



ANTIMICROBIAL ACTIVITY OF ARGENTINEAN PROPOLIS AGAINST
Staphylococcus ISOLATED OF CANINE OTITIS

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ABSTRACT

Increasing resistance toward the available antibiotics by various microbial diseases pushed veterinarian to think about alternative therapies. Propolis is a natural compound well known since ancient times for its therapeutic effects and has been employed in traditional veterinary medicine. The aim of this study was to analyze the chemical composition of 12 propolis ethanolic extracts (PEEs) from different provinces of Northern Argentina and evaluate their antimicrobial activity against *Staphylococcus* strains isolated from canine otitis. Thirteen compounds (eight flavonoids, two chalcones and three phenolic acids) were identified by TLC, spectrophotometric methods and HPLC-DAD. PEEs were effective against *Staphylococcus* strains. The highest antibacterial activities were found in samples from Tucumán province. All propolis samples were considered bacteriostatic and the selection of resistant phenotypes did not occur in presence of Argentinean-PEE. Results of the present study indicate that propolis extracts might be an effective alternative against canine otitis-causing *S. aureus* strains *in vivo*.

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1 Introduction

Propolis is a resinous substance isolated from honeybee's exudates and buds of trees and plants. These exudates are mixed with wax and some enzymes secreted by bees. Chemical composition of Propolis depends on the climatic conditions, collection time and phytogeographical characteristics of the collection site as in different ecosystems bees collect it from different plant sources, choosing appropriate representatives of the local flora (Isla et al., 2001; Park et al., 2002; Isla et al., 2005; Bankova et al., 2000; Bankova et al., 2006; Bankova et al., 2008; Kumazawa et al., 2008; Isla et al., 2009; Vera et al., 2011; Isla et al., 2012a; Isla et al., 2012b; Solórzano et al., 2012; Danert et al., 2014).

This natural product has been employed extensively since ancient times, and it's continuously use till today as a popular remedy in traditional human medicine, as well as, in veterinary medicine. Use of propolis in veterinary medicine, and the effectiveness in preventing and treatment of different pathologies has been documented by various researchers (Albanese et al., 2002; Orsolich et al., 2005; Fan et al., 2013).

Bacterial resistance is associated with the excessive use of antimicrobial agents. In recent years, the bacterial resistance both, in animal as human, have drawn much attention by its impact on health (Guardabassi et al., 2004; Phillips et al., 2004; Aarestrup, 2005).

Canine otitis was included as the principal group of diseases in veterinary medicine and is highly prevalent; 21.7–42% of dogs have been reported to be infected (Oliveira et al., 2006; Ramalho et al., 2007). Canine otitis develops due to predisposing factors and the action of microorganisms that belong to the normal microbiota flora of the auricular area. These infections can exhibit a high level of antibiotic resistance (Lyskova et al., 2007; Pedersen et al., 2007) and thus requires alternative therapies such as the use of natural products. In this sense, Argentinean propolis extracts may be used as a natural antimicrobial for its treatment.

The aim of this study was to analyze the chemical composition of propolis extracts from different provinces of Northern Argentina and evaluate their *in vitro* antimicrobial activity against *Staphylococcus* strains isolated from canine otitis.

2 Materials and methods

2.1 Propolis samples

A total of 12 samples were collected from four provinces of Northern Argentina: Salta, Tucumán, Jujuy and Misiones. Five were from Salta i.e. Department Metán (SA-163-INTA), Department Anta (SA-350-INTA, SA-193-INTA, SA-349-INTA), and Department Cachi (SA-104-INTA). Five were from Tucumán i.e. Department Tafí del Valle (T-101-INTA, T-102-INTA), Department Trancas (T-103-INTA), Department

Leales (T-206-INTA), and Department Famaillá (T-192-INTA). One was from Jujuy: Department Ledesma (Ju-100-INTA) and one from Misiones: Department Apóstoles (M-299-INTA).

2.2 Preparation of propolis ethanolic extracts (PEE)

2g of propolis were mixed with 25 mL of 80% ethanol (EtOH:H₂O, 80:20, v/v) and maintaining with shaken at 70 °C for 30 min in thermostated shaker (Vicking). After extraction, the mixture was centrifuged at 3000 xg for 10 min, and the supernatants were used for further analysis (Isla et al., 2005).

2.3 Phytochemical screening

The extracts were standardized based on phenolic compounds and flavonoid content according to IRAM-INTA normative N°15935-2.

2.3.1 Determination of Phenolic compounds

The concentrations of phenolic compounds from the PEE were determined by using the Folin-Ciocalteu method (Singleton et al., 1999). The extract solution was mixed with 0.2 mL of the Folin-Ciocalteu reagent, and 0.8 mL of 15.9% sodium carbonate. Final volume was carried to 3 mL with distilled water. The mixture was heated at 50°C for 5 min, then it kept for cooling at room temperature and absorption was measured at 765nm. Results were expressed as mg of gallic acid equivalents per mL (mgGAE/mL).

2.3.2 Flavone and flavonol content

Total flavone and flavonol content was estimated by spectrophotometric assay based on aluminum chloride complex formation (Popova et al., 2005). Results were expressed as mg of quercetin equivalents per mL (mgQE/mL).

2.3.3 Flavanone and dihydroflavonol content

Flavanone and dihydroflavonol content was determined by using DNP in acid media according to Popova et al. (2005) with modifications. DNP solution (0.5 mL of 1% DNP solution in methanol containing 2 mL 96% sulfuric acid) was added to 0.25 mL of different dilutions of extracts and heated at 50°C for 50 min. After cooling, the mixture was diluted 3:33 fold with 10% KOH and centrifuged at 1500 xg during 10 min and the supernatant was diluted six fold with methanol. Absorbance was measured at 492 nm. Results were expressed as mg of naringenin equivalents per mL (mgNE/mL).

2.4 PEE absorption spectra

The PEE (25 µL) were mixed with 2 mL of 80% EtOH, and the mixtures were scanned at wavelengths between 200 and 450 nm with a UV-Vis Beckman DU 650 spectrophotometer.

2.5 Thin layer chromatography (TLC)

The different extracts (10 µg of total phenolic compounds) were spotted on to a silica gel TLC plate (Kieselgel 60 F254 0.2mm, Merck). The plates were developed in ascending direction with toluene: CHCl₃: acetone (4.5:2.5:3.5, v/v/v) as mobile phase. TLC plates were revealed under UV light at 365 nm (UV Lamp Model GL-58 Mineralight Lamp) and Natural Products reagent (NP – 1% methanolic solution of diphenylboric acid aminoethyl ester) (Wagner et al., 1984).

