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Relation between composition, antioxidant and antibacterial activities and botanical origin of multi-floral bee pollen

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Summary

Harvested bee pollen is valuable for its nutritional value and healthy properties. This work relates the botanical origin of sixteen bee pollens from Chile with their phenolic, protein and carotenoid content, and antioxidant/antibacterial activities. Our results showed that the chemical properties of different bee pollens are associated with the plant species from which each one was derived from. Some correlations between chemical properties and botanical origin were observed. Bee pollen showed between 20.0-30.4% protein, 2.8-50.2 mg/kg carotenoids, 22.8-918.4 mg/kg phenolics, and 4.51-91.19 mmol Fe²⁺/kg pollen. Antibacterial activity was observed against all bacteria assayed even surpassing the activity of traditional antibiotics. *Brassica* sp. and *Galega officinalis* are an abundant source of antioxidants and antibacterial compounds. Other species such as those derived from fruit and endemic plants from Chile, although they occur less frequently, are also good source of these compounds. Some correlations between botanical origin and chemical, antioxidant and antibacterial properties were observed. Knowing the influence of plant species over the antioxidant or antibacterial properties of bee pollen, will allow selecting the best location for honeycombs and will allow beekeepers to differentiate and add value to their products.

Keywords: *Brassica* sp., *Galega officinalis*, *Trevoa quinquenervia*, *Prunus* sp., *Medicago* sp., FRAP, diameter of inhibition, HPLC-DAD, protein, carotenoid.

Introduction

Bee pollen corresponds to microspores of spermatophytes and entomophilous plants with flowers collected and transported by bees in their last pair of legs as granules or pollen-loads. Once in the hive, bee adds salivary enzymes (e.g., amylase, catalase) to the pollen-loads and reserved them as nutrient resource for honeycomb. Bee pollen is valuable for its nutritional value and healthful properties and is considered by many beekeepers as a mean of diversifying and increasing their income.

Bee pollen products are valuable for their nutritional value and healthful properties. As a nutrient, bee pollen provides to the human diet protein, fat and other components in lesser amount. Bee pollen presents all essential amino acids to the human diet and its content varies between 10 and 40% (BOGDANOV, 2014). Dry bee pollen presents an average protein content about 23.8% (ALMEIDA-MURADIAN et al., 2005). Fatty acids are 3%, from which about half of them are oleic (omega-3), linoleic (omega-6) and linolenic acids (omega-3) (BOGDANOV, 2014). Carbohydrates in bee pollen are mainly polysaccharides such as starch and sugars, and represent between 13 and 55 g/100 g of sample. With respect to healthful characteristics, bee pollen has been described as anti-anemic, tonic and restorative,

hormone regulator, intestinal regulator, vasoprotector, hepatoprotective, anti-atherosclerotic agent, antiallergic, anticarcinogenic, antioxidant, antibacterial and as antifungal (DENISOW and DENISOW-PIETRZYK, 2016; GRAIKOU et al., 2011).

Phenolic acids, flavonoids and pigments as β -carotene, are mainly related to the healthy properties exhibited by bee pollen such as antioxidant and antibacterial (ALOISI and RUPPEL, 2014; ALICIC et al., 2014). Phenolic acids and flavonoid glycosides are present in the nectar of flowers visited by bees, which are hydrolyzed and transferred to the bee pollen. The number and variety of phenolic acids and flavonoids are highly variable, since beekeepers mix bee pollen from different botanical origins (MORAIS et al., 2011; LEJA et al., 2007). The main group of pigments that compose bee pollen corresponds to carotenoids, especially β -carotene (17% of all carotenoids), whose concentration also depends on the botanical origin of the pollen (ALMEIDA-MURADIAN et al., 2005).

