Immunomodulatory activity of *Apis mellifera* propolis from the North of Argentina

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ABSTRACT

Propolis is a bee hive product with complex chemical composition that was used as medicine from ancient times. The immunomodulatory activity of partially purified propolis extracts (PPPEs), galangin and pinocembrin was studied through their *in vitro* effect on neutrophil chemotactic and phagocytic activities. PPPEs (15 samples from each of 10 different beehives) were more effective (around 45% and 50% for a concentration of 40 μg of PPPEs/mL of active substance) as chemotactic agents than galangin and pinocembrin (around 20 and 25%, respectively) and showed higher neutrophil phagocitic activity (270 ± 10% for beehive 10) than galangin and pinocembrin (180 ± 10 and 135 ± 9%, respectively). The highest effect of PPPEs can be attributed to a synergistic effect among components of PPPEs. The results of this study indicated that PPPEs, galangin and pinocembrin stimulate neutrophil chemotactic activity at various concentrations suggesting their possible use for patients suffering neutrophil disfunction. The nitro blue tetrazolium results indicated that the extracts and isolated flavonoids have the capacity to scavenge active radicals suggesting that they would be valuable in the treatment of diseases associated with free radical damage.

Consequently, the *in vitro* results suggest a potential value of these propolis extracts in the medical field.

1. Introduction

Propolis is a beehive product composed of resins, waxes, essential oils and bee secretions with a complex chemical composition, which was used in traditional medicine since ancient times (Ghisalberti, 1979). Their components are collected by bees from plants and used to cement and varnish the hives. Much of the literature concerning propolis was focused on their chemical constituents, biological activities and the botanical origins of the resins from which propolis are derived (Kumazawa et al., 2003; Lotti et al., 2010; Quiroga, Sampietro, Soberón, Sgariglia, & Vattuone, 2006; Santos et al., 2003; Simoes-Ambrosio et al., 2010). Moreover, propolis are used by bees to protect the colonies from diseases (Salatino, Weinstein Teixeira, Negri, & Message, 2005). Propolis

inhibit bacterial growth with a major effect on Gram-positive, and limited action on Gram-negative bacteria (De Vecchi & Drago, 2007; Nieva Moreno, Isla, Cudmani, Vattuone, & Sampietro, 1999; Sforcin, Fernandes, Lopes, Bankova, & Funari, 2000) and phytopathogenic fungal growth (Mendes Possamai, Honorio-França, Barcelos Reinaque, Luzia França, & De Souza Souto, 2013; Quiroga et al., 2006). Propolis are active on superficial mycosis (Silici, Koç, Ayangil, & Cankaya, 2005); show antiviral (Schnitzler et al., 2010), anti-inflammatory (Naito, Yasumuro, Kondou, & Ohara, 2007) and even anticancer and immunomodulatory activities (Chan, Cheung, & Sze, 2013; Scheller et al., 2003); also show immunity boosting activity (Sforcin, 2007; Simoes-Ambrosio et al., 2010), and are advised as effective natural antibiotics for some complex ailments like epididymitis, yeast infections, orchitis, gonorrhea, and syphilis; as antiviral in the case of hepatitis, canker sores and as anti-inflammatory balm in the cases of allergies, asthma and arthritis (Scheller et al., 2003). Available chemotherapeutic agents have mainly immunosuppressive action and most of them present serious problems of cytotoxicity and a variety of side effects. For instance, cytokines like interleukins and interferons are used as

immunostimulants, but are not very effective in the long term because of their cost and adverse effects. Thus, natural products are gaining importance as a source of immunomodulatory agents. Neutrophils are a key cellular element of the innate immune system (Galloway & Depledge, 2001). Following activation by immune stimuli, such as lipopolysaccharides, glycolipids and methylated DNA, neutrophils execute several specialized functions that include chemotaxis, phagocytosis, and the generation of reactive oxygen metabolites. Disturbances of these processes result in a dramatic increase in susceptibility to infections (Nathan, 2002). The term chemotaxis was introduced in 1884 by Pfeffer, who described it as directional migration of neutrophils against a chemical gradient. Neutrophils contain a potent battery of biological activities including oxidants, proteinases, and antimicrobial peptides; also produce high quantities of reactive oxygen (ROS) and nitrogen (RNS) species such as $O_2^{\bullet-}$ and $NO^{\bullet}$ mediated by the activity of oxidant-generating systems such as the NADPH oxidase and nitric synthase, respectively (Gebska, Olszanecki, & Korbut, 2005; Sheppard et al., 2005). During ingestion (phagocytosis) of foreign particles, ROS generated at the phagosome membrane are released directly into the phagosome. This process facilitates intracellular killing (Moraes, Zurawska, & Downey, 2006).