2.6 High performance liquid chromatography (HPLC)

The HPLC system consisting of a Waters 1525 Binary HPLC Pumps system with a 1500 Series Column Heater, a manual injection valve with a 20 µL loop (Rheodyne Inc., Cotati, CA) and a Waters 2998 photodiode array detector (PAD) was used to analyze the extracts. An XBridgeTM C18 column (4.6 mm × 150 mm, 5 µm; Waters Corporation, Milford, MA) was used. Gradient elution was carried out with methanol (MeOH, solvent B) and H₂O -9% acetic acid (solvent A) (conditions: 25% B–75% A from 0 to 10 min and kept at 45% B–55% A from 10 to 20 min, 70% B–30% A from 20 to 40 min, 75% B–25% A from 40 to 50 min, 100% B from 50 to 55 min) were used to separate PEE components. Flow rate was set at 0.8 mL/min. Data collection was carried out with Empower TM 2 software. The presence of phenolic compounds in extracts was confirmed by UV spectrometry (220–500 nm) in comparison with standard compounds.

2.7 Antimicrobial assays

2.7.1 Microorganisms

The microorganisms used in this study were isolated from canine otitis. Five strains i. e. *Staphylococcus haemolyticus* (n=1) (Lipron-A1), *S. aureus* (n=2) (Lipron-A2 and A3), *S. intermedius* (n =1) (Lipron-A5) and *S. aureus* ATCC strain (ATCC 29213) were used in the study. The strains were identified by the use of biochemical profiles according to the recommendations of the Manual of Clinical Microbiology (Murray et al., 1999). All organisms were maintained in brain–heart infusion (BHI medium) containing 30% (v/v) glycerol at –20 °C. Before testing, the suspensions were transferred to Mueller–Hinton agar (MHA) and aerobically grown overnight at 35 °C.

2.7.2 Antimicrobial commercial susceptibility test

Antibiotic susceptibility was determined by the disk diffusion method using commercial disk (Oxoid, UK) containing the amount of antibiotic indicated by international normative; viz Erythromycin (15 µg), Clindamycin (2 µg), Teicoplanin (30 µg), Gentamicin (10 µg), Ciprofloxacin (5 µg), Oxacillin (1 µg), Rifampicin (5 µg), Sulphamethoxazole/Trimethoprim (23.75/1.25 µg) and Vancomycin (30 µg). Resistance was defined for each case according to the inhibition zone diameter

according to CLSI, 2012a, CLSI, 2013: Erythromycin (≤ 13 mm), Clindamycin (≤ 14 mm), Teicoplanin (≤ 10 mm), Gentamicin (≤ 12 mm), Ciprofloxacin (≤ 15 mm), Oxacillin (≤ 10 mm), Rifampicin (≤ 16 mm), Sulphamethoxazole/Trimethoprim (≤ 10 mm) and Vancomycin (CIM ≥ 16 µg/ml).

2.7.3 Preparation of Inoculum

The inoculum were prepared by adjusting the turbidity of the suspension of each culture to 0.5 Mc Farland and by counting the number of colonies (10⁸ colony forming units (CFU)/mL).

2.7.4 Bioautographic assay

The different extracts (10 µg of total phenolic compounds) were spotted on to a silica gel TLC plate (Kieselgel 60 F254 0.2mm, Merck). The plates were developed in ascending direction with toluene: CHCl₃: acetone (4.5:2.5:3.5, v/v/v) as mobile phase and air-dried. Plates were covered with 2 mL of soft medium (BHI with 0.6% agar) containing 10⁵ colony forming units (CFU). Then, the plates were aerobically incubated at 35 °C for 16–20 h and sprayed with a 2.5 mg/mL MTT solution (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium) in PBS (10mM sodium phosphate buffer, pH 7, with 0.15M NaCl). The bands with antibacterial activity were identified as yellow zones against a blue background. These growth inhibition areas were compared with the R_f values of the related spots on the TLC plate revealed with NP and UV light according the methodology described in 2.5 (Nieva Moreno et al., 1999).

2.7.5 Broth microdilution method

Dilutions of PEE ranging from 10 to 640 µg GAE/mL were prepared in sterile 96 well microplates. The wells were filled with 100 µL cation-adjusted (Ca²⁺ 8 mg/L and Mg²⁺ 5 mg/L) Mueller–Hinton broth. The final inoculum was 5 × 10⁵ CFU/mL (CLSI, 2012b, CLSI, 2013) adjusted.

The inoculated microplates were aerobically incubated at 35 °C for 18 h. Bacterial growth was indicated by the presence of turbidity and a pellet on the well bottom. MICs were determined presumptively as the first well where no pellet appeared. Irreversible deletion of viability of the tested strains was confirmed by reinoculating Mueller Hinton agar plates with 10 µL of each culture medium from the microplates. MBC was determined after 18 h of incubation at 35 °C. MBC was defined as the lowest extract concentration at which 99.9% of the bacteria had been killed.

In order to determine the antibacterial effect of the PEE, the values of the ratio MBC/MIC were determined. Bactericidal effect was considered when MBC/MIC was ≤ 2, bacteriostatic effect when MBC/MIC was ≥ 4 and < 32 and tolerance when MBC/MIC was ≥ 32 (Reynaldi et al., 2010).

2.7.6 Time–kill studies

The tubes containing Mueller–Hinton broth with and without PEE were seeded with a log phase inoculum of 10^5 CFU/mL (*S. aureus* and *S. intermedius*) to a final volume of 10 mL. Different PEE concentrations (T-101-INTA, T-192-INTA and SA-104-INTA) corresponding to MIC, 2xMIC and 4xMIC values were used. Inoculated broths were incubated at 35 °C. At 0, 4, 8 and 24 h intervals after inoculation, a portion (0.1mL) was removed from each tube and was subjected to fold serial dilution. Then, 0.01 mL of every dilution was spread in MH agar plates. A time–kill curve was constructed for each isolate by using the viable cell counts determined at each interval. Bactericidal activity was defined as a reduction of 99.9% ($\geq 3 \log_{10}$ CFU/mL) of the total count of CFU/mL in the original inoculum in 4 h (NCCLS, 1999).

