported in **Table 1** using both positive and negative ionization modes. Afterward, plant and propolis extracts were qualitatively evaluated using HPLC-ESI-MS/MS (MRM), confirming profiles observed with HPLC-DAD.

2.8. Antifungal Activity. *2.8.1. Microorganisms and Media.* For the antifungal evaluation, strains from the American Type Culture Collection (ATCC), Rockville, MD, and CEREMIC (C), Centro de Referencia en Micología, Facultad de Ciencias Bioquímicas y Farmacéuticas, Suipacha 531, 2000 Rosario, Argentina, were used. The first panel was integrated by the following microorganisms: *Candida albicans* ATCC 10231, *Candida tropicalis* C 131, *Saccharomyces cerevisiae* ATCC 9763, *Cryptococcus neoformans* ATCC 32264, *Aspergillus flavus* ATCC 9170, *Aspergillus fumigatus* ATTC26934, *Aspergillus niger* ATCC 9029, *Trichophyton rubrum* C 110, *Trichophyton mentagrophytes* ATCC 9972, and *Microsporum gypseum* C 115. The second panel included 10 *Trichophyton rubrum*, and 8 *Trichophyton mentagrophytes* strains

Table 2. Minimum Inhibitory Concentrations (MIC) and Minimum Fungicidal Concentration (MFC) of Propolis Extracts, *Zuccagnia punctata* Exudate, and Compounds Isolated against Yeasts and Dermatophytes[a]

extract or compd	*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
MEP1	125	250	125	250	125	−;250	125	−;250	16	16	16	31.2	16	31.2
MEP2	125	250	125	250	125	−;250	62.5	250	16	16	16	31.2	16	31.2
MEP3	125	250	125	−;250	62.5	250	62.5	250	16	16	16	31.2	16	31.2
*Zp*DCME	62.5	62.5	62.5	62.5	31.25	62.5	31.25	31.25	16	16	8	16	8	16
1	−;250		−;250		−;250		−;250		−;250		−;250		−;250	
2	−;250		−;250		−;250		−;250		8	8	8	16	8	16
3	31.2	62.5	31.2	125	16	62.5	16	31.2	4	4	4	8	4	4
5	31.25		31.25		31.25		31.25		nd		nd		nd	
6	62.5		125		125		250		125		31.2		31.2	
9	−;250		−;250		−;250		−;250		125		62.5		62.5	
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	

[a] *Ca*, *Candida albicans* ATCC 10231; *Ct*, *Candida tropicalis* C 131 2000; *Sc*, *Saccharomyces cerevisiae* ATCC 9763; *Cn*, *Cryptococcus neoformans*; *Mg*, *Microsporum gypseum* C 115; *Tr*, *Trichophyton rubrum* C113; *Tm*, *Trichophyton mentagrophytes* ATCC 9972; ATCC, American Type Culture Collection; C, Center of Mycological Reference (Rosario, Argentina); amp B, amphotericin B; keto, ketoconazole; terb, terbinafine.

Table 3. Minimum Inhibitory Concentrations (MIC) and Minimum Fungicidal Concentration (MFC) of MEP1, MEP2, and MEP3, *Zp*DCME, and Chalcones **2** and **3** (in Micrograms per Milliliter)[a]