In the present study, the *in vitro* immunomodulatory activity of partially purified propolis extracts and two propolis isolated compounds (galangin and pinocembrin) were evaluated by neutrophil chemotaxis, and neutrophil phagocytic function studies.

2. Materials and methods

2.1. Study site and predominant plant species

Beehives were situated in the subtropical forest of 'El Siambón', Tucumán, Argentina. The vegetation of the area corresponds to the "Yungas" biogeographic province (Cabrera & Willink, 1980). Native vegetation is an evergreen forest dominated by Lauraceae, Myrtaceae, Fabaceae, Juglandaceae, Salicaceae and Nyctaginaceae. The pristine montane forest was progressively replaced by poplar, eucalyptus and mainly by pinus trees. Bees living in this area have access to native and introduced plant species. Preliminary studies on pollen content of propolis of this region showed that the resin-producing trees *Salix humboldiana*, *Pinus* and *Eucalyptus* are the most visited.

2.2. Chemicals

Analytical grade solvents were from Cicarelli Labs. (Argentina). HPLC solvents were from Sintorgan Labs. (Argentina). NaCl, phenol reagent, gallic acid, NaH_2PO_4, Na_2HPO_4, heparin, zymosan, Ficoll-Hypaque, Hank's balanced salt solution, RPMI 1640, Nitroblue Tetrazolium, Hematoxylin dye, Trypan Blue dye and Wright's stain from Sigma–Aldrich (USA). Silica gel 60 F_{254} plates, Wright's stain and vanillin were from Merck (Germany). Galangin and pinocembrin standards (HPLC quality) were from Indofine Chemical Company Inc. (Belle Mead, NJ, USA). Membrane filters (pore size 0.22 and 0.50 μm) were from Pall Life Sciences (USA).

2.3. Preparation of propolis extracts

Propolis were collected scrapping *Apis mellifera* beehives and stored in sealed containers, in the dark at 4 °C. Partially purified propolis extracts (PPPEs) were prepared according to Quiroga et al., 2006, with slight modifications: Fifteen (15) samples of propolis of each beehive of ten (1–10) different apiaries of the province of "Yungas" were collected, cut in small pieces and frozen at −20 °C. Then, they were grinded in a mortar and extracted with 96%

ethanol (1 g of propolis per 10 mL of 96% ethanol). Suspensions were kept at room temperature for 5 days in the dark with shaking and centrifuged at 13,000 g for 15 min to separate insoluble substances. The supernatant was frozen at −20 °C for at least 2 h and centrifuged at 13 000 g for 15 min to separate waxes and gums that were discarded. The solvent of the supernatant was evaporated under reduced pressure at 40 °C in a rotary evaporator until constant weight. Residues were dissolved in 96% ethanol containing 1% DMSO up to a concentration of 100 mg of gallic acid equivalents (GAE)/mL (w/v) (Singleton, Orthofer, & Lamuela-Raventos, 1999). The obtained brownish but transparent preparations were named partially purified propolis extracts (PPPEs). They were stored at 4 °C in the dark.

2.4. Analysis of phenolic compounds

Total phenolic compounds were determined by the phenol sulphuric method (Singleton et al., 1999). Results were expressed as micrograms of gallic acid equivalents/mL (μg GAE/mL).

2.5. TLC analysis

Phytochemical analysis of PPPEs were performed by thin layer chromatography (TLC) on silica gel 60 F_{254} plates with the mobile phase petroleum-ethyl acetate (7:3). Visualization was performed under visible and UV light (254 and 366 nm, UV Lamp Model UV 5L-58 Mineral Light Lamp) before and after staining with 1% methanolic 2-aminoethyl diphenyl borate reagent (flavonoids detection) (Wagner, Bladt, & Zgainski, 1984). Standards: galangin, Rf 0.52, color at 366 nm: dark yellow; pinocembrin: Rf 0.63, color at 366 nm: yellow.

