International Regulations of *Propolis* Quality: Required Assays do not Necessarily Reflect their Polyphenolic-Related *In Vitro* Activities

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Abstract: *Propolis* has been proposed as a polyphenolic-rich natural product potentially able to be used for human consumption or even for medicinal proposes. To guarantee a minimum phenolic and flavonoid content and as consequence of their related-biological activities, international requirements of *propolis* quality are commonly applied. In this work we assessed phenolic and flavonoid contents of *propolis*; the antioxidant capacity (toward peroxyl radicals and hypochlorous acid); the ability to generate nitric oxide (NO); and, finally the antimicrobial activity of 6 *propolis* samples from the VI region of Chile. Our results show that the total phenolic and flavonoid content of *propolis* samples are not always in agreement with their polyphenolic-associated *in vitro* activities. For example, P03 and P06 samples showed the lowest (25 ± 4 GAE/g *propolis*) and the highest (105 ± 3 GAE/g *propolis*) total phenolic content, respectively. This was in agreement with flavonoid content and their Oxygen Radical Absorbance Capacity (ORAC) activity. However, this dependence was not observed toward HOCl, NO release and antimicrobial activity. Based on our results, we consider that, in order to guarantee the antioxidant or antimicrobial *in vitro* effects, the international regulations of *propolis* quality should contemplate the convenience of incorporating other simple analytical test such as ORAC or antimicrobial tests.

Keywords: antimicrobial activity, antioxidant activity, flavonoid content, international rules, nitric oxide, *propolis*, total phenolic content

Practical Application: Together with the titration of polyphenols and flavonoids, it is necessary to apply other assays to guarantee antioxidant capacity (as in the ORAC method) and antimicrobial activity of *propolis* samples. These assays should be considered by authorities for including in the international regulations of *propolis* quality.

Introduction

Besides carrying antioxidant properties, food-containing polyphenols are increasingly recognized for other potentially-useful bioactivities. Among these, some polyphenols display anti-inflammatory, vasodilating and/or platelet-aggregating inhibitory properties (Pandey and Rizvi 2009). Nevertheless, studying the antioxidant properties of natural polyphenols still accounts for most of the food chemistry-oriented research on these compounds. Most reported data strongly supports the existence of dependence between the chemical structure of polyphenols and their *in vitro* antioxidant capacity (Pandey and Rizvi 2009; Castro and others 2014). Among these activities, the ability of polyphenols to scavenge reactive species such as free radicals, as well as, hypochlorous and nitrous acid, has been highlighted (Oldreive and others 1998; Kumar and Pandey 2013; Perez-Cruz and others 2013; Vione and others 2004). In the case of the reaction with nitrous acid, which could be present in the stomach cavity, it also produces nitric oxide (NO; Peri and others 2005; Gago and others 2007), ascribing to polyphenols new potential beneficial actions in gastric environments (Rocha and others 2011).

Moreover, the well-known antioxidant activity of pure polyphenols and/or their complex mixtures that many of these compounds are also able to exert an antimicrobial action against a wide array of microorganisms has already been reported (Cushnie and Lamb 2005; Rocha and others 2011; Barrientos and others 2013). In fact, several flavonoids, including apigenin, galangin, flavone and flavonol glycosides, isoflavones, flavanones, and chalcones have shown a potent *in vitro* antibacterial activity (Kumar and Pandey 2013).

Taking into account the wide range of biological activities attributed to polyphenols, many researchers have focused on the search for polyphenolic-rich natural sources. In this context, *propolis* has been proposed as a natural product potentially able to be used for human consumption or even for medicinal proposes (Toreti and others 2013). The chemical composition of *propolis* depends on the local flora; approximately, 50% of raw *propolis* is accounted for in the resin fraction (polyphenolic fraction), 30% wax, 10% essential oils, 5% pollen, and 5% of organic and inorganic compounds (Kosalec and others 2004; Montenegro and others 2004). Considering the chemical diversity of *propolis*, international rules have been developed to guarantee a minimum content of total phenolic compounds and flavonoids given that even in similar

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geographical areas *propolis* shows different chemical compositions. Among the most cited rules are the ones applied by countries such as Russia (RSFSR-317-RST-77), Bulgaria (25 72483-84 ON), Argentina (Argentine Food Code, Joint Resolution 94/2008 and 357/2008, IRAM-INTA 15935-2), Cuba (Resolution 932-88), and Brazil (Standards Technical Regulations for Securing Identity and Quality of *Propolis*; 1999; 2001; Montenegro and others 2004; Hernández and others 2005). These rules have established as assays, to determine the total phenolic and flavonoid contents, the Folin–Ciocalteu (FC) and the $Al(OH)_3$-based methodologies, respectively. Nevertheless, previous reports suggest that the FC index does not always adequately reflect the *in vitro* scavenging and antimicrobial activities of complex samples (Alarcon and others 2008; Cabral and others 2012; Perez-Cruz and others 2013). For such reasons, this work addressed the actual existence (or not) of a direct relationship between the total phenolic and flavonoid contents of various *propolis* samples (obtained from the VI region of Chile) with such *in vitro* activities. *Propolis* samples were first characterized in terms of their botanical origin; polyphenolic profile; and, total phenolic and flavonoid content. The antioxidant activity was determined employing different experimental approaches (that is, scavenging activity toward peroxyl radicals and hypochlorous acid, and NO production assays) and the antimicrobial activity of the samples assayed toward *Streptococcus pyogenes*, *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa*.

Materials and Methods

Chemicals

6-Hydroxy-2,5,8-tetramethylchroman-2-carboxylic acid (Trolox), pyrogallol-sulfonephthalein (PGR), fluorescein disodium salt (FL), 2,2'-Azo-bis(2-amidinopropane) dihydrochloride (AAPH), 2,2-diphenyl-1-picrylhydrazyl (DPPH) and all the antioxidants studied were purchased from Sigma-Aldrich (St. Louis, Mo., U.S.A.). FC, aluminium chloride, sodium nitrite, potassium iodide, sodium chloride, hydrochloridric acid, disodium hydrogen phosphate dihydrate, and sodium phosphate monobasic reagent were supplied by Merck (Darmstadt, Germany). Solvents were of High Performance Liquid Chromatography (HPLC) grade. Water was purified in a Milli-Q system (Synergy, Millipore®, Darmstadt, Germany).

