Journal of Ethnopharmacology 168 (2015) 37–44

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

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

Anti-inflammatory activity and phenolic profile of propolis from two locations in Región Metropolitana de Santiago, Chile

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ARTICLE INFO

Article history:
Received 15 January 2015
Received in revised form
21 March 2015
Accepted 22 March 2015
Available online 31 March 2015

Keywords:
Anti-inflammatory activity
Chilean propolis
Flavonoids
Phenolic profile

ABSTRACT

Ethnopharmacological relevance: Propolis has long been used as a popular folk medicine due to its wide spectrum of alleged biological and pharmaceutical properties. In Chile, propolis is widely used by folklore medicine as an anti-inflammatory agent; however, this property has not been demonstrated by scientific methods.

Aim of the study: The objective of this study was to determine the anti-inflammatory activity *in vivo* and *in vitro* and to establish the phenolic profile of propolis collected in two localities in Región Metropolitana de Santiago (RM), Chile.

Materials and methods: Propolis was collected in the areas of Caleu and Buin, RM Chile. Following that, the samples were unwaxed to obtain the global ethanolic extracts of propolis (EEPs) and, from these, the serial extracts of dichloromethane (EEP-DCMs) and ethanol (EEP-EtOHs). The topic anti-inflammatory effect was evaluated through mice ear edema induced by arachidonic acid (AA) and 12-O-tetradecanoylphorbol-13-acetate (TPA) at a dose of 3 mg/ear. Nitric oxide (NO) measurements were determined spectrophotometrically (Greiss reagent) by the accumulation of nitrite in the medium of macrophages RAW 264.7 stimulated with the lipopolysaccharide (LPS, 1 μg/mL) for 20 h at different concentrations of the EEPs, EEP-DCMs and EEP-EtOHs (6.25–50.00 μg/mL). The content of total phenols and flavonoids were determined through the methods of Folin–Ciocalteau and $AlCl_3$, respectively. The profile of phenolic compounds was determined by HPLC–UV–ESI-MS/MS.

Results: The EEP-EtOH (64%) and EEP (59%) of Buin were the most active in the inflammation induced by TPA and AA respectively, being the anti-inflammatory effect stronger than the same Caleu extracts. Regarding the release of NO, all the extracts from the Buin propolis inhibited significantly its release in a concentration-dependent manner, this inhibition was stronger than the extracts from Caleu propolis.

Conclusions: Our research shows for the first time a comparative study of the topical *in vivo* activity of two Chilean propolis. Both propolis showed *in vivo* topical anti-inflammatory activity against AA and TPA, the most active was Buin propolis and this difference is due in part to the variations in total phenols and flavonoids content and the phenolic profile. The phenols and flavonoids content of Buin propolis was higher than Caleu propolis. The extracts from Buin propolis result in a lower release of NO.

1. Introduction

Propolis is a resinous material elaborated by the bee *Apis mellifera, n.v.*, through the recollection of the exudates from different plant species. The bees use propolis to repair combs, to strengthen the thin borders of the comb, and to make the entrance of the hive watertight or easier to defend. Propolis is also used as an embalming substance to cover the carcass of a hive invader which the bees have killed but cannot transport out of the hive. The bees cover the invader with propolis and wax, and the remains are left at the bottom or on one of the walls of the hive (Toreti et al., 2013). Propolis is constituted by resinous, sticky and balsamic substances; such as waxes, essential oils and pollen, among others (Tosi et al., 2006).

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http://dx.doi.org/10.1016/j.jep.2015.03.050
0378-8741/

Folk medicine recommends the use of propolis, due to its antibacterial, anti-fungal, and anti-viral effects and its hepatoprotective and anti-inflammatory properties, to increase the resistance against infections and to treat gastroduodenal ulcers (Castaldo and Capasso, 2002). Propolis has attracted researchers' interest in the last decades because of several biological and pharmacological properties, such as immunomodulatory, anti-tumor, anti-inflammatory, anti-oxidant, anti-bacterial, anti-viral, anti-fungal and anti-parasite, among others (Sforcin and Bankova, 2011). Besides, products containing propolis have been intensely marketed by the pharmaceutical industry and health-food stores (Sforcin, 2007). The ethnopharmacological approach, combined with chemical and biological methods, may provide useful pharmacological leads about the medical effects of propolis.

In Chilean propolis, only some of these properties have been evaluated, such as anti-oxidant (Astudillo et al., 2000; Castro et al., 2014; Russo et al., 2004), anti-bacterial (Saavedra et al., 2011), anti-fungal (Herrera et al., 2010) and anti-tumoral (Russo et al., 2004), among others. However, the anti-inflammatory activity through *in vivo* and *in vitro* methods has not been investigated.

The chemical composition of propolis is complex and varied, which is why a great amount of compounds have been identified, such as: alcohols, aldehydes, phenolic acids, amino acids, chalcones, flavonoids, lignans, triterpenes, steroids and sugars, among others. However, phenolic compounds are the most abundant (Righi et al., 2013).

In this research, we present a comparative study of the *in vivo* anti-inflammatory activity of two propolis recollected from the localities of Buin and Caleu, which have similar climate and soil conditions but whose main variation was in the surrounding vegetation. Different unwaxed extracts were prepared and their topical anti-inflammatory activities were evaluated in the edema model in mice ear, induced by 12-O-tetradecanoyl-phorbol-13-acetate (TPA) and arachidonic acid (AA). The total phenols and flavonoids content, phenolic profile and inhibitory effect of the release of nitric oxide (NO) were determined for each extract.