The type and concentration of the polyphenolic compounds influence the antibacterial and antioxidant activity exhibited by bee pollen. The most important polyphenolic compounds related with these activities are vanillic acid, protocatechuic acid, gallic acid, p-coumaric acid, hesperidin, rutin, kaempferol, apigenin, luteolin, quercetin, and isorhamnetin (ALICIC et al., 2014). These compounds also serve as biochemical markers of bee pollen (TOMÁS-BARBERÁN et al., 1989) related with the botanical origin. Bee pollen rich in these compounds has shown activity against specific pathogens such as *Staphylococcus aureus* (CABRERA and MONTENEGRO, 2013), *Escherichia coli* (LIBONATTI et al., 2014; CABRERA and MONTENEGRO, 2013), *Streptococcus viridians* (CAMPOS et al., 2010; BISNO and STEVENS, 1996), and *Pseudomonas aeruginosa* (ABOUDA et al., 2011; CARPES et al., 2007).

In order to establish relationships between the botanical origin of multifloral Chilean bee pollen and their phenolic, protein and carotenoid content, and antioxidant/antibacterial activities, we present a characterization by HPLC-DAD of the phenolic compounds present in their extracts, their botanical origin, and a quantification of their total carotenoid and protein content. *In vitro* antioxidant and antibacterial activities were evaluated by Ferric Reducing Antioxidant Power (FRAP) and determining the zone of inhibition against *E. coli*, *S. aureus*, *P. aeruginosa*, and *S. pyogenes*, respectively. Characterization on antioxidant and antibacterial properties present in samples of multiflora bee pollen will allow the beekeeping sector to add value to this product.

Materials and methods

Bee pollen

Sixteen samples of commercial bee pollen were purchased from local beekeepers of Central Chile between December 2013 and February 2014. Samples were lyophilized and stored at -20 °C. The determination of botanical origin was performed using palynological analysis method described at Chilean Regulation (NCh3255, 2011).

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Five grams of each type of bee pollen corbiculae were separated by color and each fraction was weighed. Then one corbiculae of each kind of bee pollen sample was crushed with alcohol to disperse the pollen grains. Several drops of red calberla were used to stain the grains allowing their observation under light microscope. To determine the botanical origin specific literature (MARTICORENA and QUEZADA, 1985; HEUSSER, 1971) and the botanical palinoteca of Botanical laboratory at Pontificia Universidad Católica de Chile were consulted.

Total protein content

Protein determination was performed by Kjeldahl method based on standard AOAC (1984). One gram of sample was weighted and homogenized. In the digestion step organic nitrogen in the sample was decomposed by a solution of concentrated sulfuric acid, sodium sulfate, cuprum dioxide and applying a temperature cycle: 120 °C for 15 min, 200 °C for 2 min, 300 °C for 2 min, and 402 °C for 40 min, on a DK 6 Kjeldahl Digestion Unit (Velp Scientifica). Sodium hydroxide was added and distillation on 3% boric acid was performed using a UDK 129 Kjeldahl Distillation Unit (Velp Scientifica). Titration was performed with 0.1 M hydrochloric acid. Conversion factor used was 6.25.

Total carotenoid content

Carotenoid extraction was performed weighting 4 g of bee pollen, milled and sonicated in an ultrasonic bath for 15 minutes with 20 mL of petroleum ether-acetone mixture (1:1 v/v). The extract was transferred to a separator funnel and washed with 60 mL of distilled water. The aqueous phase was discarded and the organic portion was passed through 2 g of anhydrous sodium sulfate. The whole process was repeated until the sample showed no coloration. Finally, the extract was evaporated to dryness under a stream of nitrogen, reconstituted in 2 mL of butanol and quantified by HPLC-DAD at 440 and 480 nm.

Bee pollen extracts

Bee pollen extraction process was based and adapted from LEBLANC et al. (2009). Ten grams of multiflora bee pollen were mixed with 10 mL of distilled water and ultrasonicated for one hour. The mixture was centrifuged at 6000 rpm for 20 minutes and the supernatant was stored at 4 °C in darkness. This process was repeated 5 times. The collected supernatants were combined and filtered using qualitative paper (Whatman No. 2). Finally they were evaporated (rotary evaporator Buchi R-210) and the dry extract was reconstituted with 10 mL of ultrapure water, filtered (EDLAB CA syringe filter 0.45 mm) and stored at -20 °C.