Ethanolic extracts preparation (EEP)

Six *propolis* samples from the VI region of Chile were provided as raw material by the beekeepers association (Apiunisexta A.G) collected in late spring/early summer, and stored at –20 °C in the dark until use. *Propolis* ethanolic extracts (EEP, 21 mg/mL) were prepared by adding 20 mL of 70% ethanol to 1.5 g of raw *propolis* previously milled. The suspension was heated to 50 to 60 °C for 30 min under agitation and then filtered. This procedure was repeated twice for each sample, and the collected extracts were combined to a final volume of 70.0 mL. EEP stored at –20 °C in the dark until use (Silva and others 2011).

Botanical analysis of propolis

The botanical analysis was determined according to the methodology described by Montenegro and others (1992). Subsequently, they were counted and identified plant structures (pollen grains, trichomes, and vessels). The identification was made by comparing the different structures with relevant literature (Heusser 1971; Montenegro 1984; Erdtman 1986), with photographs and permanent preparations available in the Laboratory of Botany (Department of Plant Sciences, Faculty of Agronomy and Forest Engineering, Pontificia Univ. Católica de Chile, Santiago, Chile), and the proportion of each of the total structures counted were estimated.

Total Phenolics Determination

The total phenolics content (TP) was determined according to the FC method (Singleton and Rossi 1965; Singleton and others 1999). A diluted solution of EEP (50 μg/mL in ethanol) was mixed with 2.5 mL of the FC reagent 1:10 (v/v) and 2.0 mL of a solution of Na_2CO_3. After 60 minutes, the absorbance of the resulting blue solution was measured at 760 nm using an Agilent 8453 UV-visible spectrophotometer (Palo Alto, Calif., U.S.A.). Quantification was done by linear regression from a calibration curve constructed from gallic acid (5 to 50 μM). Results are expressed as milligrams of gallic acid equivalents per gram of *propolis* (GAE/g). Values are reported as mean ± standard deviations (SD) of 3 independent determinations.

Flavonoids Determination

Flavonoids content was estimated according to an aluminum chloride method based on the procedure described by Woisky and Salatino (1998). A diluted solution of EEP (100 μg/mL in ethanol) was mixed with 0.5 mL of 2% $AlCl_3$ ethanol solution. After 60 minutes at room temperature, the absorbance was measured at 420 nm using an Agilent 8453 UV-visible spectrophotometer (Palo Alto). Total flavonoid contents were calculated as milligrams of quercetin equivalents per gram of *propolis* (QE/g) from a calibration curve (5 to 30 μM quercetin). Values are reported as mean ± SD of 3 independent determinations.

DPPH radical scavenging assay

DPPH assay was carried out using a modified method described by Brand-Williams and others (1995) (Chen and others 2003). Briefly, the EEP solution was diluted at 50 μg/mL and mixed with an ethanolic solution of DPPH (100 μM). The tubes were stored in the dark for 40 minutes and absorbance was measured at 517 nm using an Agilent 8453 UV-visible spectrophotometer (Palo Alto). Results were expressed as a percentage decrease with respect to control values and presented as mean ± SD of 3 independent determinations.

ORAC (Oxygen Radical Absorbance Capacity) determinations

The consumption of FL or pyrogallol red (PGR), associated to their incubation with AAPH, was estimated from fluorescence (F) and absorbance (A) measurements, respectively. Values of (F/F_0) or (A/A_0) were plotted as a function of time. Integration of the area under the curve (AUC) was performed up to a time such that (F/F_0) or (A/A_0) reached a value of 0.2. These areas were employed to obtain ORAC values, according to Eq. (1).

$$\mathrm{ORAC} = \frac{(\mathrm{AUC} - \mathrm{AUC}_0)}{(\mathrm{AUC}_{\mathrm{Trolox}} - \mathrm{AUC}_0)} f\,[\mathrm{Trolox}] \qquad (1)$$

where AUC is an area under the curve in presence of EEP samples, integrated between time zero and that corresponding to 80% of the probe consumption; AUC_0 is the area under the curve for control; AUC_{Trolox} is the area under the curve for Trolox; f is the dilution factor of the sample, equal to the ratio between the total volume of the AAPH-PGR or AAPH-FL solution and the added sample volume; Trolox is the molar concentration (Lopez-Alarcon

and Lissi 2006). Results are expressed as mM equivalents of Trolox (TE) per gram of *propolis* (TE/g). Values are reported as mean ± SD of 3 independent determinations.

Consumption of PGR induced by hypochlorite

PGR and hypochlorite stock solutions (1 mM) were prepared daily in a chelex-treated phosphate buffer 75 mM, pH 7.4. The concentration of hypochlorite was determined at pH 12.0 by UV-visible spectroscopy employing an extinction coefficient of 350/M/cm at 292 nm (Halliwell and Gutteridge 2007). Working solutions were prepared as follow: To a solution containing PGR (15 μM) with or without EEP, was added an aliquot of hypochlorite (30 μL, 1 mM) to obtain a final hypochlorite concentration of 10 μM in a 3mL of final experiment volume. The consumption of PGR was evaluated from the progressive absorbance decrease measured at 540 nm in a thermostatized cuvette of a Hewlett Packard 8453 (Palo Alto) UV-visible spectrophotometer (Perez-Cruz and others 2013). The PGR-HOCl values are relative to Trolox activity and reported as mean ± SD of 3 independent determinations.

NO measurement

The time course of NO production under acidic pH was followed electrochemically using the ISO-NOP (World Precision Instruments). A $NaNO_2$ solution containing H_2SO_4 and KI 1:1 (0.1 M) was used to calibrate the electrode according to the manufacturer's instructions. The reaction mixture containing diluted solution of EEP (0, 2.0, 3.7, and 5.4 μg/mL) and $NaNO_2$ (200 μM) in simulated gastric juice (SGJ, 34 mM NaCl, adjusted at pH 2.0 with hydrochloridric acid) was incubated at 37 °C in a thermostatized cell, and NO was followed continuously for over an hour. Similar experiments were carried out employing $NaNO_2$ (20 to 200 μM) and diluted solution of EEP (5.4 μg/mL). Values are reported as nM of NO generated (mean ± SD) of 3 independent determinations.

HPLC analysis

EEP was injected in a HPLC Agilent 1200 with a G1315D DAD detector equipped with a C_{18} Hibar (Merck) and eluted with a mobile phase containing KH_2PO_4 10 mM pH 2.6 (solvent A) and acetonitrile (solvent B). The gradient was 30% to 35% A (0 to 12 min) and 65% B (12 to 105 min) at a flow rate of 0.8 mL/min. The chromatographic elution of phenolic compounds was followed at 254, 290, 320, and 350 nm, and their UV spectra were recorded. The identification of phenolic components was evaluated by comparing their retention times and a spectrum obtained with those standards, and a peak confirmation was obtained by spiking the EEP with the phenolic components. Calibration curves from 25 to 300 μM were obtained.