2.7.7 Selection of propolis-resistant *S. aureus* cells

S. aureus strains were transferred every 24 h for approximately 60 generations into fresh MH broth media containing sub-lethal doses of PEE (T-206-INTA). The propolis T-206 was selected for this assay by its higher antibacterial activity than other propolis samples. The sub-lethal dose was defined as the concentration of ethanolic extract that reduced the bacterial growth rate in at least 50%, but only caused less than 20% decrease in the maximum OD_{560nm} of *S. aureus* cultures. A concentration of 2.5 $\mu\text{g/mL}$ was used for isolate A3. After each transfer, the cultures were incubated at 35 °C for 12 h. To verify the selection of resistant cells, the sensibility of each isolate to propolis was re-evaluated every 20 transfers by microdilution assays as described previously (Santana et al., 2012).

2.8 Statistical analysis

Results are mean values obtained from at least three independent experiments. Differences between values were analyzed by Tukey test using InfoStat software. Values with a common letter are not significantly different ($p > 0.5$).

3 Results and discussion

3.1 Phytochemical screening

Most PEE showed a high content of phenolic compounds, between 13.2 to 23.5 g/100 g of phenolic compounds were reported (Table 1). These values were similar to those reported for propolis from Brazil, China, Uruguay and other propolis from Argentina (Bonvehí et al., 1994; Nieva Moreno et al., 2000; Bedascarrasbure et al., 2004; Chaillou et al., 2004; Isla et al., 2009; Lima et al., 2009; Lozina et al., 2010a). Amongst various PEP extract, SA-163-INTA, M-299-INTA, T-103-INTA showed lowest phenolic compounds concentrations, these values were similar to reported by Chaillou et al. (2004) for propolis from Santiago del Estero (Argentina). T-206-INTA was the sample that showed the lowest phenolic compounds content (1.72 g/100g of propolis) with similar

values to those reported by Lozina et al. (2010a) for propolis from Chaco (Argentina).

Regarding the quantification of flavones and flavonols content (Table 1), T-101-INTA (27.48 g/100g), T-102-INTA (18.27 g/100g) and SA-350-INTA (16.08 g/100g) propolis showed the highest concentration of both, while samples of M-299-INTA, T-206-INTA and Ju-100-INTA showed the lowest contents (0.13; 0.24; and 0.38 g/100g, respectively). The samples from T-101, T-102, SA-350, SA-349 and Ju-100 showed the highest values of flavanones and dihydroflavonols concentration (12.82; 9.57; 8.56; 8.62 and 10.46 g/100g, respectively) while SA-163, T-103 and M-299 samples showed the lowest values, and it was between 3.00 and 1.38 g/100g (Table 1), these findings shows similarly with the results of Isla et al. (2009) those who described same type of values for the propolis from San Juan. These results revealed that quantitative chemical composition of propolis depends on the phytogeographical region where the hives are located.

The absorption profiles of T-101, T-102, T-192, SA-350, SA-349 and SA-104 propolis samples were similar with wide bands between 230 and 315 nm, a small band at 330 nm and a peak at 360 nm. The absorption spectra of T-103, T-206, SA-163, SA-193, Ju-100 and M-299 propolis samples were different from each other. According with TLC and HPLC-DAD patterns the PEEs were classified in five different groups. Group I: T-101, group II: T-206, Group III: SA-193, Group IV: T-102, T-103, T-192, SA-163, SA-350, SA-349 and SA-104 and Group V: Ju-100 and M-299. HPLC-DAD analysis revealed a complex chemical composition (Figure. 1). Three phenolic acids viz chlorogenic acid (C1), p-coumaric acid (C2), ferulic acid (C3); eight flavonoids viz hesperidin (C4), naringenin (C5), hesperetin (C6), kaempferol (C7), apigenin (C8), 7- hydroxiflavanone (C9), pinocembrin (C10), chrysin (C11), and two chalcones viz 2',4'- dihydroxychalcone (C12), 2',4'-dihydroxy 3-methoxychalcone (C13) were identified from the analyzed samples (Figure.1). Presence of these compounds were also reported by various researcher from propolis samples (Kumazawa et al., 2004; Gardana et al., 2007; Zhou et al., 2008; Lima et al., 2009; Agüero et al., 2010; Isla et al., 2012b; Righi et al., 2013).

3.2 Antimicrobial activity

All canine otitis isolates were susceptible to oxacillin, (Table 2). Resistance to ciprofloxacin, teicoplanin and trimethoprin/sulphamethoxazole was also reported in study. The screening of antimicrobial activity by contact bioautography which was used for qualitative antibacterial activity detection, demonstrated that ethanolic extracts have antibacterial properties. T-101 showed two inhibition bands coincident with characteristic dark brown bands from chalcones by TLC/NP-UV (Nieva Moreno et al., 2005, Zampini et al., 2005). The highest number of bands with antibacterial activity was observed in T-206 and SA-104 propolis. In T-206 and SA-104 samples, the bands with $R_f < 0.8$ belong to flavonoids.