2.6. HPLC analysis of propolis

Two main products (**1** and **2**) were isolated from PPPEs by reversed phase high performance liquid chromatography (RP-HPLC) on a gradient HPLC Gilson system (Villiers Le Bell, France) equipped with 118 UV-VIS. Detector was set at 254 and 340 nm; flow rate was of 0.7 mL/min. Rheodyne injector fitted with a loop of 20 μL. An IB-SIL C 18 column (5 μm, 250 × 4.6 mm ID) Phenomenex column (Torrance, California, USA) at 25 °C was used. The elution gradient was performed with solvent A (1% v/v formic acid in water) and solvent B (1% v/v formic acid in a mixture water-acetonitrile 1:1 v/v): t = 0 min, 0% B, t = 45 min 100% B. Chromatographic peaks were detected at 254 and 340 nm. Fractions corresponding to peaks (**1** and **2**) were collected, dried by lyophilisation and dissolved in methanol for further experiments. In order to obtain enough quantity of material, multiple injections were carried out employing semi-preparative IB-SIL5 C18 column (5 μm, 250 × 10 mm ID) from Phenomenex and a Rheodyne injector fitted with a 500 μL loop adjusting the flow rate at 2.8 mL/min. Fractions corresponding to peaks **1** and **2** were collected, dried by lyophilisation, dissolved in methanol and analysed by TLC. The purity of these compounds was verified by analytical HPLC experiments. Reference compounds, commercially obtained (galangin and pinocembrin) were co-chromatographed with the isolated substances to confirm their HPLC retention times. UV–visible spectra recorded on a Beckman DU 650 spectrophotometer and TLC, suggested pinocembrin (**1**) and galangin (**2**) were de isolated substances.

2.7. MS and NMR spectrometers

Electron impact mass spectra (EI-MS) were performed on a Hewlett–Packard 5970 Series mass spectrometer in assays using

isobutene as a reagent gas to generate the molecular ion $[MH]^+$. Electronic energy was 40 eV at 200 °C. The heating was between 50 °C and 40 °C at 100 °C. min^{-1}. Mass spectrometers were scanned over an m/z range of 100−630 Da. Data were processed using XCalibur 1.3 software (Austing, TX, USA). ^1H (200 MHz) and ^{13}C (50 MHz) NMR spectra were recorded on a Bruker NMR with a Bruker AC 200 console (Bruker, Germany). Tetramethylsilane (TMS) was used as the internal reference (δ 0.00) for ^1H and ^{13}C NMR spectra measured in DMSO-d_6.

2.8. Characterization of the isolated compounds

Compound **1** (pinocembrin); white powder; mp 194−195 °C; UV (MeOH) λ_{max} values are 225 and 290 nm; ^1H NMR δ (ppm) values, 20 °C, in CD_3OD are 2.77 (1H, dd, J = 17.2, 3.2 Hz, H-3a), 3.06 (1H, dd, J = 12.8, 17.2 Hz, H-3b), 5.44 (1H, dd, J = 3.2, 12.8 Hz, H-2), 5.52 (1H, d, J = 2.2 Hz, H-6), 6.01 (1H, d, J = 2.2 Hz, H-8), 7.41 (5H, m, H-2′ -6′); ^{13}C NMR δ (ppm) values, in CD_3OD are 25 °C, δ 40.45 (C-3), 80.17 (C-2), 95.94 (C-8), 96.84 (C-6), 102.69 (C-10), 127.47 (C-2′/6′), 129.39 (C-4′) 129.46 (C-3′/5′), 139.59 (C-1′), 163.59 (C-9), 164.41 (C-5), 167.62 (C-7), 196.75 (C-4).