Antimicrobial activity

Antimicrobial activity was assayed against human pathogenic microorganisms, *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC27853), *Staphylococcus aureus* (ATCC25923), and *Streptococcus pyogenes* (ISP 364-00), obtained from the Chilean National Institute of Public Health (ISP) activated 24 h before the experiment in soy agar medium. Antimicrobial effect of *propolis* extracts was determined by using agar-well diffusion, disc-diffusion, and broth dilution methods. Bactericidal activity of each extract was assessed by time-kill curves. Suspensions of the tested microorganisms were spread onto the surface of Mueller–Hinton agar plates. The wells of 6-mm diameter were cut from the agar and filled with diluted solution of EEP (100 μL, 0.43 g/mL). The inoculated plates were incubated at 37 °C for 24 h. Antimicrobial activity was evaluated by measuring the diameter of the growth inhibition zone around the well.

Disk diffusion method. For the disk diffusion assay (Vanden and others 1991), petri dishes containing Mueller-Hinton Agar plates were inoculated with microbial suspensions. Disks of filter paper of 5 mm diameter were impregnated with 5 μL of each dilution of EEP (0.43 g/mL) and placed on the agar surface. Discs of ampicillin were used as positive controls. The plates were incubated overnight at 37 °C and the diameters of any resulting zones of growth inhibition were measured.

Broth microdilution method. Minimum inhibitory concentrations (MIC) of the *propolis* sample were determined using the standard micro dilution techniques. Two control tubes were maintained for each test batch. These included antibiotic control (a tube containing *propolis* and growth medium without inoculum) and organism control (a tube containing bacterial suspension). MIC values were taken as the lowest concentration of compound (highest dilution) that produced no visible bacterial growth (no turbidity) when compared with the control tubes after 24 h of incubation at 37 °C.

Statistical analyses

All data represents the mean values ± SD of at least 3 independent experiments, each conducted in triplicate. Pearson parametric correlation ana-lyzes was carried out using Origin Pro 8 software. A value of $P < 0.05$ was considered to be significant.

Results and Discussion

The search for phenolic compounds with antioxidant properties, potentially able to prevent or decrease the damage triggered by oxidative stress, has received increasing attention during the last few decades. The beneficial health effects associated with the consumption of fruits and vegetables rich in antioxidant phenolics has been broadly recognized, but in the case of cardiovascular health, particular attention though recently been focused on the ability of phenolics such as hydroxytyrosol, present in extra virgin olive oil, and epicatechin, present in cocoa (Vauzour and others 2010). Given its natural characteristics and its richness in polyphenols, *propolis* has also gained attention as its consumption could also contribute toward the aim of providing additional phenolics in our diets.

Characterization of propolis samples

Six propolis samples, collected from the VI region of Chile, were studied. Table 1 shows the botanical origin of such propolis samples, as determined by micro-morphological analysis of pollen and epidermal. As it is presented, excepting sample P01, species introduced in Chile such as Galega officinalis L. (galega), Brassica sp. (cabbage, cauliflower, broccoli, brussel sprouts), and Medicago sativa L. (alfalfa) were the main plant species found in our propolis samples. Interestingly, endemic species in Chile, such as Quillaja saponaria (quillay), Aristotelia chilensis (maqui), Lithrea caustica (litre), were found in a minor percentage. These species are well-known for their high phenolic content (especially Aristotelia chilensis), thus it could be expected that they provide a higher phenolic content than G. officinalis, Brassica sp., and M. sativa to propolis samples.

Table 2 shows the total phenolic and flavonoid content of *propolis* samples, as well as the DPPH consumption values. Total phenolic content, determined by FC assay, varied between 25 and 105 mg GAE/g *propolis*.

Table 1–Floral composition of *propolis* samples studied from VI Region, Chile.

Sample	Predominant species
P01	*Lithrea caustica* (18%), *Raphanus sativus* (13%), *Galega officinalis* (12%), *Retanilla trinervia* (9%), *Satureja gilliesi* (7%), *Quillaja saponaria* (6%), Cactaceae (5%), *Proustia sp.* (5%), *Aristotelia chilensis* (5%), *Eucalyptus sp.*(3%), *Vicia sp.*(3%), NN (2%), *Schinus sp. (2%), Baccharis sp.* (1%), *Trevoa quinquenervia* (1%)
P02	*Galega officinalis* (24%), *Quillaja saponaria* (11%), *Lithraea caustica* (9%), *Medicago sativa* (8%), *Trifolium repens* (6%), *Meliotus indicus* (6%), *Luma/myrceugenia* (4%), *Escallonia sp.* (4%), *Eucalyptus sp.* (4%), *Azara sp* (3%), *Trevoa quinquenervia* (3%), *Prunus sp* (3%) *Retanilla trinervia* (2%), *Haplopappus sp.* (2%), *Acacia sp* (2%), *Baccharis sp* (2%)
P03	*Galega officinalis* (43%), *Brassica sp* (12%), *Quillaja saponaria* (8%), *Azara sp.* (9%), *Chenopodiaceae* (4%), *Trifolium repens* (3%), *Trevoa quinquenervia* (3%), *Melilotus indicus* (3%), *Aristotelia chilensis* (2%), *Tristerix sp.* (2%) *Sophora/Proustia/Baccharis* (2%), *Buddleja/Castanea* (1%)
P04	*Galega officinalis* (39%), *Brassica sp.* (11%) *Azara sp.*(9%), *Quillaja saponaria* (6%), *Lotus uliginosus* (5%), *Medicago sativa* (4%), *Luma/Myrceugenia* (3%), *Trifolium repens* (3%), *Melilotus indicus* (3%), *Retanilla trinervia* (2%), *Baccharis sp.* (2%), *Acacia sp.* (2%)
P05	*Galega officinalis* (40%), *Brassica sp.* (10%), *Medicago sativa* (7%), *Trevoa quinquenervia* (7%), *Luma/Myrceugenia* (6%), *Quillaja saponaria* (5%), *Escallonia sp.* (3%), *Eucalyptus sp.*(3%), *Ophyosporus sp.* (3%), *Melilotus indicus* (3%), *Schinus sp.* (3%), *Trifolium repens* (2%), *Aristotelia chilensis* (2%), *Lithraea caustica* (2%), *Hypochaeris/Tarazacum* (1%)
P06	*Galega officinalis* (35%), *Brassica sp.* (13%), *Azara sp.* (10%), *Quillaja saponaria* (9%), *Medicago sativa* (4%), *Aristotelia chilensis* (4%), *Trifolium repens* (3%), *Trevoa quinquenervia* (3%), *Melilotus indicus* (3%), *Medicago polymorpha* (2%), *Luma/Myrceugenia* (2%),*Tristerix sp.* (2%), *Trifolium pratense* (2%), *Convolvulus sp.* (1%) *Gutirrezia/Haplopappus* (1%), *Eucalyptus sp.* (1%)

Table 2–Content of phenolic and flavonoid compounds in EEP, and DPPH consumption.