2. Materials and methods

2.1. Propolis samples

The samples were recollected during spring in 2011, the hives free of diseases were located in Buin (33°38′43.84″S; 70°39′59.69″O) and Caleu (33°00′12″S; 70°59′37″O), Región Metropolitana de Santiago (RM), Chile. The harvest was done with plastic meshes. Then, the meshes were preserved at −4 °C for 4 h; after this period, the meshes were flexed to release the propolis. Afterwards, the different samples were stored at −20 °C protected from the light.

Buin propolis presented a homogenous aspect, a rigid consistency with brown and green hues. Caleu propolis presented a heterogenous aspect, a rigid consistency, and green and yellow hues.

Buin and Caleu are known for having a Mediterranean weather, long dry season and rainy winter. It is a warm temperate weather in which winter rains are concentrated between May and August. The main difference between both locations is in the vegetation surrounding the hives, identified by the taxonomist Sebastian Teiller. In Buin, there were found: *Agapanthus africanus* (n.v. agapanto), *Argyranthemum frutescens* (n.v. paraquet), *Cyperus alterniflorus* (n.v. paragüita), *Escallonia illinita* (n.v. barraco), *Lavandula angustifolia* (n.v. lavanda), *Medicago sativa* (n.v. alfalfa), *Nerium oleander* (n.v. laurel de flor), *Otholobium glandulosum* (n.v. culén), *Populus* spp. (n.v álamo), *Salvia microphylla* (salvia) *and Verbena officinalis* (n.v. verbena); and in Caleu: *Santolina chamaecyparissus* (n.v. manzanillera), *Acacia caven* (n.v. espino), *Trifolium repens* (n.v. trébol blanco), *Cynodon dactylon* (n.v. chépica), *Lotus corniculatus* (n.v. lotera), *Plantago lanceolata* (n.v. siete venas), *Populus* spp. (n.v. álamo) *and Quillaja saponaria* (n.v. quillay).

2.2. Preparation of propolis extracts

To elaborate the global ethanolic extracts of propolis (EEPs), initially a process of unwaxing was carried out in the crude propolis employing temperature cycles, according to the methodology proposed by Alencar et al. (2007) and Kalogeropoulos et al. (2009). 400 g of raw propolis from each locality was weighed, homogenized in a mortar and added to 750 mL of ethanol. Following this, the ethanolic mixture was introduced in a thermoregulated bath at 70 °C for 30 min; afterwards, it was cooled at room temperature and refrigerated at −20 °C for 12 h. Finally, it was filtered and the supernatant was preserved. This procedure was repeated 3 times with the aim of extracting the wax exhaustively. Once the propolis was unwaxed, the extracts were put in a rotary evaporator at 60 °C until the solvent was completely eliminated, obtaining the EEP.

The serial extracts were elaborated through successive extractions of the EEP with the solvents dichloromethane (DCM) and ethanol (EtOH), resulting in the extracts EEP-DCM y EEP-EtOH, respectively. Each extraction was carried out until the EEPs were completely exhausted; between each extraction the material was dried at room temperature before adding the new dissolvent. The serial extracts were concentrated in a rotary evaporator at reduced pressure.

2.3. In vivo topical anti-inflammatory activity

Two inflammatory agents, AA and TPA, were used to estimate the probable anti-inflammatory action mechanism of the propolis under study. The reference drugs used were indomethacin and nimesulide against TPA and AA, respectively.

All animal experiments were performed according to the ethical guidelines suggested by the "International Norms for the Biomedical Investigation with Animals", elaborated by the Council of International Organizations (1990) and the bio-ethics norms of the Commission of the Instituto de Salud Pública de Chile (ISP) and the Facultad de Ciencias Químicas y Farmacéuticas of the Universidad de Chile (CBE2012-4).

Adult male CF-1 mice (20–25 g), obtained from the stock at the ISP, were used to assess the anti-inflammatory effect. All animals were housed in a climate and light-controlled room with a 12 h light–dark cycle, fasted overnight before the day of the assays, with free access to water. For each of the samples under study, the anti-inflammatory activity was evaluated in two groups. One group of 8 treated mice and the other of 16 control mice. After 5 min of sample treatment (3 mg/ear of the EEP, EEP-DCM and EEP-EtOH), mice received 5 μg of TPA or 2 mg of AA (Sigma, St. Louis, MO, USA) as pro-inflammatory agents, dissolved in 20 μL of acetone (solvent does not interfere with the assay). Control subjects only received TPA or AA at the same concentration. Both, the sample and the TPA or AA, were applied to the inner (10 μL) and outer (10 μL) surfaces of the right ear. The left ear only received acetone. Mice were sacrificed by cervical dislocation (after 6 h of TPA and 1 h of AA), and a 6 mm diameter section of the right and left ears were cut and weighted. The weight differences between both ear sections correspond to the edema value. Topical anti-inflammatory effect (EA) was evaluated according to the following equation: $\%EA = [W_c - W_s/W_c] \times 100$; where W_c and W_s are the difference median values of the weights of the right and the left ear sections of the control (W_c) and the treated animals (W_s) respectively (Delporte et al., 2003).