Compound **2** (galangin): yellow powder mp 214−215 °C; UV (MeOH) λ_{max} values are 267 and 370 nm: ^1H NMR δ (ppm) values, in CD3OD are 12.43 (1H, s, 5-OH), 10.74 (1H, s, 7-OH), 9.59 (1H, s, 3-OH), 8.20 (2H, dd, 2′, 6′-H), 7.62 (2H, dd, 3′, 5′-H), 7.53 (1H, m, 4′-H), 6.43 ($J_{H8/H6}$ = 1.5 Hz, d, 1H, 8-H), 6.21 (1H, d, 6H); ^{13}C NMR δ (ppm) values, in CD_3OD, are 178.1 (4-C), 169.1 (5-C), 166.2 (7-C), 159.7 (2-C), 136.4 (3-C), 130.2 (1′C), 128.5 (3′, 5′-C), 127.9 (4′-C), 126.1 (2′, 6′-C), 103.2 (10-C), 98.1 (6-C), 97.8 (8-C).

Spectral data and the published literature exactly attested the structure of compound **2** as galangin (3,5,7-trihydroxyflavone (Maria, Cristina, Olga, & Rosa 2007), and compound **1** as pino-cembrin (5,7-dihydroxyflavanone) (Adelman et al., 2007; Bick, Brown, & Hillis, 1972; Neacsu et al., 2007).

2.9. Neutrophil isolation

Neutrophils were isolated from peripheral venous blood of adult male (AB group, 40−45 years old) healthy, nonsmoking, without medication volunteers. Twenty mL of fresh blood was added with 4 mL of 4.5% dextran B in 0.9% saline (10 mM sodium phosphate buffer with 0.9% NaCl, pH 7.5) and 500 units of heparin. After gently shaken the mixture was allowed to stand for 50 min. The supernatant rich in leucocytes was separated from erythrocytes by centrifuging at 2000 g. Neutrophils were separated from mononuclear cells by Ficoll-Hypaque density gradient centrifugation (Ferrante & Thong, 1980). Neutrophils from the bottom of the density gradient were washed twice, by centrifugation, with Hank's balanced salt solution (HBSS), suspended in RPMI 1640 medium at a concentration of 10^6 neutrophils/mL and used as targets for chemotactic assays. Other preparation was suspended at 10^6 neutrophils/mL in HBSS for NBT reduction test (Freeman & King, 1972). Preparations contained >95% neutrophils, as assessed by staining with Wright's stain.

2.10. Preparation of activated serum solutions

Venous blood was kept at room temperature for 15 min and then at 4 °C for 30 min. The supernatant was separated after centrifuging at 2000 g for 15 min at 4 °C. Zymosan (5 mg/mL) for positive control, dissolved in RPMI 1640 was added to the serum and the preparation was left to stand at 37 °C for 60 min. The upper phase was separated by centrifuging at 2000 g at 4 °C for 10 min. The activated serum was kept at −20 °C until use.

2.11. Neutrophil locomotion and chemotaxis test

The chemotactic activity of PPPEs and the two flavonoids was evaluated by the leading front technique according to Wilkinson (Wilkinson, 1981) with some modifications. The lower compartment of the chemotactic chamber (5 mL beaker) was filled with RPMI 1640 as control (chamber 1); zymosan activated serum diluted 1:10 with RPMI 1640 (as positive control in chamber 2) and the other chambers (3, 4, 5 and 6) with different final concentrations (10, 20, 40, and 100 µg GAE/mL) of test samples. The upper compartment (a tuberculin 1 mL syringe with a filter of 3 µm pore size (Millipore, glued to its lower end) was wet with the solvent and filled with neutrophil cell suspension (10^6 cells/mL in RPMI 1640). Then, the upper compartment was placed into the lower compartment ensuring that the fluid level in the upper chamber was the same as in the lower to avoid gradient disturbances. Filters were allowed to wet from the top before putting them in the lower compartment. The system was incubated at 37 °C for 180 min. Then, the upper compartment was removed and inverted to empty the fluid. After some minutes filters were detached and their lower surfaces were fixed with 70% ethanol for 2 min and then stained with haematoxylin dye for 5 min. Filters were observed under microscope using 100 × lens and the number of neutrophil cells reached to the lower surface of filters was counted.