Total phenolic compounds

Colorimetric determination of the phenolic content was evaluated by the Folin-Ciocalteu reaction (FC). Assays were performed on bee pollen extracts. The absorbance at 765 nm of the mixture of 200 µL extract, 50 µL Folin-Ciocalteu reagent, 150 mL of 20% w/v sodium carbonate solution (Na₂CO₃) and 600 mL of ultrapure water was measured in triplicate after 30 minutes of reaction. A calibration curve was constructed with gallic acid concentrations between 10 and 50 mg/mL.

Antioxidant activity

The antioxidant activity was determined by the Ferric Reducing Antioxidant Power (FRAP) assay. 200 µL of pollen extract was mixed with 1.8 mL of FRAP reagent, after 15 minutes in the dark the absorbance was measured at 593 nm. FRAP reagent was prepared as

follow: 25 mL of acetate buffer, 2.5 mL TPTZ solution (10 mmol/L of TPTZ in HCl 40 mmol/L) and 2.5 mL of 20 mM FeCl₃·6H₂O. A calibration curve was calculated with known solutions of FeSO₄·7H₂O in a concentration range of 20 and 100 µg/mL. In order to compare the antioxidant power of these samples, this test was performed to a blueberry sample, which is a recognized natural source of antioxidant compounds.

Identification and quantification of flavonoids and phenolic acids

The identification and quantification of flavonoids and phenolic acids on bee pollen extracts were performed by high performance liquid chromatography with a diode array detector based on BENZIE and STRAIN (1996). Elite Merck LaChrom HPLC Hitachi was used in a reverse phase column (LiChroCART RP-18) with a mobile phase of aqueous formic acid 5% (v/v) and methanol at constant solvent flow of 1 mL/min at 30 °C. Samples were injected manually. Chromatograms were monitored at 290 and 340 nm. A calibration curve was made with high purity standards and area of peaks found with the EZChrom Elite v.3.3.1 (Scientific Software Inc. 1988-2005; Agilent 2005-2008) program.

Antibacterial activity

The antibacterial activity of bee pollen extracts was evaluated by diameter of inhibition against *Escherichia coli* ATCC-25922, *Staphylococcus aureus* ATCC-25923, *Pseudomonas aeruginosa* ATCC 27853 and *Streptococcus pyogenes* I.S.P. 364-00 (Supplied by Chilean Public Health Institute). Diameter of inhibition was determined using the standard reported by CLSI (2006): bacterial strains were inoculated on Mueller Hinton agar for 24 hours at 37 °C. After that time, colonies were selected and diluted in saline solution to a concentration of 10⁻³ UFC by visual comparison with a standard of 0.5 McFarland (1.5×10⁸, Becton & Dickinson Company, USA). Once strains were swab on the agar, 6 mm diameter holes were made, and 100 µL of each extract were deposited in each hole. Petri dishes were incubated between 18 to 24 hours at 37 °C until measurements. The inhibition diameter that appeared around each hole was measured. Tetracycline, ampicillin and chloramphenicol were used as controls.

Statistical analysis

Statistical analysis of the results was performed using a one-way variance analysis (ANOVA) followed by Tukey HSD method with 95% (p<0.05) level of confidence and computed by STAT-GRAPHICS Centurion XV software 15.02.05. Samples were analyzed in triplicate. Correlations between results were made using the Pearson's correlation coefficient (r) (p<0.05).

Results and discussion

The botanical origin described the presence of different plant sources used by bees to produce the bee pollen. This description permitted to classify them as native/non-native/mixed and unifloral/bifloral/multiflora bee pollen (NCh 3255, 2011) (Tab. 1). Floral species found in the samples are closely related with the geographic location of hives.

The analyzed samples of bee pollen were predominantly derived from non-native floral species and frequently from only one of them. The majority of samples analyzed corresponded to non-native unifloral (nine), followed by non-native multifloral, mixed multifloral, and non-native bifloral (two samples of each), and native unifloral (one). Among the samples analyzed, *Galega officinalis* predominated in thirteen samples and *Brassica* sp. was present in eleven samples. *Brassica* sp. accounted for 34% of the average weight of each sample,

Tab. 1: Botanical origin and classification of samples. Data represent analyses of 300 pollen grains counted in 5 distinct optical areas in three different samples.