isolate		MEP1		MEP2		MEP3		*Zp*DCME		**2**		**3**		terb
		MIC_{100}	MFC	MIC_{100}	MFC	MIC_{100}	MFC	MIC_{100}	MFC	MIC_{100}	MFC	MIC_{100}	MFC	
T. rubrum	C 110	16	16	16	16	16	31.2	16	16	3.9	7.8	1.9	7.8	0.06
T. rubrum	C 135	31.25	31.25	31.25	31.25	16	16	8	16	3.9	7.8	3.9	7.8	0.06
T. rubrum	C 136	16	16	16	16	16	16	8	16	3.9	7.8	3.9	7.8	0.06
T. rubrum	C 137	16	16	16	16	16	16	8	16	3.9	3.9	1.9	3.9	0.06
T. rubrum	C 139	16	16	16	16	16	16	16	16	1.9	7.8	1.9	7.8	0.12
T. rubrum	C 140	31.25	31.25	31.25	31.25	31.25	62.5	16	32	1.9	3.9	1.9	1.9	0.03
T. mentagrophytes	C 108	16	16	16	16	16	16	16	16	3.9	3.9	1.9	7.8	0.06
T. mentagrophytes	C 364	16	16	16	16	16	16	8	16	3.9	15.6	1.9	7.8	0.06
T. mentagrophytes	C 539	16	16	16	16	16	16	8	16	3.9	15.6	3.9	7.8	0.06
T. mentagrophytes	C 738	31.25	31.25	31.25	31.25	31.25	31.25	16	32	1.9	1.9	1.9	3.9	0.06
T. mentagrophytes	C 943	16	16	16	16	16	16	32	32	3.9	7.8	1.9	7.8	0.06
T. mentagrophytes	C 726	61.25	31.25	31.25	31.25	31.25	31.25	32	32	3.9	7.8	3.9	7.8	0.12

[a] Tested against clinical isolates of the dermatophytes *T. rubrum* and *T. mentagrophytes*. MIC_{100}, concentration of compound that inhibited 100% of the growth control; Terb, terbinafine.

(voucher numbers in **Table 3**). 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^{-1} (*16*).

2.8.2. Antifungal Susceptibility Testing. The minimum inhibitory concentration (MIC) of each extract or compound was determined by using broth microdilution techniques according to the guidelines of the Clinical and Laboratory Standards Institute (*16*). MIC values were determined in RPMI-1640 medium (Sigma, St. Louis, MO) buffered to pH 7.0 with MOPS (Remel, Lenexa, KS). Microtiter trays were incubated at 35 °C for yeasts and hialohyphomycetes and at 28−30 °C for dermatophyte strains in a moist, dark chamber, and 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 2-fold diluted with RPMI medium from 256 to 0.98 $\mu g\ mL^{-1}$ (final volume = 100 μL) to reach a final dimethyl sulfoxide (DMSO) concentration of ≤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, and amphotericin B were used as positive controls. The end point was defined as the lowest concentration of drug resulting in total inhibition of visual growth compared to the growth in the control wells containing no antifungal compound. 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. 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 (*17*).

3. RESULTS AND DISCUSSION

3.1. Antifungal Activity of Methanolic Propolis Extracts and Isolated Compounds. The MeOH extracts (MEP1, MEP2, and MEP3) of the three collected raw propolis samples from the province of Tucumán were first assayed for antifungal properties with the microbroth dilution method following the guidelines of CLSI (*16*) at concentrations of ≤250 $\mu g\ mL^{-1}$.

The panel of fungi included yeasts (*C. albicans*, *C. tropicalis*, *S. cerevisiae*, and *C. neoformans*), *Aspergillus* spp. (*A. flavus*, *A. fumigatus*, and *A. niger*), and the dermatophytes *T. mentagrophytes*, *T. rubrum*, and *M. gypseum*. The antimicrobial activity is considered to be very interesting in the case of MICs < 100 $\mu g\ mL^{-1}$ for extracts and 10 $\mu g\ mL^{-1}$ for isolated compounds (*18*). Results (**Table 2**) show that all dermatophytes and yeasts tested were inhibited by the different propolis extracts with MIC values between 16 and 125 $\mu g\ mL^{-1}$. On the contrary, species of the *Aspergillus* genus were not sensitive to propolis extracts.

	R_1	R_2	R_3	R_4
1: Izalpinin	OH	OCH_3	H	H
5: Galangin	OH	OH	H	H
7: Rhamnocitrin	OH	OCH_3	H	OH
8: 3-hydroxy -7,8-dimethoxy flavone	OH	OCH_3	OCH_3	H

	R_1	R_2	R_3
4: 7-hydroxyflavanone	H	OH	H
6: Pinocembrin	OH	OH	H
9: 7-Hydroxy-8-methoxyflavanone	H	OH	OCH_3
10: Pinostrobin	OH	OCH_3	H

	R
2: 2′,4′-dihydroxy-3′-methoxychalcone	OCH_3
3: 2′,4′-dihydroxychalcone	H

Figure 1. Main antifungal chalcones and flavonoids from propolis (methanolic extracts) and *Zuccagnia punctata* exudate from the province of Tucumán (Argentina).