2.12. Nitro blue tetrazolium (NBT) reduction test

Nitro Blue Tetrazolium (NBT) reduction test was applied according to Baehner and Nathan (1968) with modifications. A neutrophil suspension (10^6 cells/mL, with a viability index of at least 95%) was prepared in 0.5 mL of HBSS solution in 13 different tubes. PBS (0.1 mL) and 0.1 mL of latex (un-opsonized ingerible particles, 1 µm diameter) suspension was added to the first tube (standard). Assay mixtures were prepared by replacing the PBS by 0.1 mL of different concentrations of PPPEs, galangin or pinocembrin with the same final concentrations as used in the chemotaxis experiments (10, 20, 40 and 100 GAE/mL, final concentration). Finally 0.2 mL of 0.15% NBT was added to each tube and all preparations were incubated at 37 °C for 30 min, in the laminar air-flow unit in dark conditions. A control consisting in all reagents but neutrophil suspension was made. The experiment was repeated three times. Reactions were stopped with 1 mL of 0.5 N HCl and the reduced dye, blue formazan, was solubilized with 3 mL of 2 M potassium hydroxide in 0.1% DMSO and measured spectrophotometrically at 515 nm. Results were expressed as the difference in optical density (515 nm) among the stimulated neutrophils and the control; and were considered as an index of intracellular killing activity of neutrophils. Sample activities were calculated as the percentage ratio of the compounds relative to the positive control (latex). All reagents were prepared under sterile conditions obtained by 0.22 µm filtration.

2.13. Neutrophils viability

To determine the cytotoxicity of propolis components, neutrophils were prepared as if for chemotaxis and suspended in HBSS with several dilutions of PPPE, galangin or pinocembrin (controls) ranging from 10 to 100 µg GAE/mL. Appropriate controls were also assessed for effects on cell viability: cell control, ethanol, and DMSO. After 30 min incubation at 37 °C, cells were collected and viability was visually assessed by Trypan Blue dye exclusion test. All tests were performed by duplicate. The concentration of the study agents showing a viability of 90% or more were selected for the experimentation.

2.14. Statistical analysis

Data were analyzed using one-way ANOVA followed by Tukey's post hoc test. In cases, where a vehicle (either ethanol or DMSO) was used, the results were also compared with that of the vehicle control. Results were mentioned as mean ± SD. The level of significance for all analysis was taken as $p < 0.05$.

3. Results

PPPEs and their galangin and pinocembrin constituents isolated by HPLC (Fig. 1) showed neutrophil chemotactic activity at all assayed concentrations (Banskota et al., 1998). The highest mean number of neutrophils on membranes were for samples 7 - 10, producing the maximal chemotactic stimulus the concentration of 40 μg GAE/mL in the assay mixture, while the action of galangin and pinocembrin was less effective at the same concentration (Table 1). This behaviour is an evidence of the differential chemoattractant activity of propolis components from different places in the same phytogeographical region in Argentina and also the difference among galangin, pinocembrin and PPPEs. All values were compared with the selected standard, i.e. zymosan activated serum for a positive control, and RPMI 1640 for migration without stimulus (295 ± 18 and 14 ± 0.4, respectively). A clear relation of dose−response of neutrophil migration with growing concentrations of PPPEs was observed till 40 μg GAE/mL. A similar result was observed for the flavonoids being galangin a better stimulant than pinocembrin for neutrophil migration at 40 μg GAE of each compound/mL in the assay.

PPPEs, galangin and pinocembrin were evaluated with the NBT reduction test over a concentration range from 0.1 to 1 μg GAE/mL. All concentrations increased the formation of formazan crystals

Fig. 1. HPLC chromatogram of partially purified propolis extract (PPPE) of a sample of beehive Nro. 10, recorded at: a) 290 nm and b) 340 nm. Pinocembrin: 1, and galangin: 2.

indicating superoxide generation as compared to the control. The maximal stimulation of neutrophil phagocytic activity was present in samples 7−10 (191 ± 2; 189 ± 2; 194 ± 3 and195 ± 2%, respectively) at a concentration of 40 μg GAE/mL, while the phagocytic activity determined for galangin and pinocembrin, 182 ± 2% and 173 ± 3%, respectively, was at a concentration of 100 μg GAE/mL for each product (Table 2). Neutrophils stimulated with ingerible particles (latex) and PPPEs or flavonoids, separately, were incubated with the oxidised dye (NBT). After centrifugation, the reduced dye (formazan) was extracted from the pellet. Elevated formazan production (deposits) suggested that NBT can migrate within the cells and be reduced. This assay is the universally accepted method for defining the superoxide anion production (Baehner & Nathan, 1968).