Sample	Predominant pollen (>45%)		Secondary pollen (16 - 45%)		Important minor pollen (3 - 15%)		Minor pollen (<3%)		Classification
	Specie	%	Specie	%	Specie	%	Specie	%	
1	<i>Brassica</i> sp.	57.8	<i>Galega officinalis</i>	42.2					Non-native unifloral
2			<i>Eschscholzia californica</i>	34.8	<i>Medicago sativa</i>	12.2	<i>Apiaceae</i>	2.0	Non-native bifloral
			<i>Brassica</i> sp.	30.6	<i>Schinus</i> sp.	10.2	Fungal spores	2.0	
							<i>Olea europaea</i>	8.2	
3			<i>Schinus</i> sp.	37.5			<i>Mutisia</i> sp.	2.5	Mixed multifloral
			<i>Brassica</i> sp.	22.9			<i>Medicago polymorpha</i>	0.4	
							<i>Medicago sativa</i>	0.4	
4	<i>Galega officinalis</i>	50.0	<i>Asteraceae raphanus</i> sp.	26.0 24.0					Non-native unifloral
5	<i>Medicago sativa</i>	58.0	<i>Galega officinalis</i>	39.6			<i>Hypochaeris/Taraxacum</i>	2.4	Non-native unifloral
6			<i>Brassica</i> sp.	36.5	<i>Galega officinalis</i>	15.6			Non-native multiflora
			<i>Medicago polymorpha</i>	27.1					
			<i>Convolvulus</i> sp.	20.8					
7	<i>Brassica</i> sp. <i>Galega officinalis</i>	51.0 46.9					<i>Adesmia</i> sp.	2.0	Non-native unifloral
8	<i>Prunus</i> sp. <i>Trevoa quinquenervia</i>	52.2 45.7					<i>Maytenus boaria</i>	2.2	Native unifloral
9	<i>Brassica</i> sp.	58.3	<i>Sonchus</i> sp.	22.9	<i>Oxalis</i> sp. <i>Papilionaceae</i>	12.5 4.2	<i>Asteraceae</i>	2.1	Non-native unifloral
10			<i>Brassica</i> sp. <i>Galega officinalis</i> <i>Dysopsis</i> sp.	32.7 26.5 20.4	<i>Medicago sativa</i> <i>Hypochaeris/Taraxacum</i>	8.2 8.2	<i>Trifolium</i> sp. <i>Asteraceae</i>	2.0 2.0	Non-native multifloral
11	<i>Brassica</i> sp.	72.9			<i>Actinidia deliciosa</i> <i>Fabaceae</i>	12.5 4.2	<i>Galega officinalis</i> <i>Asteraceae</i> <i>Hypochaeris/Taraxacum</i> <i>Quillaja saponaria</i> <i>Vicia</i> sp.	2.1 2.1 2.1 2.1	Non-native unifloral
12	<i>Galega officinalis</i>	74.5	<i>Brassica</i> sp.	19.6			Fern spores <i>Trifolium repens</i> <i>Fabaceae</i>	2.0 2.0 2.0	Non-native unifloral
13			<i>Convolvulus</i> sp. <i>Convolvulus</i> sp. <i>Brassica</i> sp.	43.2 38.6 16.0			<i>Chenopodiaceae</i>	2.3	Non-native bifloral
14	<i>Chenopodiaceae</i>	58.0	<i>Convolvulus</i> sp.	24.0	<i>Brassica</i> sp. <i>Clarkia tenella</i>	12.0 4.0	<i>Chenopodiaceae</i>	2.0	Non-native unifloral
15	<i>Cactaceae</i>	51.2	<i>Galega officinalis</i>	34.1	<i>Amaranthaceae</i>	12.2	<i>Tecophilaceae</i>	2.4	Mixed multifloral
16	<i>Medicago</i> sp.	62.5			<i>Asteraceae</i> <i>Mirtaceae</i> <i>Galega officinalis</i> <i>Malvaceae</i>	10.4 10.4 8.3 8.3			Non-native unifloral

while *Galega officinalis* accounted for 40%. This indicates that *Brassica* sp. and *Galega officinalis* are important sources of pollen collection. The *Asteraceae* family was the least frequently detected in the samples analyzed, comprising only 2% of the average sample weight when present.