2.4. Inhibitory activity of NO release

2.4.1. Cell culture

The murine macrophage cell line RAW 264.7 was maintained in DMEM Ham´s F-12 medium supplemented with 10% fetal bovine serum, L-glutamine (1 mM), penicillin (100 U/mL), streptomycin (100 μg/mL), gentamicin (40 μg/mL), acquired in Life and Technology (Barcelona, Spain) and L-arginine (1 mM, Sigma, St. Louis, MO, USA) in a humidified 5% CO_2 atmosphere (Díaz-Viciedo et al., 2008; Girón et al., 2010).

2.4.2. MTT assay for cell viability

Cytotoxicity studies were assessed by the mitochondrial-dependent reduction of the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) to formazan. Macrophages were plated at a density of 3.25×10^5 cells/well in 96-well plates. To determine the appropriate non-toxic concentrations for cells, cells were incubated in the presence of different concentrations of propolis samples (6.25–100.00 μg/mL) for 24 h, before they reacted with MTT (2 mg/mL in phosphate-buffered saline PBS, Sigma, St. Louis, MO, USA) at 37 °C for 1 h. The reaction product, formazan, was extracted with dimethyl sulphoxide (DMSO) and the absorbance was read at 550 nm. Assays were performed in triplicate, and results were expressed as the percent reduction in cell viability compared to untreated control cultures for at least three independent experiments (Cuadrado et al., 2012; Girón et al., 2010).

2.4.3. Inhibitory activity of NO release in RAW 264.7 macrophages

Macrophage cells were seeded in 96-well plates and were incubated with 1 μg/mL of lipopolysaccharide (LPS, Sigma, St. Louis, MO, USA) at 37 °C for 20 h in the presence of test samples or vehicle. The release of NO into phenol red-free medium was determined from the amount of accumulated nitrite, measured spectrophotometrically with the Griess reagent (Sigma, St. Louis, MO, USA). The compound N-(3-[Aminoemethyl]benzyl) acetamidine (1400W, Sigma, St. Louis, MO, USA) was used as positive control. The absorbance at 540 nm was compared to a $NaNO_2$ standard (Cuadrado et al., 2012; Girón et al., 2010).

2.5. Determination of total phenolic content in the propolis extracts

To determine the amount of total phenols present in the EEP, EEP-DCM and EEP-EtOH of each propolis, the method Folin–Ciocalteau was used according to what was suggested by Cicco et al. (2009). 100 μL of hydroalcoholic solutions (methanol/water, 70:30 v/v) of the extracts (1 mg/mL) were mixed with 100 μL of the reactive Folin–Ciocalteau (Merck, Germany), after 2 min, 800 μL of Na_2CO_3 at 5% p/v were added. This mixture was heated in a bath at 40 °C for 20 min, then the absorbance was measured in a spectrophotometer Unicam UV–vis. The samples were evaluated in triplicate. The obtained absorbances were interpolated in a calibration curve ($y=0.1044x+0.0097$, $R^2=0.9995$, $F_{calculated}$ 0.3445 < F_{Table} 3.7083) of galic acid (2–7 μg/mL). The results were expressed as *g* equivalents of galic acid/100 g of dry extract of propolis.

2.6. Determination of flavonoids content in the propolis extracts

The $AlCl_3$ was employed to determine the content of flavonoids. Hydroalcoholic solutions (methanol/water, 70:30 v/v) were elaborated from the propolis extracts (1 mg/mL). Afterwards, 0.5 mL of the hydroalcoholic solutions of propolis were mixed with 0.5 mL of an $AlCl_3$ (0.5% p/v) solution, then it was brought to volume with methanol at 25 mL. The mixtures were left for 30 min the dark, then were placed in a quartz cuvette, and the absorbances at 465 nm were read in a spectrophotometer UV/vis Spectromer Unicam UV3 (Popova et al., 2004). The results were interpolated in the calibration curve ($y=0.0087x-0.002$, $R^2=0.9964$, $F_{calculado}$ 0.7456 < F_{Tabla} 3.2592) previously constructed with quercetin (2–6 μg/mL). The results were expressed as *g* equivalent of quercetin/100 g of dry propolis extract.

2.7. Identification of propolis extracts by HPLC–UV–ESI-MS/MS

The propolis extracts were analyzed by HPLC Agilent 1100 (Agilent Technologies Inc., CA-USA) connected to the ion electrospray trap mass spectrometer Esquire 4000 ESI-IT (Bruker Daltonik GmbH, Germany). For the separation of HPLC, a column Symmetry C18 (Waters, MA, USA) 5 μM of 250×4.6 mm^2 was used. The separation of 20 μL of blank (methanol) and the propolis extracts (6000 ppm) was done at room temperature using a gradient system composed by the binary phases (A) formic acid 0.1% v/v in water and (B) formic acid 0.1% v/v in acetonitrile. The elution gradient was: 0–3 min 25% B, 3–10 min 30% B, 10–40 min 40% B, 40–60 min 60% B y 60–92 min 90% B. The flow employed was of 1.2 mL/min. The UV detection was made using a wave length of 290 nm. The ionization process (nebulization) using electrospray was done at 3.000 V assisted by nitrogen as the nebulizer gas at a temperature of 365 °C, pressure of 60 psi and flow of 10 L/min. The mass spectrometers were acquired in negative polarity. The analysis of the chromatograms and mass specters was done using the program Data Analysis 3.2 (Bruker Daltonik GmbH, Germany). The identification of the compounds was done revising the scientific literature (Castro et al., 2014; Falcão et al., 2010; Gardana et al., 2007; Medana et al., 2008; Pellati et al., 2011; Righi et al., 2013) and the data base MassBank. The relative amount of each compound in each extract was calculated. The molecular ion of each compound was selected by selective ion mode and then its area was integrated; the peak area of each compound of the EEPs was assigned as the 100% of abundance in order to calculate the relative percentage with their respective EEP-DCM and EEP-EtOH. With the aim of comparing the relative percentage between both propolis, the peak areas of the common compounds were compared. The 100% was assigned to the content of each compound in the Buin EEP and, therefore, the relative percentage obtained corresponded to the area of the peak of each compound from the Caleu extracts divided by the area of the Buin EEP multiplied by 100.