4. Discussion

Several studies suggested that propolis chemical composition as well as its biological activities depend from the vegetation where beehives are placed and the season of propolis collection (Greenaway, May, Scaysbrook, & Whatley, 1991; Marcucci & Bankova, 1999; Santos et al., 2003; Sforcin, Orsi, & Bankova, 2005; Simoes-Ambrosio et al., 2010). With this information, and our own results on propolis from Amaicha del Valle (Nieva Moreno, Isla, Sampietro, & Vattuone, 2000), we performed the study of the immunomodulatory activity of propolis of different places of the subtropical montane forest of Argentina, where propolis is extensively used as a traditional medicine. Propolis are rich in flavonoids. Flavonoids consumed in the everyday diet of humans and animals in general, display many biological effects including immunomodulatory activity (Glusker & Russi, 1986). Neutrophilic polymorphonuclear leukocytes (neutrophils) play a primary function in the innate immune defence of humans and animals against microorganisms and other invaders (Stites, 1987). Their role is achieved through a sequence of events culminating with fagocytosis of pathogens or foreign antigens (Nathan, 2002). Neutrophil defence dysfunction mainly in chemotaxis, phagocytosis and intracellular killing activity, was found to be related with several infectious and other complications (Lehrer, Ganz, Selstad, Babior, & Curmutte, 1988). Neutrophils can detect the chemotactic substances at nanomolar concentrations and move towards them. Zymosan is frequently used as a reference chemoattractant agent in many *in vivo* and *in vitro* bioassays (Basaran, Ceritoglu, Undeger, & Basaran, 1997). Moreover, among polymorphonuclear cells, neutrophils are the most common cell type studied, as neutrophil migration mimics inflammation *in vivo*. Schneider and co-workers have demonstrated quercetin modulation of human neutrophil migration (Schneider, Berton, Spisani, Traniello, & Romeo, 1979). The effect of flavonoids on murine peritoneal exudate PMNs was examined by the chemotaxis chamber method and showed that kaempherol and quercetin significantly enhanced both direct and random migration at concentrations of 1−100 μM; then, it was suggested that the position and number of hydroxyl substitutions in flavonoids might be important for the observed activity (Kenny, Balistreri, & Torney, 1990; Sharma et al., 1996). According to our results galangin, pinocembrin and PPPEs showed a significant enhancement of neutrophil migration. Galangin and pinocembrin are phytoconstituents present in edible plants and found in a significant amount in honey and propolis. They show pharmacological effects on many biological systems (Patel, Patel, Gadewar, & Tahilyani, 2012; Rasul et al., 2013). Another important factor for the enhancement of neutrophil migration is a possible synergism among components of the PPPE mixtures that potentiate their activity and/or there are other components of PPPEs that also have chemotactic activity with an additive or synergistic effect. This
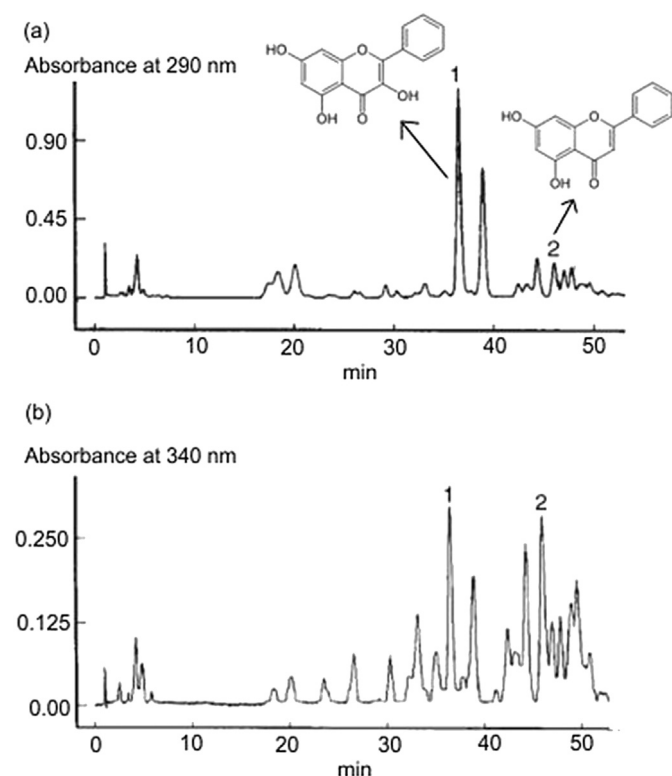
Table 1
Effect of partially purified propolis extracts (PPPEs) from 'El Siambón', Tucumán, Argentina and the isolated flavonoids on chemotactic activity. Mean number of neutrophils per field in the negative control (interaction with RPMI 1640) = 14.25 ± 0.40. Mean number of neutrophils per field in the positive control (interaction with zymosan activated serum) = 295.51 ± 18.