The dermatophytes *M. gypseum*, *T. mentagrophytes*, and *T. rubrum* were the most susceptible species, with MICs = 16 μg mL^{-1}. The yeasts *C. albicans*, *C. tropicalis*, *S. cerevisiae*, and *C. neoformans* were moderately inhibited, with MIC values between 62.5 and 125 μg mL^{-1}.

Interestingly enough, propolis extracts were not only fungistatic but fungicidal as well, with low MFC values against dermatophytes (16−31.2 μg mL^{-1}).

Because the three MEP extracts displayed MIC values of <20 μg mL^{-1} against the dermatophytes of the first panel (see **Table 2**), they were tested against a second panel containing six clinical isolates of each of the species *T. mentagrophytes* and *T. rubrum* (**Table 3**), to gain insight into their spectrum of activity.

The three extracts displayed very strong activities against this second panel with MICs = 16 μg mL^{-1} in 8 of the 12 clinical strains; 31.25 μg mL^{-1} in an additional 3 of 8, and between 31.25 and 61.25 μg mL^{-1} in the remaining strain. In addition, propolis extracts were fungicideal against all strains (MFC range = 16−62.5 μg mL^{-1}), a condition highly appreciated in antifungal drugs to avoid recurrence.

These results undoubtedly give scientific support to the traditional use of propolis in Tucumán province for fungal-related infections, because it is worth considering that the signs or symptoms that are related to traditional antifungal use are mainly related to the easy to see skin or mucosal conditions (tineas, athlete's foot, dandruff, etc). 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 (*19*).

The pure compounds identified during the bioassay-guided fractionation of propolis were also assessed for antifungal effect (**Table 2**). Izalpinin (**1**) was inactive against all fungi tested, whereas compounds **5**, **6**, and **9** were moderately active, with MICS ranging from 31.25 to 250 μg mL^{-1}. Instead, chalcones **2** and **3** were shown to be the most active metabolites: chalcone **2** showed a selective and strong activity against *M. gypseum*, *T. mentagrophytes*, and *T. rubrum*, with MICs = 8 μg mL^{-1}; chalcone **3** showed a broader antifungal spectrum and a stronger activity than **2**, inhibiting 8 of the 10 tested fungal species. The lowest MICs (4 μg mL^{-1}) were displayed against dermatophytes, although *S. cerevisiae* and *C. neoformans* were inhibited with low MICs, too. Interestingly enough, compounds **2** and **3** were shown to be fungicidal with MFCs between 4 and 16 μg mL^{-1} against the three dermatophytes. These chalcones have been recently reported as the main antifungal compounds from *Z. punctata* (*20*) when tested in the same panel of fungi.

Considering that *Z. punctata* grows abundantly in the same geographical area where beehives are placed, we presumed that *Z. punctata* could be the botanical origin of the studied propolis. These results give additional support to the antifungal activity of the medicinal plant *Z. punctata*, an endemic species from Argentina (*21*).