Total protein content

Total protein of the samples ranged between 20.0 and 30.4%, with an average of 25.4% (Fig. 1). The values observed are similar to the amounts reported in literature (ALMEIDA-MURADIAN et al., 2005; BOGDANOV, 2014; BALKANSKA and IGNATOVA, 2012). This result

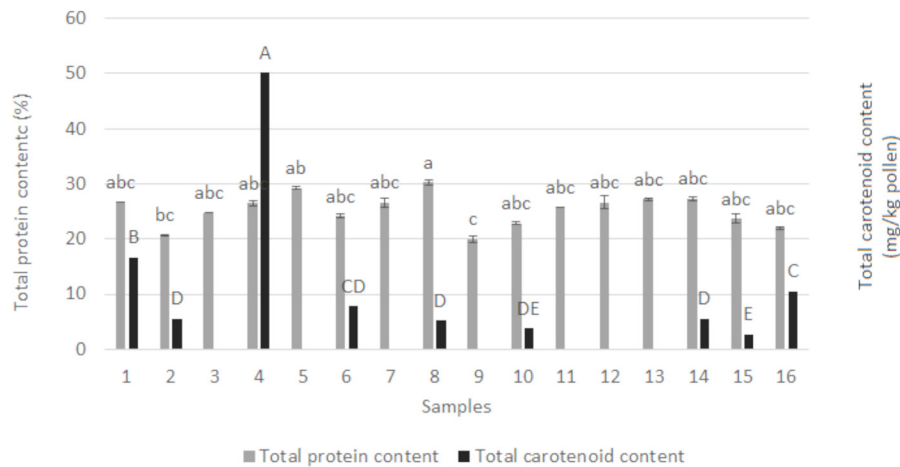


Fig. 1: Total protein and carotenoid content of samples (mean \pm SD; $n = 3$). In each column different letters imply significant differences ($p < 0.05$).

confirms that bee pollen could be a good source of vegetable protein replacing dietary animal sources such as meat (20% protein content, SCHMIDT et al., 1985), that currently are highly criticized for causing or increasing the likelihood of developing diseases (WHO, 2015; BERNSTEIN et al., 2010). Bee pollen is even a better vegetable protein than quinoa (12-23% protein content, JAMES, 2009).

The samples composed by *Prunus* sp. 52.2% / *Trevoa quinqueruvia* 45.7% (sample 8) and by *Medicago sativa* 58.0% / *Galega officinalis* 39.6% (sample 5) have the highest protein contents. These results are in agreement with VANDERPLANCK et al. (2014), whom reported 25.8% of protein in *Prunus* sp. bee pollen. There is not reported protein content of bee pollen from *Trevoa quinqueruvia*. ANDRADA and TELLERÍA (2005) reported that *Medicago sativa* has 22% of protein content and according to PEIRETTI and GAI (2006) *Galega officinalis* has 20%. Moreover, samples such as *Eschscholzia californica* 34.8% / *Brassica* sp. 30.6% / *Medicago sativa* 12.2% / *Schinus* sp. 10.2% / *Olea europaea* 8.2% (sample 2), and *Brassica* sp. 58.3% / *Sonchus* sp. 22.9% / *Oxalis* sp. 12.5% (sample 9) samples have poorest content. These results are also expected since FORCONE et al. (2013) reported 21.1% of protein content in bee pollen from *Eschscholzia californica*.

Regarding the correlation of the protein content with the botanical origin, no relation was found with a confidence level of 95% ($p < 0.05$). This indicates that the protein content is not dependent on any particular species. It is also observed that the bee pollen samples did not present significant difference in protein content between them ($p < 0.05$).