μg GAE/mL of PPPEs, galangin, pinocembrin in the assay	10	20	40	100[1]
Beehive Number	Mean number of neutrophils per field ± standard deviation[2]			
1	120.30 ± 0.03[a,3]	145.50 ± 0.10[a]	201.25 ± 0.10[a]	199.65 ± 0.12[a]
2	119.50 ± 0.15[a]	139.80 ± 0.15[a]	199.25 ± 0.08[a]	200.82 ± 0.10[a]
3	109.50 ± 0.07[a]	145.45 ± 0.10[a]	200.45 ± 0.10[a]	198.85 ± 0.10[a]
4	121.09 ± 0.09[a]	139.55 ± 0.10[a]	199.25 ± 0.09[a]	195.25 ± 0.09[a]
5	119.07 ± 0.05[a]	145.45 ± 0.20[a]	199.27 ± 0.08[a]	201.10 ± 0.10[a]
6	120.30 ± 0.02[a]	145.50 ± 0.15[a]	202.50 ± 0.10[a]	199.75 ± 0.10[a]
7	120.30 ± 0.02[a]	179.75 ± 0.20[b]	269.08 ± 0.10[b]	250.80 ± 0.25[b]
8	159.50 ± 0.17[b]	180.75 ± 0.10[b]	278.70 ± 0.08[b]	260.25 ± 0.10[b]
9	171.09 ± 0.10[b]	199.51 ± 0.10[b]	287.25 ± 0.20[b]	278.20 ± 0.10[b]
10	172.07 ± 0.04[b]	179.90 ± 0.15[b]	298.25 ± 0.01[bc]	289.10 ± 0.15[b]
Isolated flavonoids				
Galangin	105.39 ± 0.10[c]	138.12 ± 0.10[a]	165.98 ± 0.21[c]	160.80 ± 0.15[c]
Pinocembrin	96.09 ± 0.15[c]	120.23 ± 0.15[ac]	150.25 ± 0.14[c]	145.25 ± 0.19[c]

[1] Micrograms of PPPEs, galangin and pinocembrin in the assay mixture; expressed as gallic acid equivalents.
[2] Values are presented as the mean of fifteen samples obtained from the same beehive ± standard deviation.
[3] The same letter, in the same column, indicates that the values are not significantly different at 0.05 level.

behaviour is an evidence of the differential chemoattractant activity of propolis derived from different places in the same phytogeographical region in Argentina. PPPEs have a higher immunostimulatory effect on neutrophils than galangin and pinocembrin, showed to be a good chemoattractant and increase significantly the neutrophil intracellular phagocytic activity. It worth to mention that propolis and their components also influence some enzyme systems involved in the immune response and the generation of inflammatory processes (Middleton & Kandaswami, 1993; Simoes-Ambrosio et al., 2010).

Neutrophil phagocytic and intracellular killing activities are the main functions of neutrophils in host resistance. This process is evidenced by the property of the tetrazolium salts that can rapidly penetrate into intact cells and directly into subcellular membranes with dehydrogenase activity, where they are converted to colored formazan derivatives (Seidler, 1991). Consequently, they were used (formazan derivatives) as indicators of the reducing system. Account was taken of the property of tetrazolium salts replace the natural final acceptor (oxygen) in the biological redox process and are reduced to formazan derivatives by receiving electrons enzymatically from substances of the hydrogen transport system. Kaempherol and quercetin have been shown to modulate the oxidative burst of stimulated human neutrophils (Busse, Kopp, & Middleton, 1984).