2.8. Statistical analysis

The significance of the results (p) of the *in vivo* assay was determined using Kruskall–Wallis test and Mann–Whitney test was used for the individual comparisons. One-way ANOVA and Tukey post-hoc multiple comparison tests were used to analyze data from the *in vitro* assay. The differences were considered significant for $p \leq 0.05$.

3. Results and discussion

3.1. In vivo topical anti-inflammatory activity

The results are summarized in Table 1. All extracts of propolis showed topical anti-inflammatory activity. Generally, they were more active against TPA than AA. In the same way, against both anti-inflammatory agents, the extracts from Buin propolis were more active than the extracts from Caleu propolis.

The model of ear edema induction in mice, using different inflammatory agents (TPA and AA), is widely used to identify the probable topical anti-inflammatory effect of a substance under

Table 1
Topical anti-inflammatory effects, assessed by the mice ear edema induced by arachidonic acid (AA) and O-tetradecanoyl-phorbol-13-acetate (TPA), total phenolic and flavonoids content of propolis extracts.

Propolis extracts	Topical anti-inflammatory effects			Phenolic contents	
	Dose (mg/ear)	% $EA_{AA} \pm$ SEM	% $EA_{TPA} \pm$ SEM	Total phenolic (% w/w)	Flavonoids (% w/w)
EEP_{Caleu}	3	8 ± 8	50 ± 3*	$24.3 \pm 0.6^{b,c,d,e,f}$	$3.1 \pm 0.6^{d,e,f}$
$EEP\text{-}DCM_{Caleu}$	3	0 ± 6	36 ± 4*	$20.8 \pm 0.6^{a,c,d,e,f}$	$2.5 \pm 0.4^{d,e,f}$
$EEP\text{-}EtOH_{Caleu}$	3	26 ± 7*	42 ± 3*	$14.6 \pm 0.4^{a,b,d,e,f}$	$2.1 \pm 0.2^{d,e,f}$
EEP_{Buin}	3	59 ± 7*	55 ± 5*	$31.5 \pm 0.4^{a,b,c,f}$	$10.9 \pm 0.2^{a,b,c,e,f}$
$EEP\text{-}DCM_{Buin}$	3	38 ± 7*	61 ± 2*	$30.9 \pm 0.4^{a,b,c,f}$	$7.6 \pm 0.5^{a,b,c,d,f}$
$EEP\text{-}EtOH_{Buin}$	3	28 ± 6*	64 ± 4*	$36.4 \pm 0.6^{a,b,c,d,e}$	$14.8 \pm 0.4^{a,b,c,d,c}$
NIM	1	$\uparrow 49 \pm 4$*	n.d	n.d	n.d
IND	0.5	n.d	$\uparrow 93 \pm 3$*	n.d	n.d

↑ maximum effect; EEP global ethanolic extract of propolis; EEP-DCM serial dichloromethane extract of propolis; EEP-EtOH serial ethanolic extract of propolis; EA_{AA} topical anti-inflammatory effect against AA; EA_{TPA} topical anti-inflammatory effect against TPA; NIM nimesulide; IND indomethacin; n.d not determined. Results total polyphenolic and flavonoids are presented as mean ± SD ($n=3$) and calculated as galic acid and quercetin equivalents, respectively.

* $p \leq 0.05$ respect to control.
[a] Significative difference compared with EEP_{Caleu}.
[b] Significative difference compared with $EEP\text{-}DCM_{Caleu}$.
[c] Significative difference compared with $EEP\text{-}EtOH_{Caleu}$.
[d] Significative difference compared with EEP_{Buin}.
[e] Significative difference compared with $EEP\text{-}DCM_{Buin}$.
[f] Significative difference compared with $EEP\text{-}EtOH_{Buin}$.

study and its probable action mechanism (Gábor, 2003). TPA induced inflammation develops more slowly than AA induced inflammation. TPA is a powerful tumor promoting agent found in croton oil (*Croton tiglium* L.). The topical administration of TPA provokes an acute edema with leukocyte infiltration, acting through the triggering of the protein kinase C (PKC), which is Ca^{2+} and phospholipids dependent. PKC plays an important role in the signal transduction of a great variety of substances that trigger cellular and proliferation functions. Active PKC acts at different levels, activating the nuclear factor kappa B (NF-κB). This transcription factor promotes the expression of several pro-inflammatory agents, such as cyclooxygenase 2 (COX-2) and inducible nitric oxide synthase (NOS-2), inflammatory cytokines such as interleukin-1 (IL-1), IL-2, IL-6, IL-8 and the tumor necrosis factor-alpha (TNF-α), which is another pro-inflammatory and host defense cytokine. By contrast, the anti-inflammatory response to AA is quicker and is produced by an increased activity of myeloperoxidase and elastase, due to neutrophils' quick arrival after applying this inflammatory agent (Gábor, 2003).