Total carotenoid content

Carotenoids were observed in nine bee pollen samples, which ranged between 2.8 and 50.2 mg/kg of pollen with 12.0 mg/kg of pollen in average (Fig. 1). The values obtained in our samples were lower than those ranged between 10 - 200 mg/kg reported in the literature from other multiflora bee pollen samples (ALMEIDA-MURADIAN, 2005; MĂRGĂOAN et al., 2010). This difference can be explained by the wide difference in carotenoid content between genus, families and species.

Significant differences ($p < 0.05$) were observed in total carotenoid content, where the samples of *Galega officinalis* 50% / *Asteraceae* 26.0% / *Raphanus* sp. 24.0% (sample 4) and *Brassica* sp. 57.8% / *Galega officinalis* 42.2% (sample 1) presented the highest carotenoid content. Meanwhile the sample composed by *Cactaceae* 51.2% / *Galega officinalis* 34.1% / *Amaranthaceae* 12.2% / *Tecophilaceae* 2.4% (sample 15) showed the lowest content that indicates that bee

pollen from these species are poor as carotenoid sources. It is possible that the high content present in sample 4 was due to the presence of bee pollen from *Galega officinalis* since bee pollen from *Brassica* sp has been reported as very poor in carotenoids (STANCIU et al., 2016) but also exists a high variation in carotenoid content inside species that compose this genus (JAHANGIR et al., 2009). OLIVEIRA et al. (2009) and BOBIS (2014), has been reported that *Asteraceae* and *Raphanus* sp. are sources of high carotenoid content. There is no information about carotenoid contents of bee pollen from *Cactaceae*, *Amaranthaceae* and *Tecophilaceae* families but probably have lower contents.

A positive correlation was found between carotenoid content and *Asteraceae* ($r=0.92$; $n=5$; samples 4, 9, 10, 11, 16) and *Raphanus* sp. ($r=0.95$; $n=1$; sample 4). A moderate interdependence between carotenoid content and *Galega officinalis* was observed ($r=0.45$; $n=13$; samples 1, 3, 4, 5, 6, 7, 10, 11, 12, 13, 14, 15, and 16). However, it is not possible to affirm that the presence of these species really correlated with the carotenoid content since there is no information about these parameters in samples of bee pollen from these species.

Identification and quantification of flavonoids and phenolic acids

Six phenolic acids (p-coumaric, chlorogenic/caffeic, ferulic, sinapic, and cinnamic acids) and two flavonoids (kaempferol, luteolin) were identified by liquid chromatography with diode array (Tab. 2). p-Coumaric acid was presented in all samples and chlorogenic/caffeic and ferulic acids were presented in ten samples. The most frequent flavonoid was kaempferol and the least frequent was luteolin. Kaempferol was presented in six samples and luteolin in only one. Samples with the highest polyphenolics concentrations (i.e., phenolic acid +flavonoids) were *Medicago* sp. 62.5% (sample 16, 918.42 mg/kg) and *Brassica* sp. 51.0% / *Galega officinalis* 46.9% (sample 7, 638.63 mg/kg).

These results differ from phenolic acids and flavonoids concentration previously reported for similar botanical origin bee pollen. The presence of kaempferol in bee pollen derived from *Brassica* sp. has been previously reported (FATRCOVÁ-ŠRAMKOVÁ et al., 2013). It has been previously observed that bee pollen from *Brassica napus* subsp. *napus* L. contains luteolin (FATRCOVÁ-ŠRAMKOVÁ et al., 2013). However, only one of the samples in this study that contain this botanical origin (*Brassica* sp. 51.0% / *Galega officinalis* 46.9% / *Adesmia* sp. 2%) presents luteolin, with a concentration 10 times higher than that reported by FATRCOVÁ-ŠRAMKOVÁ et al. (2013). Apigenin, a common flavonoid present in bee pollen with a biological activity was not found in any sample. This variability in bee pollen

Tab. 2: HPLC-DAD profile of bee pollen samples evaluated (mean \pm SD; $n = 3$). In each column different letters imply significant differences ($p < 0.05$). Nd: Non-detected (under detection threshold).