The activation of the non-specific immune system by immunomodulatory agents of any origin is a response of the body against diverse substances with stimulating capacity. Nevertheless, with respect to propolis and propolis components there is a need of systematic studies to fundament their possible pharmacological applications.

Our results indicated that the immunomodulatory activity of PPPEs, galangin and pinocembrin from propolis collected from beehives of the southernmost extension of the subtropical Andean montane forest of Argentina, may be attributed to the synergistic effect of groups of propolis constituents. The absence of toxicity of the PPPEs indicates that these propolis and the isolated compounds can be a useful source for treating patients suffering from some immune disorder.

5. Conclusions

As consequence of the ancient and extensive use of propolis as medicine and medical food, it is important to investigate their bioactivities.

The in vitro results show evidence that neutrophils recognize propolis extracts and isolated flavonoids as stimulants of their

Table 2
Nitroblue Tetrazolium (NBT) Test: Enhance of reduced neutrophils after treatment with partially purified propolis extracts (PPPEs) from 'El Siambón', Tucumán, Argentina and the isolated flavonoids (galangin and pinocembrin).

μg GAE/mL of PPPEs, galangin, pinocembrin in the assay	10	20	40	100[1]	Control*
Number of beehives	Intracellular killing activity (%)[2]				
1	115 ± 4[a,3]	119 ± 5[a]	115 ± 3[a]	115 ± 5[a]	15 ± 2[a]
2	110 ± 3[a]	116 ± 5[a]	115 ± 4[a]	114 ± 3[a]	17 ± 1[a]
3	113 ± 2[a]	115 ± 4[a]	115 ± 4[a]	115 ± 5[a]	15 ± 2[a]
4	112 ± 3[a]	115 ± 2[a]	115 ± 6[a]	114 ± 2[a]	16 ± 2[a]
5	116 ± 3[a]	115 ± 6[a]	116 ± 4[a]	115 ± 3[a]	18 ± 1[a]
6	117 ± 4[a]	115 ± 4[a]	116 ± 3[a]	153 ± 5[b]	16 ± 2[a]
7	168 ± 2[b]	179 ± 2[b]	191 ± 2[b]	185 ± 2[c]	18 ± 1[a]
8	162 ± 3[b]	187 ± 4[c]	189 ± 2[b]	185 ± 3[c]	16 ± 1[a]
9	168 ± 5[b]	189 ± 3[c]	194 ± 3[b]	182 ± 2[c]	19 ± 2[a]
10	171 ± 4[b]	185 ± 2[c]	195 ± 2[b]	179 ± 3[c]	19 ± 2[a]
Isolated flavonoids					
Galangin	168 ± 3[b]	170 ± 5[b]	174 ± 2[b]	182 ± 2[c]	16 ± 2[a]
Pinocembrin	162 ± 2[b]	165 ± 2[b]	168 ± 2[b]	173 ± 3[b]	16 ± 1[a]

*Control contained all reagents but neutrophil suspension.
[1] Micrograms of PPPEs, galangin and pinocembrin in the assay mixture; expressed as gallic acid equivalents.
[2] Values are presented as the mean of fifteen samples obtained from the same beehive ± standard deviation.
[3] Within each column, means followed by the same letter are not significantly different at 0.05 level (LSD test).

movement, at different rates, according to their attraction capacity.

Partially purified propolis extracts (PPPEs) showed the maximal chemoattractant stimulus, while the action of galangin and pinocembrin was less effective at the same concentration. The highest effect of PPPEs can be attributed to a synergistic effect of components of the PPPEs.

PPPEs and flavonoids have statistically significant effect on chemotaxis suggesting that they stimulate the immune system.

The NBT reduction test showed the production of formazan indicating the stimulation of neutrophil phagocytic activity by PPPEs and flavonoids.

It is important that the studies on the biological activities of propolis of different regions of Argentina and their components be continued, opening new areas of therapeutic applications of propolis.

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