Farooqui and Farooqui (2010) described that propolis exerts anti-inflammatory activity not only by decreasing prostaglandin (PG) and leukotriene (LT) generation due to the inhibition of the expression and activities of COXs and lipoxygenases (LOXs), but also by retarding the gene expression of NOS-2, the inhibition TNF-α mediated by NF-κB, and reducing the immune response in T cells. They concluded that the inhibition of NF-κB activation may be the molecular basis of the anti-inflammatory properties of propolis. These results correlate with ours, since the propolis of Caleu and Buin were more active against the inflammation induced by TPA than by AA, since – as it was previously explained – the inflammation induced by TPA occurs through the activation of NF-κB (Gábor, 2003).

3.2. Inhibitory activity of NO release in RAW 264.7 macrophages

Inflammation is a central feature of many pathological conditions. The pathogenesis of the inflammatory response involves the sequential activation of signaling molecules, among which PGs and NO are well-known key inflammatory mediators, generated, respectively, by COX-2 and NOS-2. In murine macrophage RAW 264.7 cells, LPS induces NOS-2, and then NO release. Therefore, this macrophage cell line provides an excellent model for drug screening and to evaluate potential inhibitors on the pathway leading to the induction of NOS-2. The reactive free radical NO synthesized by NOS-2 is a major macrophage-derived inflammatory mediator and has also been reported to be involved in the development of inflammatory diseases (Díaz-Viciedo et al., 2008).

The assays of cellular viability confirmed that in concentrations between 6.25 and 50.00 μg/mL, Caleu propolis extracts were not cytotoxic and between 6.25 and 25.00 μg/mL, Buin propolis extracts were not cytotoxic (data not shown). Regarding the results of NO release, all the extracts of propolis inhibited significantly and in a dose-dependent way the production of this mediator. The Buin propolis extracts present higher values of inhibition: EEP, EEP-DCM and EEP-EtOH in a concentration of 25.00 μg/mL demonstrated an inhibition of NO release in a percentage of 73.2%, 55.3% and 67.8%, respectively. For their part, the EEP, EPP-DCM and EEP-EtOH of Caleu, in the same concentration, showed to inhibit the release of NO in a percentage of 35.5%, 45.4% and 31.7%, respectively. Therefore, the anti-inflammatory effect against TPA of said extracts could be in part linked to their capacity to inhibit of NOS-2.

The positive control (1400W in a concentration of 12.50 μg/mL), which is a selective inhibitor of NOS-2, reached an inhibition of NO liberation of 90.0%.

In the bibliography, it is possible to find propolis from different origins that inhibit the liberation of NO, for example, Han et al. (2002), evaluated the release of NO in RAW 264.7 cells, activated by LPS and treated with a Korean EEP, determining that the maximum effect of inhibition was achieved at 100 μg/mL (78.9%). Song et al. (2002) determined that a Korean EEP at concentrations of 12.5, 25.0 and 50.0 μg/mL could significantly inhibit the liberation of NO in RAW 264.7 cells stimulated during 24 h with interferon gamma (IFN-γ). Subsequently, Blonska et al., (2004) determined that an EEP from Poland was able to inhibit in a 55.8% the liberation of NO at a concentration of 30 μg/mL in macrophages J74A.1 activated with LPS. They also proved that the main inhibitory component of the NO release were chrysin, galangin, kaempferol and quercetin. These components were all present in the propolis samples studied in this work (see Tables 2 and 3).

3.3. Determination of total phenolic in the extracts of propolis

Table 1 presents the results that determine the total content of total phenols. The results show that the extracts elaborated with

Table 2
HPLC–ESI-MS^2 (IT) data obtained for the analysis of propolis of Caleu (EEP, EEP-DCM and EEP-EtOH) constituents in the negative ion mode.