Sample	Chlorogenic + Caffeic acid (mg/kg)	Ferulic acid (mg/kg)	Sinapic acid (mg/kg)	p-Coumaric acid (mg/kg)	Cinnamic acid (mg/kg)	Kaempferol (mg/kg)	Luteolin (mg/kg)
t_R (min)	6.90 + 7.06	11.74	9.09	8.42	11.74	12.67	11.96
Λ (nm)	340	290	340	290	340	340	340
1	18.16 \pm 0.73 ^{ab}	Nd	Nd	4.02 \pm 0.16 ^a	Nd	Nd	Nd
2	18.07 \pm 0.72 ^{ab}	5.66 \pm 0.23 ^b	Nd	92.01 \pm 3.68 ^{bc}	8.93 \pm 0.36 ^d	Nd	Nd
3	Nd	Nd	Nd	287.37 \pm 11.50 ^g	Nd	Nd	Nd
4	Nd	Nd	Nd	109.91 \pm 4.40 ^c	Nd	Nd	Nd
5	Nd	Nd	Nd	458.52 \pm 18.34 ⁱ	Nd	Nd	Nd
6	Nd	Nd	Nd	337.05 \pm 13.48 ^h	Nd	Nd	Nd
7	Nd	9.97 \pm 0.40 ^d	Nd	255.17 \pm 10.21 ^{ef}	Nd	57.49 \pm 2.29 ^c	316.00 \pm 2.64 ^a
8	26.94 \pm 1.08 ^{bc}	Nd	Nd	71.55 \pm 2.86 ^b	Nd	Nd	Nd
9	Nd	13.35 \pm 0.53 ^e	Nd	4.02 \pm 0.16 ^a	Nd	344.20 \pm 13.76 ^d	Nd
10	29.79 \pm 1.19 ^c	26.33 \pm 1.05 ^h	72.95 \pm 2.92 ^a	286.21 \pm 11.45 ^{fg}	Nd	17.11 \pm 0.68 ^b	Nd
11	111.60 \pm 4.46 ^f	22.61 \pm 0.90 ^g	89.67 \pm 3.59 ^b	169.60 \pm 6.78 ^d	Nd	63.68 \pm 2.55 ^c	Nd
12	43.37 \pm 1.73 ^d	4.09 \pm 0.16 ^a	22.28 \pm 0.89 ^c	82.19 \pm 3.29 ^{bc}	Nd	Nd	Nd
13	86.11 \pm 3.44 ^e	5.48 \pm 0.22 ^b	Nd	73.28 \pm 2.93 ^b	7.30 \pm 0.29 ^b	5.33 \pm 0.21 ^a	Nd
14	45.16 \pm 1.81 ^d	7.50 \pm 0.30 ^c	9.12 \pm 0.36 ^d	191.91 \pm 7.68 ^d	6.49 \pm 0.26 ^a	Nd	Nd
15	11.29 \pm 0.45 ^a	6.72 \pm 0.27 ^{bc}	51.84 \pm 2.07 ^e	243.21 \pm 9.73 ^e	Nd	19.23 \pm 0.77 ^b	Nd
16	258.92 \pm 10.36 ^g	20.58 \pm 0.82 ^f	Nd	630.92 \pm 25.24 ^j	8.00 \pm 0.32 ^c	Nd	Nd

is derived by the variability of phenolic compounds produced by plants, which depends on the stress conditions, geographic location and vegetation around the apiaries, which conditioned the flowering (MORAIS et al., 2011; LEJA et al., 2007).