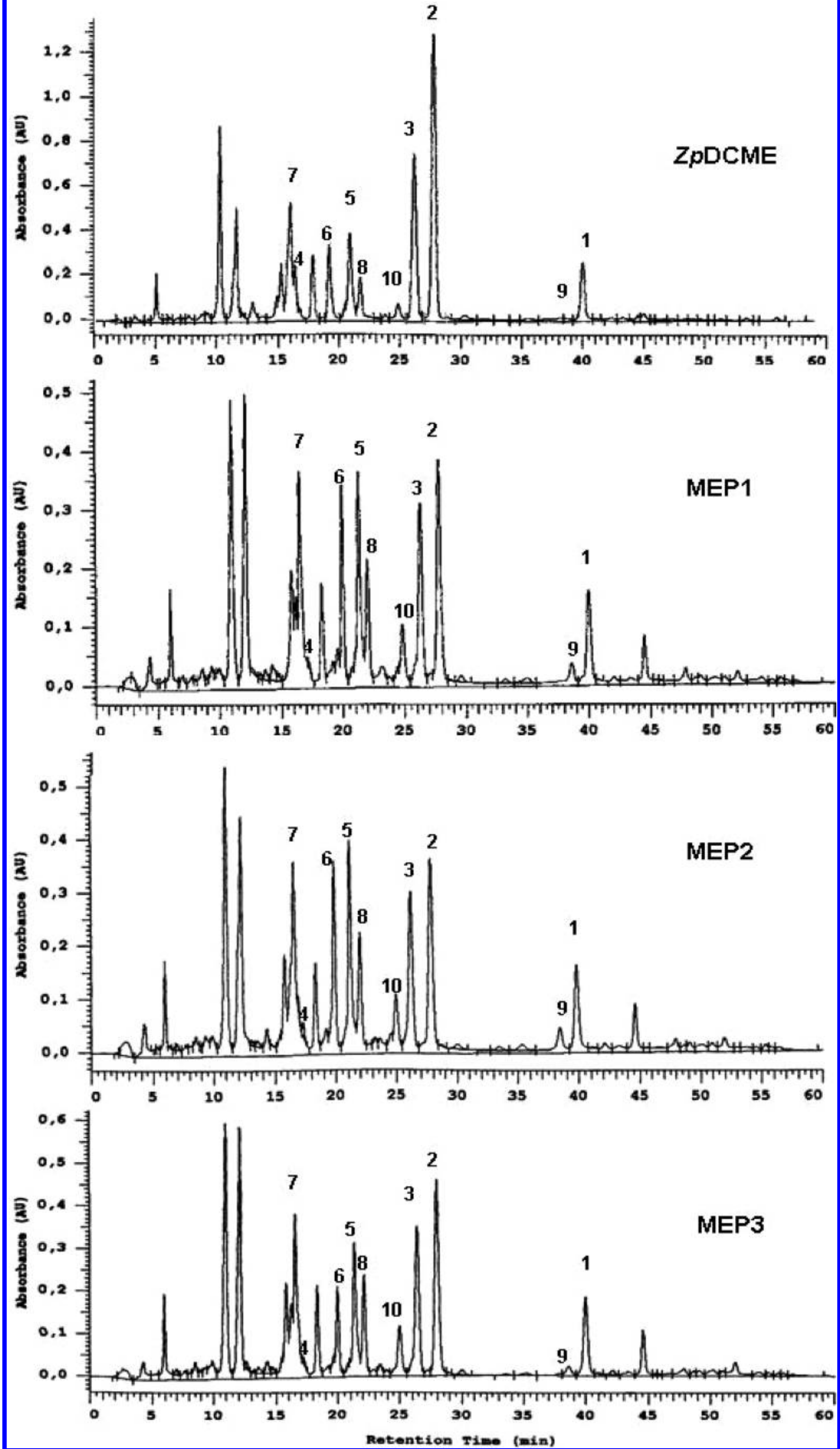

Figure 2. HPLC-DAD traces of *Zp*DCME, MEP1, MEP2, and MEP3. Peaks: 1, izalpinin; 2, 2′,4′-dihydroxy-3′-methoxychalcone; 3, 2′,4′-dihydroxychalcone; 4, 7-hydroxyflavanone; 5, galangin; 6, pinocembrin; 7, rhamnocitrin; 8, 3-hydroxy-7,8-dimethoxyflavone; 9, 7-hydroxy-8-methoxyflavanone; 10, pinostrobin.