	Compounds (EEP)	Tr (min)	M_W (g/mol)	$[M-H]^-$ (m/z)	Fragmentation MS^2 (m/z)	Semiquantification respect to EEP		Reference
						EEP-DCM %	EEP-EtOH%	
1	Caffeic acid O-glucoside	1.8	342	341	178, 149, 135	n.d	n.d	[a]
2	7,8-Dihydroxy-6-methoxycoumarin	2.1	208	207	164, 191, 108	n.d	n.d	[a,b]
3	Vanillin	4.0	152	151	135, 108	n.d	n.d	[a,b]
4	Quercetin-O-glucoside	4.2	464	463	301, 161, 271	n.d	304	[a]
5	Apigenin-O-rutinoside	5.9	578	577	269	n.d	n.d	[a]
6	Ferulic acid	6.2	194	193	149, 133, 177	n.d	434	[a,b]
7	Quercetin-3-methyl-ether	6.8	316	315	300, 271, 151	n.d	150	[a]
8	Ethoxy benzoic acid	7.1	166	165	149, 135	80	n.d	[a]
9	Quercetin	12.4	302	301	151, 178	n.d	57	[a,b]
10	Quercetin-3-methyl-ether	13.3	316	315	300, 271, 151	n.d	65	[a]
11	Kaempferol	15.4	286	285	151	n.d	n.d	[a,b]
12	Pinobanksin	15.7	272	271	253, 225, 151	107	n.d	[a]
13	Luteolin-methyl-ether	16.4	300	299	284, 255	n.d	n.d	[a]
14	Quercetin-dimethyl-ether	16.9	330	329	314, 299	n.d	n.d	[a]
15	Pinocembrin-5-methyl-ester	19.5	270	269	254, 227	n.d	n.d	[a]
16	Drupanin	20.8	232	231	187, 133	120	360	[a]
17	Quercetin-dimethyl-ether	22.0	330	329	314, 299	191	358	[a]
18	Chrysin	26.7	254	253	209, 143	n.d	n.d	[a,b]
19	Caffeic acid benzyl ester	27.5	270	269	134, 178	54	200	[a]
20	Pinocembrin	30.3	256	255	213, 211, 151	n.d	n.d	[a]
21	Galangin	33.1	270	269	213, 252, 197	237	440	[a,b]
22	Pinobanksin-3-O-acetate	33.4	314	313	253, 271	n.d	216	[a]
23	Methoxy-chrysin	35.7	284	283	269	79	n.d	[a]
24	Pinobanksin-3-O-acetate	35.9	314	313	253, 271	6	163	[a]
25	Chrysin	51.7	254	253	209, 143	44	21	[a,b]

n.d: undetected; EEP global ethanolic extract of propolis; EEP-DCM serial dichloromethane extract of propolis; EEP-EtOH serial ethanolic extract of propolis. The semiquantification was carried out in respect to EEP and the relative percentages obtained correspond to the areas of the peaks of the EEP-DCM and EEP-EtOH extracts divided by the areas of EEP × 100.

[a] Reference.
[b] Data base.

the propolis from Buin presented the highest content of phenolic compounds and flavonoids. EEP-EtOH was the extract with the most amount of total phenols and flavonoids. Adding to this, it is noteworthy that all the extracts from Buin propolis showed a greater anti-inflammatory activity *in vivo* and inhibition of NO release than the extracts from Caleu propolis.

The content of phenolic compounds in the different extracts obtained from the Buin and Caleu propolis are greater than the ones determined by Mohammadzadeh et al. (2007), who studied propolis from different areas in Iran. They contents of total phenols that they found were from 3.0% to 8.0%, using the same methodology. Silva et al., (2012) analyzed the hydroalcoholic extracts of 3 samples of propolis from different regions in Portugal, and they determined that the total phenol content was between 14.2% and 2.5%; these percentages are also lower than those obtained with the elaborated extracts from propolis of Buin and Caleu. However, the total phenol content of the propolis from Buin and Caleu are lower to the content of total phenols from propolis from the south of Chile (San Vicente de Tagua-Tagua), where results show a total phenol content of 63.9% (Russo et al., 2004).

3.4. Determination of flavonoids content in the propolis extracts

Regarding flavonoids content (Table 1), it should be noted that the method of evaluation of flavonoids, the formation of a colored complex with $AlCl_3$, allows the determination of flavones and flavonols (Popova et al., 2004). The Buin propolis presented, in all its extracts, a higher content of flavonoids regarding the extracts from Caleu propolis.

The flavonoids content of the propolis extracts from Buin were higher than those reported by Chang et al. (2002) who determined the content of flavonoids in six propolis samples: three samples from Taiwan, one from Brazil and two from China. The highest content was in one of the Chinese samples, 7.8%, and the lowest content corresponded to the Taiwanese sample, 2.3%. Socha et al., (2015) analyzed samples of propolis from various regions of Poland and found that the flavonoids content varied in 3.5–6.2%, these were significantly lower than the flavonoids content of the extracts elaborated with Buin propolis.

3.5. Identification of propolis extracts by HPLC–UV–ESI-MS/MS

Tables 2 and 3 present the phenolic profile of Caleu and Buin propolis, respectively. 12 common phenolic compounds were found between both propolis studied (quercetin, quercetin-3-methyl-ether, kaempferol, pinobanksin, quercetin-dimethyl-ether, luteolin-methyl-ether, drupanin, caffeic acid benzyl ester, pinocembrin, galangin, pinobanksin-3-O-acetate, and chrysin).

A semiquantification was done, using as reference Buin's EEP (*i.e.*, 100% was assigned to EEP of the Buin), of all the common compounds (data not shown).

By comparing the relative percentages in some compounds present in the extracts obtained from Buin propolis, we can highlight that the contents of quercetin and kaempferol were higher in the EEP-EtOH, unlike caffeic acid benzyl ester, pinocembrin, galangin, pinobanksin-3-O-acetate and caffeic acid phenethyl ester (CAPE), which where predominant in Buin's EEP-DCM. We relate to all extracts of the Caleu propolis, these obtained the minor relative percentages of the compounds found in both propolis.

In Chile, there are few studies referring to the chemical compositions of our propolis. Some of these investigations: (a) Muñoz et al. (2001), identified the compounds present in a propolis from the central zone of Chile (Colliguay, Región de Valparaíso), and found pinocembrin, galangin and kaempferol, among others, (b) Herrera et al. (2010), evaluated 6 commercial extracts of propolis obtained from the south zone of Chile (Temuco, IX Región de la Araucanía),

Table 3
HPLC–ESI-MS^2 (IT) data obtained for the analysis of propolis of Buin (EEP, EEP-DCM and EEP-EtOH) constituents in the negative ion mode.