Sample	Total polyphenol (mg GAE/g *propolis*)	Flavonoid (mg QE/g *propolis*)	DPPH (%)
P01	74 ± 1	62 ± 2	84 %
P02	91 ± 4	38 ± 3	60 %
P03	**25 ± 4**	**21 ± 2**	**18 %**
P04	81 ± 3	53 ± 3	**85 %**
P05	85 ± 2	53 ± 3	84 %
P06	**105 ± 3**	**77 ± 1**	84 %

Values represent the mean ± SD ($n = 3$). Extracts with the lowest and highest polyphenol and flavonoid concentrations and DPPH values are shown in bold. Values of DPPH consumption represent the average of 3 independent experiments. The standard deviation of these results was lower than 5%.

In the presence of polyvinylpolypyrrolidone (PVPP; Bridi and others 2014), an agent employed to remove phenolic compounds, the FC index showed a low influence of nonphenolic compounds (between 1% and 10%). As is shown in Table 2 (which depicts FC data without the pretreatment with PVPP), among all samples, total phenolic content of P03, and P06 showed the lowest and highest values, respectively. From the analysis of this data, it appears that only the P03 sample does not satisfy the international regulations, which commonly sets a 50 mg GAE/g of *propolis* as a minimum value for total phenolic content. In the case of flavonoid content, values between 21 and 77 mg QE/g *propolis* were estimated. In a similar way that total phenolic content, P03 and P06 samples showed the lowest and highest values, respectively. Nevertheless, as international rules for flavonoid content have established as a minimum value, 5 mg of QE/g of *propolis*, all studied samples (including P03) satisfied this requirement. We used the DPPH methodology as an alternative way to indirectly study and compare the phenolic content of *propolis* samples. Results, included in Table 2, show that the consumption of DPPH varied between 18% and 85%, P03 being the sample with the lowest value.

To get more insights about the chemical characterization of our *propolis* samples, we determined the concentration of the most representative polyphenols (cinnamic acids, flavonols, flavone, and flavanone); the results are depicted in Table 3.

In spite of the fact that all samples were obtained from the same region and that they have a similar pattern of botanical origin, different polyphenolic profiles and concentrations were observed. In contrast to the total phenolic content, determined by the FC assay, when using HPLC technique, samples P03 and P06 showed a similar content of polyphenols (50.9 and 50.4 mg/g of *propolis*, respectively) Table 3. This data would imply that the polyphenols of the P03 sample have a lower capacity to reduce the Folin reagent than the polyphenols present in P06. Such capacity could be associated with the number of hydroxyl groups of the phenolic compounds present in this sample. In fact, it is known that for pure compounds and also complex mixtures, Folin's index correlates to the number of phenolic hydroxyl groups present in the chemical structure of the molecules (Campos and others 2013). In this context, it has been reported that the compounds found in P03, such as caffeic acid, ferulic acid, and myricetin have a similar FC index than those found in P06 sample, as quercetin, and apigenin among others (Ma and Cheung 2007; Campos and others 2013). This suggests that the FC index of a P06 sample is mostly associated with the presence of other nonidentified polyphenolic compounds. This is not surprising because it is difficult to have pure standards of all polyphenolic compounds present in a natural extract. For this reason it seems more appropriate, for total phenolic quantification, to use the FC methodology.

Scavenging activity toward peroxyl radicals (ORAC assay)

We studied the antioxidant capacity of the samples by the ORAC methodology. For this purpose we employed FL (ORAC-FL) and pyrogallol red (ORAC-PGR) as probes. The former assay gives values mainly associated with the stoichiometry of the reaction between antioxidants and hydrosoluble peroxyl radicals (generated from the AAPH thermolysis). Although the latter index (ORAC-PGR) is related to the reactivity of the phenolic compounds toward peroxyl radicals (Lopez-Alarcon and Lissi 2006). We have previously reported that the ORAC-PGR/ORAC-FL ratio represents the quality of the antioxidants compounds contained in a particular complex mixture (Poblete and others 2009). Figure 1 shows the kinetic profiles of FL (Figure 1A) and pyrogallol red (Figure 1B) protection afforded by P07 and P03, respectively. From this kind of data, ORAC values

Table 3–Polyphenols of *propolis* extracts determined by HPLC.

Compounds	Rt/min	P01	P02	P03	P04	P05	P06
Caffeic acid	5.4	10.8 ± 0.1	–	5.9 ± .2	1.7 ± 0.2	–	2.4 ± 0.0
p-Coumaric	7.4	11.3 ± 0.2	2.0 ± 0.2	–	3.8 ± 0.1	3.3 ± 0.1	3.9 ± 0.1
Ferulic acid	7.7	4.2 ± 0.3	–	10.0 ± 0.2	1.3 ± 0.0	–	0.8 ± 0.1
Cinnamic acid	18.3	–	–	–	0.9 ± 0.0	–	1.5 ± 0.1
Total cinnamic acids		26.3 (55.1%)	2.0 (100%)	15.9 (31.2%)	7.7 (57.0%)	3.3 (18.3%)	8.6 (17.1%)
Quercetin	15.3	–	–	–	–	5.8 ± 0.4	2.5 ± 0.2
Luteolin	14.4	21.4 ± 1.8	–	15.3 ± 0.1	–	–	–
Myricetin	9.5	–	–	19.7 ± 0.3	–	–	–
Apigenin	22.3	–	–	–	3.5 ± 0.1	–	6.2 ± 0.1
Crysin	64.0	–	–	–	–	–	4.3 ± 0.2
Pinobanskin	24.0	–	–	–	2.3 ± 0.2	4.0 ± 0.2	15.0 ± 0.4
Pinocembrin	71.2	–	–	–	–	4.9 ± 0.0	16.3 ± 0.5
Total flavonoids		21.4 (44.9%)	–	35.0 (68.8%)	5.8 (43.0%)	14.7 (81.7%)	41.8 (82.9%)
Total		47.7	2.0	50.9	13.5	18.0	50.4