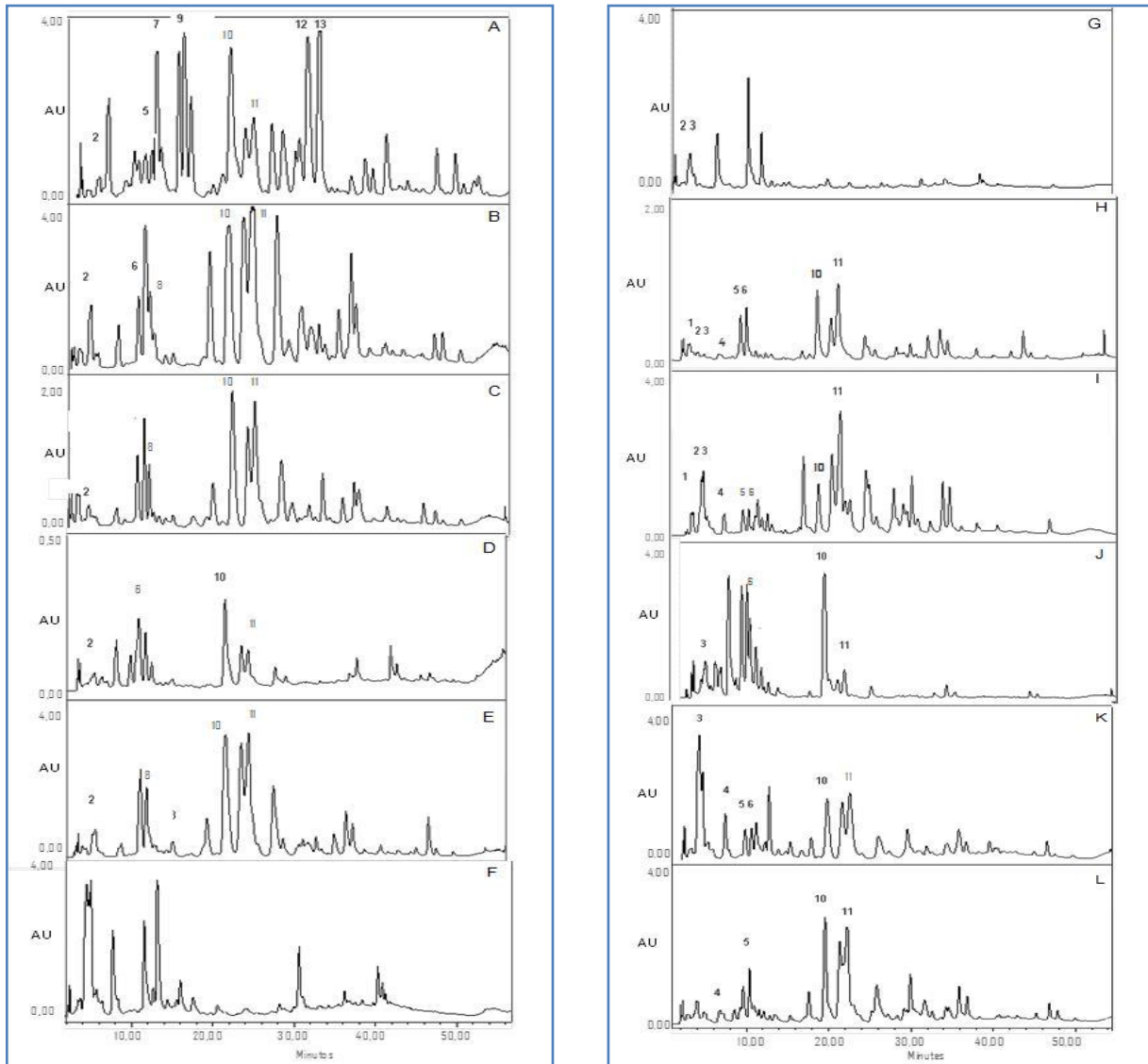


Figure 1 PEE HPLC profiles :

A) T-101. B) T-102. C) T-103. D) T-206. E) T-192. F) Ju-100. G) M-299. H) SA-163. I) SA-350 J) SA-193. K) SA-349. L) SA-104.

The identified compounds were:

1) Chlorogenic acid, 2) p-coumaric acid, 3) Ferulic acid, 4) Hesperidin, 5) Naringenin, 6) Hesperetin, 7) Kaempferol, 8) Apigenin, 9) 7-hydroxyflavanone, 10) Pinocembrin, 11) Chrysin, 12) 2', 4' dihydroxychalcone, 13) 2', 4' -dihydroxy 3- methoxychalcone

Table 1 Total phenolic compounds, flavones/flavonols and flavanones/dihydroflavonols content present in PEEs.

Samples INTA	Phenolic compounds			Flavones and Flavonols			Flavanones and Dihydroflavonols		
	(mgGAE/ml)	(mg/g) ^a	% (p/p)	(mgQE/ml)	(mg/g) ^a	% (p/p)	(mgNE/ml)	(mg/g) ^a	% (p/p)
T-101	18.86 ± 0.37	235.75 ± 4.64	23.57 ± 0.46 ^a	21.99 ± 1.23	274.87 ± 18.37	27.48 ± 1.54 ^a	10.26 ± 0.45	128.25 ± 5.64	12.82 ± 0.56 ^a
T-102	16.83 ± 0.16	210.37 ± 2.00	21.03 ± 0.20 ^b	14.62 ± 2.49	182.75 ± 15.10	18.27 ± 1.10 ^b	7.66 ± 0.30	95.75 ± 3.80	9.57 ± 0.38 ^{b,c}
T-103	6.80 ± 0.21	85.00 ± 2.60	8.50 ± 0.27 ^c	2.58 ± 0.10	32.25 ± 1.25	3.22 ± 0.12 ^{c,f}	2.40 ± 0.07	30.00 ± 0.91	3.00 ± 0.10 ^g
T-206	1.38 ± 0.04	17.25 ± 0.54	1.72 ± 0.05 ^g	0.19 ± 0.01	2.37 ± 0.10	0.24 ± 0.01 ^g	4.11 ± 0.20	51.37 ± 2.44	5.13 ± 0.24 ^{c,f}
T-192	10.58 ± 0.74	132.25 ± 9.28	13.22 ± 0.93 ^d	4.30 ± 0.04	53.70 ± 0.55	5.37 ± 0.05 ^d	3.66 ± 0.37	45.75 ± 4.57	4.57 ± 0.46 ^f
SA-163	3.96 ± 0.10	49.50 ± 1.10	4.95 ± 0.11 ^f	1.35 ± 0.04	16.87 ± 0.52	1.69 ± 0.05 ^{f,g}	1.11 ± 0.03	13.87 ± 0.35	1.38 ± 0.04 ^h
SA-350	14.21 ± 0.50	177.62 ± 5.97	17.76 ± 0.60 ^c	12.87 ± 0.06	160.87 ± 0.72	16.08 ± 0.07 ^c	6.85 ± 0.10	85.62 ± 1.27	8.56 ± 0.13 ^d
SA-193	11.43 ± 0.43	142.87 ± 5.39	14.28 ± 0.54 ^d	2.60 ± 0.10	32.50 ± 1.30	3.25 ± 0.13 ^{c,f}	4.54 ± 0.24	56.75 ± 3.00	5.67 ± 0.30 ^e
SA-349	11.58 ± 0.38	144.75 ± 4.73	14.47 ± 0.47 ^d	2.05 ± 0.05	25.62 ± 0.70	2.56 ± 0.07 ^f	6.90 ± 0.18	86.25 ± 2.29	8.62 ± 0.23 ^{c,d}
SA-104	11.55 ± 0.28	144.37 ± 3.56	14.43 ± 0.36 ^d	3.41 ± 0.08	42.62 ± 1.00	4.26 ± 0.10 ^{d,e}	3.80 ± 0.18	47.50 ± 2.22	4.75 ± 0.22 ^{c,f}
Ju-100	14.28 ± 0.02	178.50 ± 0.22	17.85 ± 0.02 ^c	0.31 ± 0.10	3.87 ± 0.10	0.38 ± 0.01 ^g	8.37 ± 0.33	104.62 ± 4.11	10.46 ± 0.41 ^b
Mi-299	5.88 ± 1.17	73.50 ± 2.30	7.35 ± 1.46 ^e	0.11 ± 0.01	1.37 ± 0.04	0.13 ± 0.01 ^g	2.32 ± 0.34	29.00 ± 4.24	2.90 ± 0.42 ^g

^a mg per g of propolis. Data are expressed as means ± standard deviations (n = 4)

Table 2 Minimum inhibitory concentration (MIC), minimum bacterial concentration (MBC) and relation MBC/MIC of PEE of four regions of Argentina.