	Compounds (EEP)	Tr (min)	M_W (g/mol)	$[M-H]^-$ (m/z)	Fragmentation MS^2 (m/z)	Semiquantification respect to EEP		Reference
						EEP-DCM %	EEP-EtOH %	
1	Dihydroxyphenyl caffeate	2.1	282	281	134, 161, 178	69	228	[a]
2	Esculetin	3.7	178	177	133	10	9	[a,b]
3	7,8-Dihydroxy-6-methoxycoumarin	3.9	208	207	164, 191, 108	6	n.d	[a,b]
4	Dihydroxyphenyl caffeate	4.1	282	281	134, 161, 178	103	277	[a]
5	p-coumaric acid	5.5	164	163	119	132	79	[a,b]
6	Dicaffeoyl quinic acid	6.3	516	515	353	n.d	831	[a]
7	Esculetin	10.1	178	177	133	114	n.d	[a,b]
8	Quercetin	12.1	302	301	151, 178	62	298	[a,b]
9	Pinobanksin-5-methyl-ether	13.0	286	285	267, 239, 252	90	31	[a]
10	Quercetin-3-methyl-ether	13.3	316	315	300, 271, 151	58	158	[a]
11	Apigenin	14.5	270	269	225, 151	73	136	[a,b]
12	Kaempferol	15.3	286	285	151	78	178	[a,b]
13	Pinobanksin	15.6	272	271	253, 225, 151	42	41	[a]
14	Luteolin-methyl-ether	16.4	300	299	284, 255	92	96	[a]
15	Quercetin-dimethyl-ether	16.8	330	329	314, 299	76	76	[a]
16	Galangin-5-methyl-ether	18.2	284	283	268, 239, 211	91	48	[a]
17	Pinobanksin-5-methyl-ether-3-acetate	18.8	328	327	285, 267	84	28	[a]
18	Quercetin-7-methyl-ether	20.2	316	315	300, 165	111	78	[a]
19	Drupanin	20.8	232	231	187, 133	n.d	613	[a,b]
20	Galangin-5-methyl-ether	21.2	284	283	268, 239, 211	36	48	[a]
21	Quercetin-dimethyl-ether	22.6	330	329	315, 299	98	60	[a]
22	Caffeic acid prenyl ester	26.2	248	247	135, 179	106	31	[a]
23	Chrysin	26.8	254	253	209, 143	78	67	[a,b]
24	Caffeic acid prenyl ester	27.2	248	247	135, 179	88	28	[a]
25	Caffeic acid benzyl ester	27.4	270	269	135, 179, 161	104	37	[a]
26	Pinocembrin	30.1	256	255	213, 211, 151	112	32	[a]
27	Galangin	31.0	270	269	213, 252, 197	105	59	[a,b]
28	Pinobanksin-5,7-dimethyl-ether	32.4	300	299	165, 285, 253	99	69	[a]
29	Caffeic acid phenylethyl ester (CAPE)	33.0	284	283	178, 135	103	38	[a]
30	Pinobanksin-3-O-acetate	33.4	314	313	253, 271	104	30	[a]
31	Caffeic acid phenylethyl ester (CAPE)	35.5	284	283	178, 135	105	31	[a]
32	Caffeic acid cinnamyl ester	43.0	296	295	178, 135, 211	104	43	[a]
33	Chrysin-5,7-dimethyl-ether	43.2	284	283	267, 165	n.d	n.d	[a]
34	Pinobanksin-3-O-propionate	43.8	328	327	253, 271, 209	147	26	[a]
35	Pinobanksin-3-O-butyrate	47.4	242	241	253	95	15	[a]
36	Pinobanksin-3-O-pentanoate	52.1	356	355	253	98	16	[a]
37	Pinobanksin-3-O-hexanoate	59.1	370	369	253, 271	84	17	[a]

n.d: undetected; EEP global ethanolic extract of propolis; EEP-DCM serial dichloromethane extract of propolis; EEP-EtOH serial ethanolic extract of propolis. The semiquantification was carried out in respect to EEP and the relative percentages obtained correspond to the areas of the peaks of the EEP-DCM and EEP-EtOH extracts divided by the areas of EGE × 100.

[a] Reference.
[b] Data base.

finding 35 compounds, among which there were caffeic acid, quercetin, kaempferol, apigenin, pinocembrin, galangin and CAPE and (c) Castro et al. (2014), who identified 30 compounds in propolis from Buin, Cajón del Maipo, Caleu, Curacaví, Lo Cañas and Pirque, only pinobanksin and pinobanksin-3-O-pentanoate were common in all samples.

Generally, our results regarding to the phenolic profile of Buin and Caleu EEPs match which was reported by Castro et al. (2014). However, in our study we found unidentified compounds in both propolis. For the Caleu propolis, in this investigation, it has been identified for the first time the presence of acid caffeic acid O-glucoside, 7,8-dihydroxy-methoxy-6-coumarin, quercetin-O-glucoside, apigenin-O-rutinoside, quercetin-3-methyl-ether, quercetin, kaempferol, quercetin-dimethyl-ether, pinocembrin-5-methyl-ester, drupanin, caffeic acid benzyl ester, galangin and metoxy-chrysin. In the same way, in this investigation it is reported for the first time for Buin propolis: dihydroxyphenyl caffeate, p-coumaric acid, dicaffeoyl quinic acid, apigenin, kaempferol, luteolin-methyl-ether, galangin-5-methyl-ether, pinobanksin-5-methyl-ether-3-acetate, drupanin, pinobanksin-5,7-dimethyl-ether, caffeic acid cinnamyl ester, chrysin-5,7-dimethyl ether and pinobanksin-3-O-hexanoate.