Data are expressed in mg/g *propolis* for EEP and the values represent the mean ± SD ($n = 3$).
Rt, retention time.

were determined; obtained results are depicted in Table 4. As shown, ORAC-PGR values varied between 0.2 and 2.3, whereas ORAC-FL varied between 0.4 and 4.1 mM Trolox equivalents (TE) / g *propolis*. In a similar way to that seen with TP, P03 and P06 samples showed the lowest and the highest scavenging activity, respectively. In fact, the ORAC index of P06 was near 10 times higher than that of P03. Despite this difference, both samples showed similar ORAC-PGR/ORAC-FL ratios (near 0.5) implying that their phenolic compounds have comparable reactivity toward peroxyl radicals. This ORAC-PGR/ORAC-FL value means that half of the phenolic compounds of the samples are able to protect the PGR probe. In addition, complex matrixes with recognized antioxidant capacity, such as red wine, have shown similar ratio values (Lopez-Alarcon and others 2011).

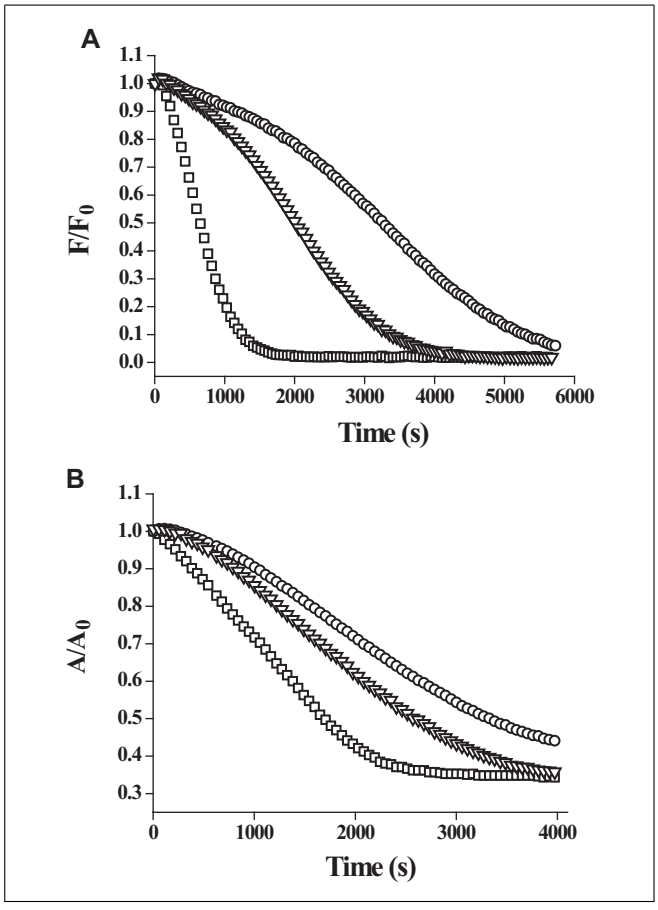

Figure 1–(A) Kinetic profiles of fluorescein (FL) consumption followed by fluorescence technique ($\lambda_{exc} = 485$ nm and $\lambda_{em} = 512$ nm for λ_{ex} and λ_{em}, respectively). □, control; ▽, 1.0 μg/mL *propolis*; O, 2.1 μg/mL *propolis*. (B) Consumption of pyrogallol red (PGR) followed by visible spectroscopy at 540 nm. □, control; ▽, 48 μg/mL *propolis*; O, 71 μg/mL *propolis*.

Scavenging activity toward hypochlorous acid (HOCl) and NO production of propolis

Figure 3 depicts results on the scavenging activity of *propolis* samples toward HOCl. This was evaluated by competitive kinetics employing PGR as a probe (HOCl-PGR index). As shown in Figure 3, P06 presented the highest activity, however, P01 and P04 also showed high values. By contrast, the lowest HOCl-PGR values were determined for a P02 sample, followed by P03. We determined the ability of *propolis* samples to produce NO during their incubation with nitrous acid (HONO). The time-course of NO production was determined during the incubation of aqueous solutions of *propolis* extracts (at pH 2.0) with HONO (rigorously nitrite in acid medium). All samples showed similar kinetic profiles of NO generation, typical results for P04 are presented in Figure 2. As shown, NO concentration showed a fast increase followed by a plateau. This kinetic behavior was in agreement with the previous reports about the reaction of HONO with complex samples such as red wine and apple extracts (Peri and others 2005; Gago and others 2007). From data extracted from the plateau region (Figure 2) we determined the maximum NO concentration generated by a particular *propolis* sample in the presence of different HONO concentrations (these values are presented in Table5). Interestingly, P04 was the sample with the highest NO production, followed by P06 which showed a slightly higher value than P03. It has been demonstrated that polyphenols react efficiently with HOCl and HONO (Firuzi and others 2004; Gago and others 2007). The reaction toward these reactive species involves complex mechanisms of polyphenol oxidation, as well as, chlorination (for HOCl) and nitrosation (for HONO) of aromatic rings. For example, the complexity of the chemistry of HONO is reflected in the fact that, in aqueous solutions HONO generates, through different equilibria, reactive intermediates such as N_2O_3, $\bullet NO_2$, N_2O_4, and NO. The generation of such species explains that the reaction of HONO with polyphenols is characterized not only

Table 4–ORAC-PGR values, ORAC-FL values, and ORAC-PGR/ORAC-FL ratio of EEP.

Sample	ORAC-PGR[a]	ORAC-FL[a]	ORAC PGR /ORAC FL
P01	0.7 ± 0.1	1.9 ± 0.2	0.37
P02	0.3 ± 0.1	2.7 ± 0.3	0.11
P03	0.2 ± 0.1	0.4 ± 0.1	**0.50**
P04	1.0 ± 0.1	2.4 ± 0.2	0.42
P05	1.2 ± 0.1	3.9 ± 0.4	0.31
P06	2.3 ± 0.1	4.1 ± 0.2	**0.56**

[a]Values expressed as mM equivalentes Trolox (TE) / g própolis. Results are shown as the mean ± SD ($n = 3$). Extracts with the highest ORAC-PGR/ORAC-FL ratio are shown in bold.

Table 5–Production of NO (nM) from nitrite (200 μM) and different concentrations of *propolis* (2.0 mg/mL, 3.7 mg/mL, 5.4 mg/mL).