Microorganisms	MIC, MBC (µgGAE/mL), MBC/MIC												Phenotype
	T-101	T-102	T-103	T-206	T-192	SA-163	SA-350	SA-193	SA-349	SA-104	Ju-100	M-299	
<i>S. haemolyticus</i> (Lipron-A1)	80/320/4	20/320/16	40/640/16	10/320/32	80/320/4	40/320/8	40/640/16	20/>640	40/>640	20/160/8	160/>640	160/>640	ErySCLiSTS VanSGenSCipR OxaSRfaSTMSS
<i>S. aureus</i> (Lipron-A2)	40/160/4	20/320/16	20/320/16	10/80/8	40/160/4	40/320/8	20/640/32	40/>640	40/640/16	20/160/8	80/>640	80/320/4	ErySCLiSTS VanSGenSCipS OxaSRfaSTMSS
<i>S. aureus</i> (Lipron-A3)	80/320/4	20/320/16	20/320/16	10/160/16	80/320/4	40/320/8	20/320/16	40/>640	40/640/16	20/80/4	160/>640	80/320/4	ErySCLiSTS VanSGenSCipS OxaSRfaSTMSS
<i>S. intermedius</i> (Lipron-A5)	40/160/4	20/640/32	20/320/16	10/80/8	40/160/4	40/320/8	20/320/16	40/640/16	40/640/16	40/160/4	80/>640	80/320/4	ErySCLiSTR VanSGenSCipS OxaSRfaSTMSS
<i>S. aureus</i> (ATCC 29213)	20/320/16	10/640/64	80/640/8	40/640/16	20/160/8	10/640/64	10/640/64	10/640/64	10/>640	10/160/16	nd	nd	

Erythromycin (Ery), Clindamycin (Cli), Teicoplanin (T), Vancomycin (Van), Gentamicin (Gen), Ciprofloxacin (Cip), Oxacillin (Oxa), Rifampicin (Rfa), Sulphamethoxazole/trimethoprin (TMS); Sensitive (S), Resistant (R), nd: Not determined.

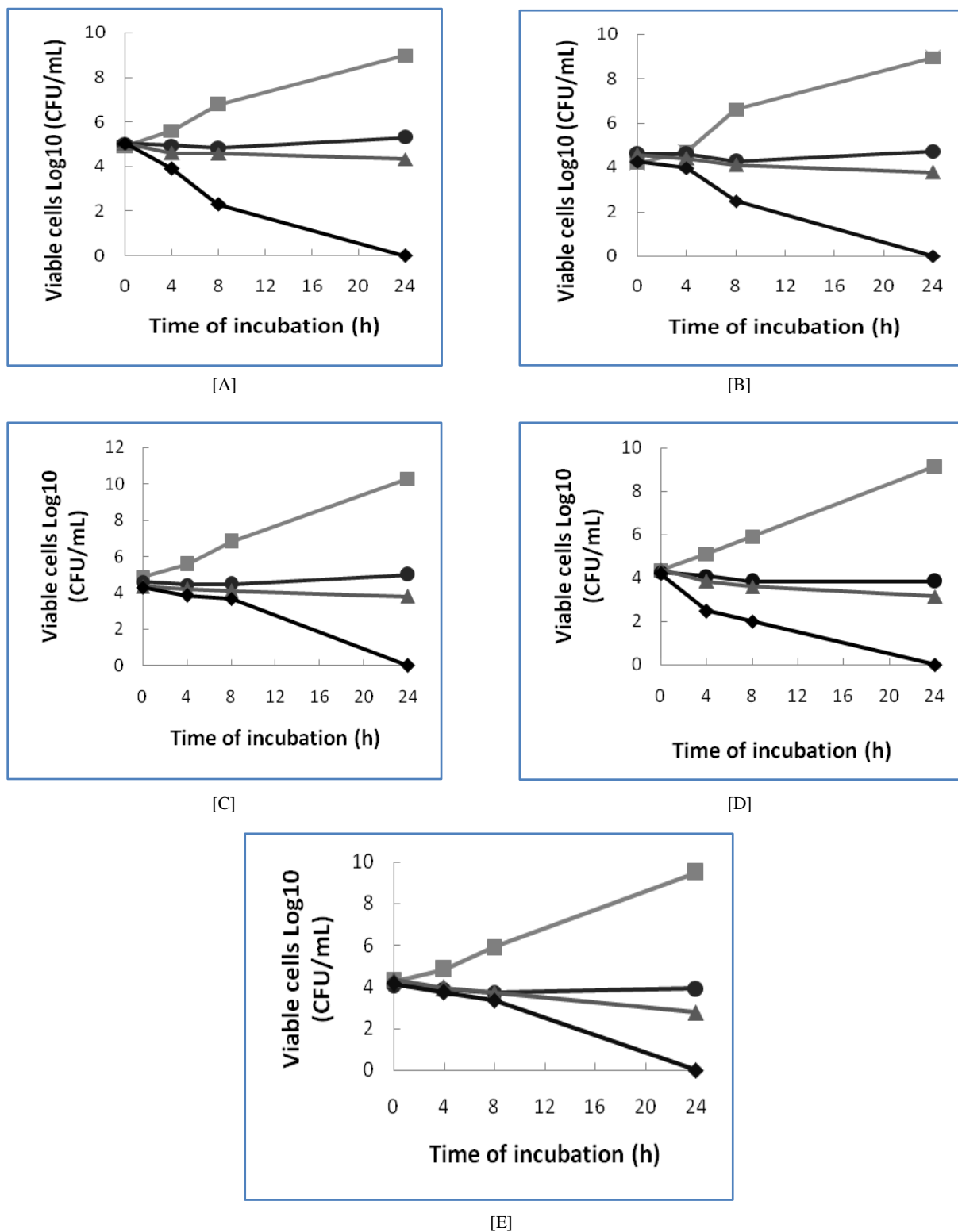


Figure 2 Killing curves corresponding to two strains of *Staphylococcus* against different concentrations of propolis extracts 80 (▲) and 160 (◆) µg/mL in comparison with a growth control without antibiotic (■). A) PEE-T-101 against *S. aureus* Lipron-A2 B) PEE-T-101 against *S. aureus* Lipron-A5 C) PEE-T-192 against *S. aureus* Lipron-A5 D) PEE-SA-104 against *S. aureus* Lipron-A2 E) PEE-SA-104 against *S. aureus* Lipron-A5.