Phenolic compounds have been said to be responsible, especially CAPE and flavonoids, for the biological activities of propolis (Wagh, 2013). Farooqui and Farooqui (2010) established that CAPE, quercetin and chrysin are the main cause of the anti-inflammatory activity of propolis. Woo et al. (2005) investigated the effect of chrysin on the expression of COX-2 in LPS-activated RAW 264.7 cells. They reported that chrysin significantly suppresses the LPS-induced COX-2 protein and mRNA expression in a dose-dependent manner. Blonska et al. (2004) studied hydroxyflavones, such as chrysin, galangin, kaempferol and quercetin, that potently reduce the iNOS mRNA level in J774A.1 macrophages. The most potent inhibitor of NO production was chrysin. Nagaoka et al. (2003) concluded that CAPE and its analogs were the most potent inhibitor production of NO. Mirzoeva and Calder (1996) studied the *in vivo* anti-inflammatory effect of propolis and its components, among them CAPE, which was the strongest inhibitor of the pro-inflammatory mediators triggered by AA. These results are related to those reported by Borrelli et al. (2002), who determined that the anti-inflammatory effects of propolis, both in chronic and acute models of inflammation, were due to the presence of CAPE. Adding to this, Rossi et al. (2002) showed that propolis and its components (pinocembrin, CAPE, galangin and the caffeic, ferulic, cinammic and cholorogenic acids) inhibit the activity of COX-1 and COX-2 directly proportional to its concentration. Of all the compounds evaluated, CAPE and galangin contributed to the anti-inflammatory activity of propolis; however, CAPE's effect was greater.

Others compounds present in Buin and Caleu propolis, with anti-inflammatory effects were: caffeic acid benzyl ester, caffeic acid prenyl ester (Uwai et al., 2008), ferulic acid, p-coumaric acid (Fernández et al., 1998), apigenin, kaempferol, quercetin-7-methyl-ether (Kim et al., 2004), dicaffeoyl quinic acid (Peluso et al., 1995), caffeic acid cinnamyl ester (Nagaoka et al., 2003), pinocembrin (Rasul et al., 2013; Soromou et al., 2012) and vanillin (Makni et al., 2011).

It is conceivable that the observed action of propolis can be due, at least in part, to the content of CAPE and flavone derivatives, some of them: apigenin, chrysin, galangin, kaempferol and quercetin are frequently found in propolis (Blonska et al., 2004; Calixto et al., 2003; Kim et al., 2004; Nagaoka et al., 2003; Rossi et al., 2002), and are present in a higher relative percentage in the extracts obtained from Buin propolis than Caleu propolis and would contribute to the higher anti-inflammatory effect of Buin extracts in respect to the Caleu extracts. It should be noted that Castro et al. (2014) determined the anti-oxidant activity, and in this study it was found that the EEP of Buin propolis had the highest anti-oxidant effect in comparison to the other propolis studied. And so, since the anti-oxidant activity of the EEP obtained from Buin propolis was higher than it was for the Caleu propolis, this would in part justify the higher anti-inflammatory activity that we found in Buin propolis, since it is known that the inflammatory process produces free radicals (Farooqui and Farooqui, 2010).

As it was previously pointed out, the localities of Buin and Caleu have similar climate and soil conditions and their main variation is the surrounding vegetation. Near the hives in Buin and Caleu were plant species of the genus *Populus*. The bud exudates of poplar trees and poplar type propolis also have similar qualitative compositions, but they may be very different quantitatively. It has been reported that chrysin, galangin, pinocembrin, quercetin, kaempferol and some phenolic acids (CAPE and p-coumaric acid) are the predominant bioactive constituents present in poplar type propolis and poplar tree buds (Rubiolo et al., 2013; Toreti et al., 2013). These compounds were identified in Buin and Caleu propolis.

4. Conclusions

Our results show the significant differences between the anti-inflammatory activity of the Buin and Caleu propolis, adding to the differences in their phenolic profile. Buin propolis exhibited a higher total phenol and flavonoids content regarding Caleu propolis, it was also the most active as a topical anti-inflammatory against the inflammatory agents TPA and AA, and was a more potent inhibitor of NO.

Although both propolis presented 12 common phenolic compounds, in Buin propolis they are present in a higher relative percentage, which could explain in part the higher anti-inflammatory effect of this propolis.

Our research show for the first time a comparative study of the anti-inflammatory topical activity *in vivo* of two Chilean propolis.

Acknowledgments

We are grateful to CONICYT AT-2012 No. 241472 (Comisión Nacional de Investigación Científica y Tecnológica, Chile), FIDUM (Fondo de Investigación y Desarrollo, Universidad Mayor), Facultad de Farmacia of the Universidad Complutense de Madrid and FONDECYT 1130155. We thank the Instituto de Salud Pública de Chile for providing the experimental animals. Special thanks to Magister Carla Sallés D. for her assistance in writing this paper.

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