Sample	[2.0 mg/mL]	[3.7 mg/mL]	[5.4 mg/mL]
P01	2.4 ± 0.03	2.7 ± 0.02	3.1 ± 0.05
P02	2.9 ± 0.03	3.3 ± 0.09	3.7 ± 0.07
P03	2.4 ± 0.04	2.6 ± 0.07	3.3 ± 0.04
P04	4.1 ± 0.06	6.3 ± 0.09	6.9 ± 0.03
P05	2.7 ± 0.02	2.8 ± 0.01	3.1 ± 0.03
P06	2.8 ± 0.01	3.2 ± 0.02	3.4 ± 0.04

Values are presented as mean ± SD ($n = 3$). Control without *propolis* 1.2 ± 0.02 nM.

by oxidation redox processes, but also by nitrosation reactions and also the production of NO.

The mixture of mechanisms in the chemistry of HOCl and HONO and the diversity of chemical structures present in a particular sample of *propolis* imply that it is very difficult to understand why one *propolis* sample shows a higher (or lower) scavenging activity toward HOCl, or capacity to produce NO during its reaction with HONO.

Antimicrobial activity of propolis samples

In this study, we also evaluated the antibacterial activity of *propolis* samples against the human pathogenic microorganisms: *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Streptococcus pyogenes*. Agar-well diffusion, disc-diffusion, and broth dilution methodologies were employed. Figure 4A shows the mean diameters of growth inhibition zones; these kind of values (mm) were obtained for each tested strain. All samples produced inhibitory zones against all tested microorganisms. The most sensitive to EEP was *Streptococcus pyogenes* and *Staphylococcus aureus*, which showed the highest inhibition zones (29 ± 6 mm). The most resistant strain was *Pseudomonas aeruginosa* and *Escherichia coli* with growth inhibition zones of 14 mm.

The MIC of the test samples are shown in Figure 4B for all strains. MIC was determined as the lowest concentration of the *propolis* extract, which inhibited the growth of the tested microorganisms. The EEP inhibited the only growth of *Streptococcus pyogenes* and *Staphylococcus aureus* and showed a MIC 200-13400 and 200-26900 μg/mL, respectively.

We observed that our *propolis* samples did not show the same inhibitory activity on bacterial growth. Nonetheless, all of them were capable of controlling the growth of bacteria *Streptococcus pyogenes* and *Staphylococcus aureus*, indicating that probably new *propolis* studies should be focused on these 2 bacteria. It has been reported that that some flavonoids (quercetin, galangin, pinocembrin), caffeic acid, benzoic acid, and cinnamic acid present in *propolis* can act on the membrane or cell wall, causing structural and functional damage (Kosalec and others 2005; Scazzocchio and others 2006; Chaillou and Nazareno 2009). Nevertheless, our antimicrobial results did not show a statistically significant correlation with the Folin index. In fact, the coefficient correlation between the inhibition zones values of *S. pyogenes* and *S. aureus* with total phenolic content was 0.17 and −0.48, respectively. In addition, MIC values of *S. pyogenes* and *S. aureus* showed a very weak correlation with total phenolic content (0.21 and 0.19, respectively).

Correlation between *in vitro* activities and total phenolic and flavonoid content

Our results show that there is a statistically significant positive correlation between total phenolic content of *propolis* samples and ORAC-FL (0.90) and DPPH (0.83). In addition, a strong correlation was found between flavonoid content and ORAC-PGR (0.86), DPPH (0.87), and HOCl-PGR index (0.87). Nonetheless, no correlation was observed between the total phenolic content and the following methods/activities: ORAC-PGR, NO release, HOCl-PGR, ORAC-PGR/ORAC-FL ratio, and antimicrobial activity. Also, no correlation was found between flavonoid content and NO release, ORAC-PGR/ORAC-FL ratio, and antimicrobial activity. Taking into account this data, and the results

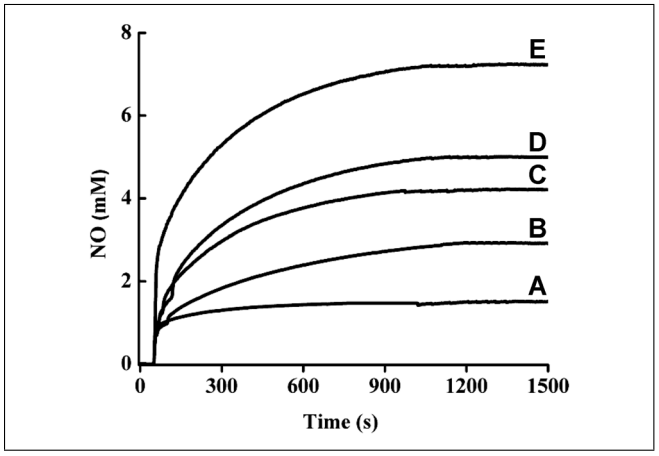

Figure 2–Time courses of NO production in diluted solution of EEP with nitrite in simulated gastric juice pH 2. Kinetic recordings for and P04 sample 5.4 mg/mL and nitrite at 50 μM (B), 100 μM (C), 150 μM (D), 200 μM (E), and control (A). Values are presented as mean ± SEM, n = 3.

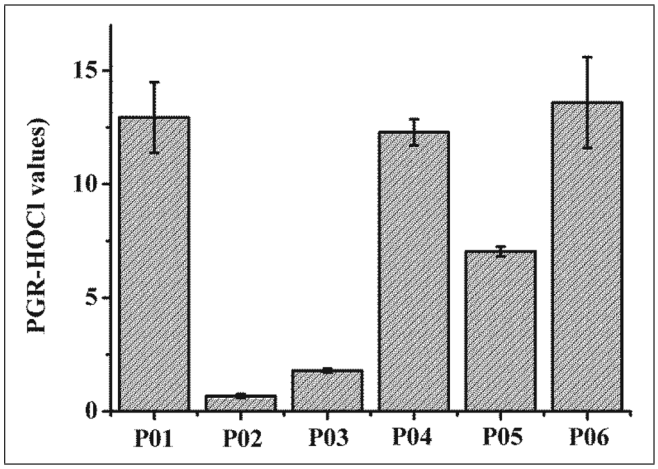

Figure 3–PGR-HOCl values (relative to Trolox activity) of *propolis* samples. Data represent the mean of at least 3 independent experiments ± Standard deviation (SD).

presented above it is clear that the total phenolic and flavonoid content of *propolis* samples, are not always in agreement with some polyphenolic-associated *in vitro* activities. Considering the international rules, sample P03 does not satisfy the minimum value of the FC index. This low value of total phenolic content of P03 was in agreement with its comparatively low ORAC-FL and PGR-HOCl indexes. However, when ORAC-PGR and the NO release (during the incubation with nitrous acid) were studied, the P03 sample showed similar values than those obtained with the other studied *propolis* samples. In addition, P03 showed a good antimicrobial activity toward *Streptococcus pyogenes* and *Staphylococcus aureus.* In the case of P06, its high polyphenolic content was in agreement with its ORAC-FL, ORAC-PGR, PGR-HOCl, and antimicrobial activity. Nonetheless, regarding its capacity to generate NO, its antimicrobial activity was similar to P01, P02, P03, and P05, but lower than P04. Therefore, from the point of view of the potential gastric effect (associated with a nitrous acid reaction) of a particular *propolis* sample, the application of the international rules should be considered carefully.