3.2. Isolation and Characterization of Antifungal Compounds from Methanolic Propolis Extracts and *Zp*DCME. Evaluation of the Botanical Origin of the Studied Propolis. Bioassay-guided isolation of the combined MEP1−3 led to the isolation of 2′,4′-dihydroxy-3′-methoxychalcone **2** and 2′,4′-dihydroxychalcone **3** as the compounds responsible for the antifungal activity (**Figure 1**). These compounds have been recently reported as the main antifungal chalcones from *Z. punctata* (*20*, *22*). Additionally, seven flavonoids (compounds **4**−**10**) were isolated and identified by spectroscopic methods (^{1}H and ^{13}C NMR) as well as by HPLC-ESI-MS/MS on propolis and *Z. punctata* exudate extracts (**Figure 1**). The identity of the different extract constituents was also established by the mass spectra of the corresponding molecular ions (MS) and confirmed by breakdown products (MS/MS) (**Table 1**). Mass spectrometry has been already used for the structural characterization of flavonoids and phenolic compounds in propolis extracts (*23*, *24*). The isolated and identified compounds comprised also four flavanones (7-hydroxyflavanone **4**, 7-hydroxy-8-methoxyflavanone **9**, pinocembrin **6**, and pinostrobin **10**) and four flavonols (izalpinin **1**, galangin **5**, rhamnocitrin **7**, and 3-hydroxy-7,8-dimethoxyflavone **8**). Compounds **1**−**10** were identified by their spectroscopic data (^{1}H and ^{13}C NMR), which are in agreement with those reported in the literature (*25*−*28*). This is the first report of izalpinin **1**, pinocembrin **6**, galangin **5**, rhamnocitrin (7-*O*-methoxykaempferol) **7**, 3-hydroxy-7,8-dimethoxyflavone **8**, and pinostrobin **10** as constituents of the medicinal plant *Z. punctata*. Compound **8** was previously obtained by methylation of 3,7-dihydroxy-8-methoxyflavone (*26*). However, to the extent of our knowledge this is the first time that **8** has been isolated from a natural source.

To determine the possible relationship of the propolis sample with the exudate constituents of *Z. punctata*, an abundant source of similar compounds in the area of the beehives, we collected fresh aerial parts of *Z. punctata* from Amaicha del Valle, province of Tucumán, and prepared an extract by dipping them in dichloromethane (*Zp*DCME). The *Zp*DCME extract was evaluated against the same fungal panel used for propolis. Results (**Table 2**) showed that *Zp*DCME extract possesses a similar antifungal activity as propolis extracts (MEP1−3) and the same spectrum of action, inhibiting all fungi tested with the exception of *Aspergillus* species.

In addition to the similar antifungal activities of propolis extracts and *Zp*DCME, the same compounds were isolated and identified from extracts of both the propolis and plant exudates.

Comparative HPLC-DAD fingerprinting of MEP1−3 and *Zp*DCME is shown in **Figure 2**.

Compounds **1**−**10** were found in all extracts. In HPLC-DAD of *Zp*DCME, MEP1, MEP2, and MEP3, two peaks with major concentrations have not been identified. These peaks correspond to polar fractions 18 (602 mg) and 19 (752 mg) from the *Zp*DCME column. Percolation by Sephadex LH-20 of both fractions with methanol shows the presence of a mixture of phenolic compounds, mainly flavonoids. Further isolation and identification of these compounds are in progress. Under our experimental conditions, the RT of compounds **1**−**10** were as follows: **1**, 40.00 min; **2**, 27.97 min; **3**, 26.40 min; **4**, 17.20 min; **5**, 21.15 min; **6**, 19.60 min; **7**, 16.90 min; **8**, 22.15 min; **9**, 38.50 min; and **10**, 24.90 min. The amounts of characterized compounds from both extracts are summarized in **Table 2**. The chalcone **2** occurs at a concentration twice as high as compound **3** in both propolis samples and the exudate of *Z. punctata* collected from the same area (province of Tucumán, Argentina). In this propolis we have found characteristic poplar-origin phenolics such as galangin and pinocembrin, which are present at low concentrations. Additionally, we report that these compounds were isolated for the first time from *Z. punctata* exudates. Thus, these results support the hypothesis that compounds present in the studied propolis could arise from the resin collected by bees from *Z. punctata*.

Considering the close similarity between the propolis composition and *Z. punctata* exudate extract and the abundance of this plant in the area where beehives are placed, we suggest that *Z. punctata* could be considered the botanical origin of the studied propolis, which reinforces evidence on the probable use of these propolises as antifungal agents as well as serving as a source of natural antifungal compounds.

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