Table 2 summarizes the MIC and MBC values as well as the ratio MBC/MIC of the different propolis extracts against Gram positive bacteria (*S. haemolyticus*, *S. aureus*, *S. intermedius*). Tucumán propolis samples showed MIC values between 10-80 µg GAE/mL and MBC values between 80-640 µg GAE/mL (Table 2). T-206 and T-102 samples were more active (MIC values 10 and 20 µg GAE/mL, respectively). The compounds identified in the T-206 sample were p-coumaric acid, hesperetin, pinocembrin and chrysin. Presence of these compounds have been reported previously as antimicrobial agents (Koo et al., 2000; Koo et al., 2002; Kosalec et al., 2003; Mandalari et al., 2007). These flavonoids were also identified from the T-102 samples. The T-206 sample showed the lowest concentration of phenolic compounds content (Table 1) and the chromatographic analyses revealed few chemical compounds, but the PEE showed the highest antibacterial activity against Gram positive bacteria. These results are in agreement with those obtained from the bioautographic assays where the highest number of bioactive compounds was shown. This shows that the quantity of total phenolic compounds or flavonoids is not the only important factor; the quality of the chemical compounds present in each PEE should also be taken in consideration.

Among the extracts of propolis collected in Salta, SA-104 and SA-350 samples demonstrated the highest bacterial growth inhibition against all the analyzed strains, with MIC values of 10-40 µg GAE/mL. MBC values were between 80-160 µg GAE/mL for SA-104 and 320-640 µg GAE/mL for SA-350 (Table 2). These results were also confirmed by those observed in the bioautography test as SA-104 tincture was one of the highest in bioactive components. The compounds identified in this propolis sample were flavonoids such as hesperidin, pinocembrin, naringenin, and chrysin, well known for their antimicrobial action (Rauha et al., 2000; Koo et al., 2000; 2002; Kosalec et al., 2003; Yi et al., 2008). The samples from Jujuy and Misiones showed the least antimicrobial activity (Tabla 2). Previous *in vitro* studies have reported the potential antibacterial activity of propolis from several regions of Argentina against human pathogens and phytopathogens (Nieva Moreno et al., 1999; Isla et al., 2005; Ordoñez et al., 2011; Vera et al., 2011; Isla et al., 2012a; Solórzano et al., 2012). Lozina et al., (2010b) reported antimicrobial activity against canine otitis strains of propolis of Mendoza in Argentina, but this is the first report of propolis from Northern Argentina against Gram positive bacteria isolated from canine otitis. Cardoso et al., (2010) evaluated the effect of propolis from Brazil against *Staphylococcus* coagulase positive (*S. aureus* and *S. intermedius*) isolated from dogs with external otitis, finding MBC values between 13.3 – 16.0 mg/mL which were higher than those presented in this study for the propolis from Northern Argentina.

Bactericidal effect was acknowledged when MBC/MIC was ≤ 2 , bacteriostatic effect when MBC/MIC was ≥ 4 and < 32 and tolerance when MBC/MIC was ≥ 32 . None propolis extract showed bactericidal effects on all analyzed canine pathogenic strains (Tabla 2). Kill-time assays were performed with PEE to confirm the bacteriostatic/bactericidal effect (Figure 2).

In the literature there were reports of resistance of *S. aureus* strains to diverse antibiotics (Zoraghi et al., 2010). Santana et al. (2012) reports that the selection of the resistance phenotype in Brazilian propolis did not occur. We assayed the resistance of *S. aureus* strains to Argentine propolis, and neither develop some resistance, as would be expected if resistant cells were present as a subpopulation in the *Staphylococcus* culture, this could be due to the complex composition of propolis, and each chemical constituent present different mechanisms of action and synergistic interactions (Salomao et al., 2008).

Conclusion

The antibacterial activity exerted by the propolis of Northern Argentina propolis extracts (mainly those from de Salta and Tucumán province) against *S. haemolyticus*, *S. intermedius* and *S. aureus*, common canine pathogenic strains, suggest their potential application as a natural antimicrobial in the treatment of canine otitis.

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References

- Aarestrup FM (2005) Veterinary drug usage and antimicrobial resistance in bacteria of animal origin. *Basic Clinical Pharmacology Toxicology* 96: 271-281.
- Agüero MB, Gonzalez M, Lima B, Svetaz L, Sanchez M, Zacchino S, Feresin GE (2010) Argentinean propolis from *Zuccagnia punctata* Cav.(Caesalpinieae) exudates: phytochemical characterization and antifungal activity. *Journal of Agricultural and Food Chemistry* 58: 194-201.
- Albanese F, Poli A, Millanta F, Abramo F (2002) Primary cutaneous extragenital canine transmissible venereal tumor with *Leishmania-laden* neoplastic cells: a further suggestion of histiocytic origin? *Veterinary Dermatology* 13: 243-246.
- Bankova VS, de Castro SL, Marcucci MC (2000) Propolis: recent advances in chemistry and plant origin. *Apidologie* 31: 3-15.
- Bankova V, Popova M, Trusheva B (2006) Plant sources of propolis: an update from a chemist's point of view. *Natural Product Communications* 1: 1023-1028.
- Bankova V, Trusheva B, Popova M (2008) New developments in propolis chemical diversity studies (since 2000). In: Orsolich N, Basic I (eds.) *Scientific evidence of the use of propolis in*

ethnomedicine. Transworld Research Network. Trivandrum, India.

Bedascarrabure E, Maldonado L, Alvarez A, Rodriguez E (2004) Contenido de fenoles y flavonoides de propóleos Argentino. *Acta Farmacéutica Bonaerense* 23: 369-372.