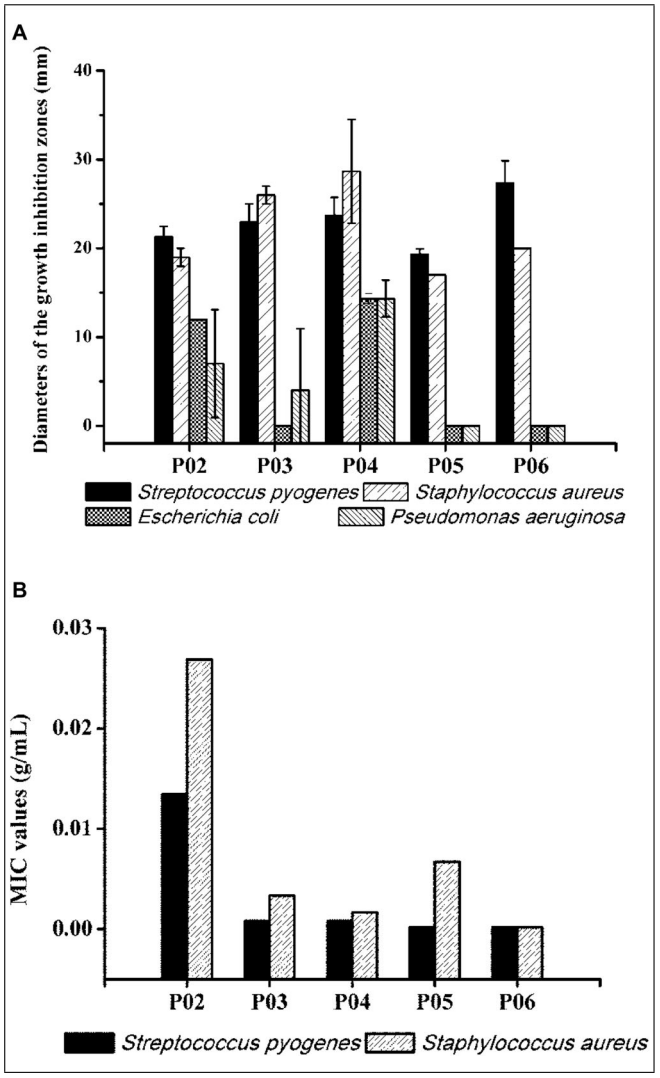

Figure 4–(A) Antimicrobial activity of *propolis* samples. Inhibition zone values are given in mm (mean ± SD). (B) Minimum inhibitory concentration (MIC) of *propolis* extracts obtained for each tested strain. Results are given in g/mL ± SD. Results are given in g/mL ± SD.

Conclusion

International regulations have established the FC index and the flavonoid content as parameters to warranty a minimum phenolic content of a sample. However, as we show in this work, such parameters are not always in agreement with *in vitro* activities related to the presence of polyphenol. For this reason, we consider that, in order to guarantee the antioxidant or antimicrobial *in vitro* effects, the international regulations on *propolis* quality should contemplate the convenience of incorporating other simple analytical tests such as ORAC or antimicrobial tests.

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References

1999. Código Alimentario Argentino, Resolución conjunta 94/2008 y 357/2008, Normas de Reglamento Técnico para Fijación de Identidad y Calidad de Propóleos, n° 52, julio 1999, Ministerio de Salud y Acción Social. In.

2001. Regulamento Técnico para fixação de identidade e qualidade de própolis, Instrução Normativa N° 3, de 19 de Janeiro de 2001, Secretaria de Defesa Agropecuária, Ministério de Agricultura de Brasil. In.

Alarcon E, Campos AM, Edwards AM, Lissi E, Lopez-Alarcon C. 2008. Antioxidant capacity of herbal infusions and tea extracts: a comparison of ORAC-fluorescein and ORAC-pyrogallol red methodologies. Food Chem 107:1114–9.

Barrientos L, Herrera CL, Montenegro G, Ortega X, Veloz J, Alvear M, Cuevas A, Saavedra N, Salazar LA. 2013. Chemical and botanical characterization of Chilean propolis and biological activity on cariogenic bacteria Streptococcus mutans and Streptococcus sobrinus. Braz J Microbiol 44:577–85.

Brand-Williams W, Cuvelier M, Berset C. 1995. Kinetics and mechanisms of antioxidant activity using the DPPH free radical method. Lebensm Wiss Technol 28:25–30.

Bridi R, Troncoso MJ, Folch-Cano C, Fuentes J, Speisky H, Lopez-Alarcon C. 2014. Polyvinylpolypyrrolidone (PVPP)-assisted folin-ciocalteu assay to assess total phenol content of commercial beverages. Food Anal Method 7:2075–83.

Cabral ISR, Oldoni TLC, de Alencar SM, Rosalen PL, Ikegaki M. 2012. The correlation between the phenolic composition and biological activities of two varieties of Brazilian propolis (G6 and G12). Braz J Pharm Sci 48:557–64.

Campos AM, Lopez-Alarcon C, Lissi E. 2013. Evaluation of the total amount of phenol groups in complex mixtures by folin's and orac-fluorescein methodologies. J Chilean Chem Soc 58:2144–7.

Castro C, Mura F, Valenzuela F, Figueroa C, Salinas R, Zuñiga MC, Torres JL, Fuguet E, Delporte C. 2014. Identification of phenolic compounds by HPLC-ESI-MS/MS and antioxidant activity from Chilean propolis. Food Res Int 64:873–9.

Chaillou LL, Nazareno MA. 2009. Chemical variability in propolis from Santiago del Estero, Argentina, related to the arboreal environment as the sources of resins. J Sci Food Agr 89:978–83.

Chen CN, Wu CL, Shy HS, Lin JK. 2003. Cytotoxic prenylflavanones from Taiwanese propolis. J Nat Prod 66:503–6.

Cushnie TPT, Lamb AJ. 2005. Antimicrobial activity of flavonoids. Int J Antimicrob Ag 26:343–56.

Erdtman G. 1986. Pollen morphology and plant taxonomy. E.J. Brill Press: Leiden, Netherlands.

Firuzi O, Mladenka P, Petrucci R, Marrosu G, Saso L. 2004. Hypochlorite scavenging activity of flavonoids. J Pharm Pharmacol 56:801–7.

Gago B, Lundberg JO, Barbosa RM, Laranjinha J. 2007. Red wine-dependent reduction of nitrite to nitric oxide in the stomach. Free Radical Bio Med 43:1233–42.

Halliwell B, Gutteridge JMC. 2007. Free radicals in biology and medicine, Fourth Edition. New York: Oxford University Press.

Hernández S, Lazo S, Junod M, Arancibia M, Flores S, Valencia A, Valenzuela V. 2005. Características organolépticas y fisico-químicas de propóleos de la provincia de ñuble, VIII región-Chile. Arch Latinoam Nutr 55:374–9.

Heusser JC. 1971. Pollen and spores of Chile. The University of Arizona Press: Tucson.

Kosalec I, Bakmaz M, Pepeljnjak S, Vladimir-Knezevic S. 2004. Quantitative analysis of the flavonoids in raw propolis from northern Croatia. Acta Pharm 54:65–72.

Kosalec I, Pelnijak S, Bakmaz M, Wladimir-Knezevic V. 2005. Flavonoid analysis and antimicrobial activity of commercially available propolis products. Acta Pharm 55:423–30.

Kumar S, Pandey AK. 2013. Chemistry and biological activities of flavonoids: an overview. Sci World J 29:16.

Lopez-Alarcon C, Lissi E. 2006. A novel and simple ORAC methodology based on the interaction of Pyrogallol Red with peroxyl radicals. Free Radical Res 40:979–85.

Lopez-Alarcon C, Ortiz R, Benavides J, Mura E, Lissi E. 2011. Use of the orac-pyrogallol red/orac-fluorescein ratio to assess the quality of antioxidants in Chilean wines. J Chilean Chem Soc 56:764–7.

Ma YT, Cheung PCK. 2007. Spectrophotometric determination of phenolic compounds by enzymatic and chemical methods – a comparison of structure-activity relationship. J Agr Food Chem 55:4222–8.

Montenegro G. 1984. Atlas de anatomía de especies vegetales autóctonas de la Zona Central. Ediciones Universidad Católica de Chile, Santiago.

Montenegro G, Gómez M, Ávila G. 1992. Importancia relativa de especies cuyo polen es utilizado por Apis mellifera en el área de la Reserva Nacional Los Ruiles, VII Región de Chile. Acta Bot Malacit 17:167–174.

Montenegro G, Mujica A, Peña R, Gómez M, Timmermann B. 2004. Similitude pattern and botanical origin of the Chilean propolis. Phyton 145–54. 53rd ANNIVERSARY.
Oldreive C, Zhao KC, Paganga G, Halliwell B, Rice-Evans C. 1998. Inhibition of nitrous acid-dependent tyrosine nitration and DNA base deamination by flavonoids and other phenolic compounds. Chem Res Toxicol 11:1574–9.
Pandey KB, Rizvi SI. 2009. Plant polyphenols as dietary antioxidants in human health and disease. Oxid Med Cell Longev 2:270–8.
Perez-Cruz F, Cortes C, Atala E, Bohle P, Valenzuela F, Olea-Azar C, Speisky H, Aspee A, Lissi E, Lopez-Alarcon C, Bridi R. 2013. Use of pyrogallol red and pyranine as probes to evaluate antioxidant capacities towards hypochlorite. Molecules 18:1638–52.
Peri L, Pietraforte D, Scorza G, Napolitano A, Fogliano V, Minetti M. 2005. Apples increase nitric oxide production by human saliva at the acidic pH of the stomach: a new biological function for polyphenols with a catechol group? Free Radical Bio Med 39: 668–81.
Poblete A, Lopez-Alarcon C, Lissi E, Campos AM. 2009. Oxygen radical antioxidant capacity (ORAC) values of herbal teas obtained employing different methodologies can provide complementary data. J Chilean Chem Soc 54:154–7.
Rocha BS, Gago B, Pereira C, Barbosa RM, Bartesaghi S, Lundberg JO, Radi R, Laranjinha J. 2011. Dietary nitrite in nitric oxide biology: a redox interplay with implications for pathophysiology and therapeutics. Curr Drug Target 12:1351–63.
Scazzocchio F, D'Auria FD, Alessandrini D, Pantanella F. 2006. Multifactorial aspects of antimicrobial activity of propolis. Microbiol Res 161:327–33.
Silva V, Genta G, Moller MN, Masner M, Thomson L, Romero N, Radi R, Fernandes DC, Laurindo FRM, Heinzen H, Fierro W, Denicola A. 2011. Antioxidant activity of uruguayan propolis. In vitro and cellular assays. J Agr Food Chem 59:6430–7.
Singleton VL, Orthofer R, Lamuela-Raventos RM. 1999. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. Oxid Antioxid 299:152–78.
Singleton VL, Rossi JA. 1965. Colorimetry of total phenolics with phosphomolybdic-phophotungstic acid reagents. Am J Enol Vitic 16:144–58.
Toreti VC, Sato HH, Pastore GM, Park YK. 2013. Recent progress of propolis for its biological and chemical compositions and its botanical origin. Evidence-Based Complementary and Alternative Medicine 2013:13.
Vanden Berghe DA, Vlietinck AJ. 1991. Screening methods for antibacterial and antiviral agents from higher plants. In: Dey PM, Harbone JB, editors. Methods in plant biochemistry. Academic Press: London. p 47–69.
Vauzour D, Rodriguez-Mateos A, Corona G, Oruna-Concha MJ, Spencer JPE. 2010. Polyphenols and human health: prevention of disease and mechanisms of action. Nutrients 2:1106–31.
Vione D, Belmondo S, Carnino L. 2004. A kinetic study of phenol nitration and nitrosation with nitrous acid in the dark. Environ Chem Lett 2:135–9.
Woisky RG, Salatino A. 1998. Analysis of propolis: some parameters and procedures for chemical quality control. J Apic Res 37:99–105.