Bonvehí JS, Coll FV, Jordà RE (1994) The composition, active components and bacteriostatic activity of propolis in dietetics. *Journal of the American Oil Chemists' Society* 71: 529-532.

Cardoso RL, Maboni F, Machado G, Alves SH, De Vargas AC (2010) Antimicrobial activity of propolis extract against *Staphylococcus* coagulase positive and *Malassezia pachydermatis* of canine otitis. *Veterinary Microbiology* 142: 432- 434.

Chaillou LL, Herrera HA, Maidana JF (2004) Estudio de propóleos de Santiago del Estero, Argentina. *Ciência e Tecnologia de Alimentos* 24: 11-15.

CLSI (2012a) Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility test for bacteria that grow aerobically; Approved Standard. Ninth Edition M7-A9 Wayne, USA.

CLSI (2012b) Clinical and Laboratory Standards Institute. Performance standards for antimicrobial Disk susceptibility test; Approved Standard- Eleventh Edition M2-A11. Wayne, USA.

CLSI (2013) Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing: Twenty-third Informational Supplement M100-S23. Wayne, USA.

Danert FC, Zampini C, Ordoñez R, Maldonado L, Bedascarrabure E, Isla MI (2014) Nutritional and functional properties of aqueous and hydroalcoholic extracts from argentinean propolis. *Natural Product Communications* 9: 167-170.

Fan Y, Lu Y, Wang D, Liu J, Song X, Zhang W, Zhao X, Nguyen L, Hua Y (2013) Effect of epimedium polysaccharide-propolis flavone immunopotentiator on immunosuppression induced by cyclophosphamide in chickens. *Cellular Immunology* 281: 37-43.

Gardana C, Scaglianti M, Pietta P, Simonetti P (2007) Analysis of the polyphenolic fraction of propolis from different sources by liquid chromatography- tandem mass spectrometry. *Journal of Pharmaceutical and Biomedical Analysis* 45: 390-399.

Guardabassi L, Schwarz S, Lloyd DH (2004) Pet animals as reservoirs of antimicrobial-resistant bacteria Review. *Journal of Antimicrobial Chemotherapy* 54: 321-332.

Isla MI, Nieva Moreno M, Sampietro A, Vattuone M (2001) Antioxidant activity of Argentine propolis extracts. *Journal of Ethnopharmacology* 76: 165-170.

Isla MI, Paredes-Guzman JF, Nieva Moreno M, Koo H, Park YK (2005) Some chemical composition and biological activity of northern Argentine propolis. *Journal of Agricultural and Food Chemistry* 53: 1166-1172.

Isla MI, Zampini IC, Ordoñez RM, Cuello S, Juárez BC, Sayago J, Bedascarrabure E, Alvarez A, Coccini F, Maldonado LM (2009) Effect of seasonal variations and collection form on antioxidant activity of propolis from San Juan, Argentina. *Journal of Medicinal Food* 12: 1334-1342.

Isla MI, Dantur Y, Zampini IC, Salas A, Danert C, Arias M, Ordoñez RM, Maldonado L, Bedascarrabure E, Nieva Moreno MI (2012a) Effect of seasonality on antimicrobial activity of San Juan (Argentina) propolis. Development of topical functionalized formulation. *Natural Product Communications* 7: 1315-1318.

Isla MI, Nieva Moreno MI, Zampini IC, Solórzano E, Danert F, Vera N, Sayago JE, Bedascarrabure E, Maldonado L, Ordoñez RM (2012b). Argentine propolis: Its flavonoid and chalcone content and its relation with the functional properties. In: Farooqui T, Farooqui A (eds.) Beneficial effects of propolis on human health and chronic diseases. 8: 161-169. Nova Science Publishers, Inc. NY. USA

IRAM-INTA Norma (2004) Extractos de propóleos. Subcomité de productos agroalimentarios del NOA. Grupo de trabajo Miel Regional. Subgrupo de Trabajo propóleos. In: IRAM (eds.) Buenos Aires. Argentina.

Koo H, Rosalen PL, Cury JA, Ambrosano GMB, Murata RM, Yatsuda R, Ikegaki M, Alencar SM, Park YK (2000) Effect of a New variety of *Apis mellifera* propolis on *Mutans streptococci*. *Current Microbiology* 41: 192-196.

Koon H, Rosalen PL, Cury JA, Park YK, Bowen WH (2002) Effects of compounds found in propolis on *Streptococcus mutans* growth and on glucosyltransferase activity. *Antimicrobial Agents and Chemotherapy* 46: 1302-1309.

Kosalec I, Bakmaz M, Pepeljnjak S (2003) Analysis of propolis from the continental and Adriatic regions of Croatia. *Acta Pharmaceutica* 53: 275-285.

Kumazawa S, Goto H, Hamasaka T, Fukumoto S, Fujimoto T, Nakayama T (2004) A new prenylated flavonoid from propolis collected in Okinawa, Japan. *Bioscience, Biotechnology and Biochemistry* 68: 260-262.

Kumazawa S, Nakamura J, Murase M, Miyagawa M, Ahn MR, Fukumoto S (2008) Plant origin of Okinawan propolis: honeybee behavior observation and phytochemical analysis. *Naturwissenschaften* 95: 781-786.

Lima B, Tapia A, Luna L, Fabani MP, Schmeda-Hirschmann G, Podio NS, Wunderlin DA, Feresin GE (2009) Main flavonoids, DPPH activity, and metal content allow determination of the geographical origin of propolis from the Province of San Juan (Argentina). *Journal of Agricultural and Food Chemistry* 57: 2691-2698.

Lyskova P, Vydralova M, Mazurova J (2007) Identification and antimicrobial susceptibility of bacteria and yeasts isolated from healthy dogs and dogs with otitis externa. *Journal of Veterinary Medicine Series A* 54: 559-563.

Lozina L, Peichoto M, Acosta O, Granero G (2010a). Estandarización y caracterización organoléptica y fisico-química de 15 propóleos argentinos. *Latin American Journal of Pharmacy* 29: 103-110.

Lozina LA, Peichoto ME, Boehringer SI, Koscienczuk P, Granero GE, Acosta OC (2010b) Efficacy of Argentine propolis formulation for topical treatment of canine otitis externa. *Arquivo Brasileiro de Medicina Veterinaria e Zootecnia* 62: 1359-1366.

Mandalari G, Bennett R, Bisignano G, Trombetta D, Saija A, Faulds C, Gasson M, Narbad A (2007) Antimicrobial activity of flavonoids extracted from bergamot (*Citrus bergamia* Risso) peel, a byproduct of the essential oil industry. *Journal of Applied Microbiology* 103: 2056-2064.

Murray, P.R., Baron, E.J., Faller, M.A., Tenover, F.C., Tenover, R.H., 1999. *Manual of Clinical Microbiology*, 7th ed. ASM, Washington, DC.

Nieva Moreno MI, Isla MI, Cudmani N, Vattuone M, Sampietro A (1999) Screening of antibacterial activity of Amaicha del Valle (Tucumán, Argentina) propolis. *Journal of Ethnopharmacology* 68: 97-102.

Nieva Moreno MI, Isla MI, Sampietro AR, Vattuone MA (2000) Comparison of the free radical-scavenging activity of propolis from several regions of Argentina. *Journal of Ethnopharmacology* 71: 109-114.

Nieva Moreno MI, Zampini IC, Ordoñez RM, Jaime GS, Vattuone MA, Isla MI (2005) Evaluation of the cytotoxicity, genotoxicity, mutagenicity, and antimutagenicity of propolis from Tucumán, Argentina. *Journal of Agricultural and Food Chemistry* 53: 8957-8962.

NCCLS (1999) National Committee for Clinical Laboratory Standards. Performance standards for antimicrobial susceptibility testing (6th ed.). Approved Standards. M2-A6, Wayne, PA.

Oliveira LC, Leite CAL, Brilhante RSN, Carvalho CBM (2006) Etiology of canine otitis media and antimicrobial

susceptibility of coagulase-positive *Staphylococci* in Fortaleza city, Brazil. *Brazilian Journal of Microbiology* 37: 144-147.

Ordoñez RM, Zampini IC, Moreno MI, Isla MI (2011) Potential application of Northern Argentine propolis to control some phytopathogenic bacteria. *Microbiological Research* 166: 578-584.

Orsolio N, Sver L, Terzic S, Basic I (2005) Peroral application of water-soluble derivative of propolis (WSDP) and its related polyphenolic compounds and their influence on immunological and antitumor activity. *Veterinary Research Communications* 29:575-593.

Park YK, Alencar SM, Aguiar CL (2002) Botanical origin and chemical composition of Brazilian propolis. *Journal of Agricultural and Food Chemistry* 50: 2502-2506.

Pedersen K, Jensen H, Finster K, Jensen VF, Heuer OE (2007) Occurrence of antimicrobial resistance in bacteria from diagnostic samples from dogs. *Journal of Antimicrobial Chemotherapy* 60: 775-781.

Phillips I, Casewell M, Cox T, De Groot B, Friis C, Jones R, Nightingale C, Preston R, Waddell J (2004) Does the use of antibiotics in food animals pose a risk to human health? A critical review of published data. *Journal of Antimicrobial Chemotherapy* 53: 28-52.

Popova M, Silici S, Kaftanoglu O, Bankova V (2005) Antibacterial activity of Turkish propolis and its qualitative and quantitative chemical composition. *Phytomedicine* 12: 221-228.

Rauha JP, Remes S, Heinonen M, Hopia A, Kahkonen M, Kujala T, Pihlaja K, Vuorela H, Vuorela P (2000) Antimicrobial effects of Finnish plant extracts containing flavonoids and other phenolic compounds. *International Journal of Food Microbiology* 56: 3-12.

Ramalho RM, Grossi R, Andriolli JL, Said RA (2007) Características clínicas dos casos de otite externa em cães atendidos em Ilheus/Bahia e sensibilidade antimicrobiana *in vitro* das bacterias isoladas. *Acta Scientiae Veterinariae, Porto Alegre* 35: 423-424.

Reynaldi FJ, Rule R, Arauz S, Alippi AM (2010) Sensibilidad *in vitro* de *Paenibacillus larvae* frente a los antibióticos oxitetraciclina, tilosina, tilmicosina y lincomicina. *Analecta Veterinari* 30: 25-29.

Righi AA, Negri G, Salatino A (2013) Comparative chemistry of propolis from eight Brazilian localities. *Evidence-Based Complementary and Alternative Medicine*.

Salomao K, Pereira PRS, Campos LC, Borba CM, Cabello PH, Marcucci MC, Castro SL (2008) Brazilian propolis: correlation between chemical composition and antimicrobial activity.

Evidence-Based Complementary and Alternative Medicine 5: 317-324.

Santana HF, Barbosa AAT, Ferreira SO, Mantovani HC (2012) Bactericidal activity of ethanolic extracts of propolis against *Staphylococcus aureus* isolated from mastitic cows. World Journal of Microbiology and Biotechnology 28: 485-491.

Singleton VL, Orthofer R, Lamuela-Raventos RM (1999) Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. Methods in Enzymology 299: 152-178.

Solórzano E, Vera N, Cuello S, Ordoñez R, Zampini C, Maldonado L, Bedascarrasbure E, Isla MI (2012) Chalcones in bioactive Argentine propolis collected in arid environments. Natural Product Communications 7: 879-882.

Vera N, Solórzano E, Ordoñez R, Maldonado L, Bedascarrasbure E, Isla MI (2011) Chemical composition of Argentinean propolis collected in extreme regions and its relation with antimicrobial and antioxidant activities. Natural Product Communications 6: 823-827.

Wagner H, Bladt S, Zgainsky E (1984) Plant Drug Analysis: A Thin Layer Chromatography.. In Springer (Eds) Berlín. Germany.

Yi ZB, Yu Y, Liang YZ, Zeng B (2008) *In vitro* antioxidant and antimicrobial activities of the extract of *Pericarpium Citri Reticulatae* of a new Citrus cultivar and its main flavonoids. LWT-Food Science and Technology 41: 597-603.

Zampini IC, Vattuone MA, Isla MI (2005) Antibacterial activity of *Zuccagnia punctata* Cav. ethanolic extracts. Journal of Ethnopharmacology 102: 450-456.

Zhou J, Li Y, Zhao J, Xue X, Wu L, Chen F (2008) Geographical traceability of propolis by high-performance liquid-chromatography fingerprints. Food Chemistry 108: 749-759.

Zoraghi R, See R, Gong H, Lian T, Swayze R, Finlay B, Brunha R, McMaster W, Reiner N (2010) Functional analysis, overexpression and kinetic characterization of pyruvate kinase from methicillin-resistant *Staphylococcus aureus*. Biochemistry 49: 7733-7747.