Regarding the correlation between phenolic acids/flavonoids and botanical origin several dependences were observed. *Medicago* showed a positive correlation with p-coumaric acid ($r=0.67$; $n=16$) that indicates high concentrations of this compound in samples that include it (i.e., samples 2, 3, 5, 6, 10, and 16). Bee pollen from *Eschscholzia californica* and *Olea europaea* has a positive correlation with cinnamic acid content ($r=0.54$, 0.54 , respectively) that is in agreement with high content of this compound at samples with these kind of bee pollen (i.e., sample 2). Samples with bee pollen from *Malvaceae* has a high correlation with chlorogenic/caffeic acid and p-coumaric acid ($r=0.67$, 0.87 , respectively) that indicates a high content of this compounds at samples that have bee pollen from these species (i.e., sample 16). Samples with *Mirtaceae* bee pollen also have a high correlation with chlorogenic/caffeic acid and p-coumaric acid ($r=0.67$, 0.87 , respectively) that is present in sample 16. Bee pollen from *Sonchus* sp., *Oxalis* sp and *Papilionaceae* have a high correlation with kaempferol ($r=0.97$, 0.97 , 0.97 , respectively; $n=16$) that is in agreement with the high content of kaempferol at sample 9. Sample 10, composed by bee pollen from *Dysopsis* sp. has a positive correlation with ferulic and sinapic acid ($r=0.57$, 0.52) that indicates a high content of this compound. Finally, bee pollen from *Actinidia deliciosa* has a positive correlation with sinapic acid ($r=0.68$) that is in agree with the higher content at sample 11.

Determination of total phenolic content and ferric reducing antioxidant power (FRAP)

Total phenolic content and ferric reducing antioxidant power (FRAP) for bee pollen samples are showed in Fig. 2. The total phenolic con-

tent of samples ranged between 6.86 and 52.99 g GAE/kg of pollen, with an average value of 12.64 g GAE/kg of pollen. These contents are higher than other reports such as values published by PASCOAL et al. (2014) and FEÁS et al. (2012) which showed ranges between 4.96 and 19.80 g GAE/kg, respectively. The sample of *Prunus* sp. 52.2% / *Trevoa quinqueruvia* 45.7% / *Maytenus boaria* 2.2% (sample 8) and *Brassica* sp. 58.3% / *Sonchus* sp. 22.9% / *Oxalis* sp. 12.5% (sample 9) have the highest values (33.34 and 52.99 g GAE/kg, respectively). MÄRGÄOAN et al. (2013) and STANCIU et al. (2016) reported a content of 8.87g GAE/kg and 7.57g GAE/kg on average respectively for bee pollen from *Prunus* sp. In addition, STANCIU et al. (2016) reports that the content of bee pollen from *Brassica* sp. is 11.62 g GAE/kg and 5.46 g GAE/kg for *Oxalis* sp. bee pollen. All values being higher than the bee pollen of another species. As for the phenolic content of bee pollen from *Sonchus* sp., *Trevoa quinqueruvia* and *Maytenus boaria*, there are no reports, however they may have a high content considering the total content of the samples. The high phenolic value of *Brassica* sp. 58.3% / *Sonchus* sp. 22.9% / *Oxalis* sp. 12.5% / *Papilionaceae* 4.2% / *Asteraceae* 2.1% (Sample 9) may be due to the high content reported at Tab. 2. *Brassica* sp. has been reported with a high content of kaempferol associated with its antioxidant activity (FATRCOVÁ-ŠRAMKOVÁ et al., 2013; LI et al., 2016). There are no reports of the presence of kaempferol in bee pollen of the other species present in the sample 8 and 9.

FRAP values ranged between 4.51 and 91.19 mmol Fe²⁺/kg pollen, with an average of 35.95 mmol Fe²⁺/kg pollen (Fig. 3). The sample of *Prunus* sp. 52.2% / *Trevoa quinqueruvia* 45.7% / *Maytenus boaria* 2.2% (sample 8) has the highest FRAP value with 91.19 mmol Fe²⁺/kg pollen. This value was higher than the FRAP value of blueberry, a very well known natural antioxidant (between 58.99 and 63.41 Fe²⁺ mmol/kg). However, compared to the FRAP values found in literature (5.36 mM Fe²⁺/g, MARGHITAS et al., 2009 and 21 mM eq. Fe²⁺/g, MONTENEGRO et al., 2013), our results are much smaller (less

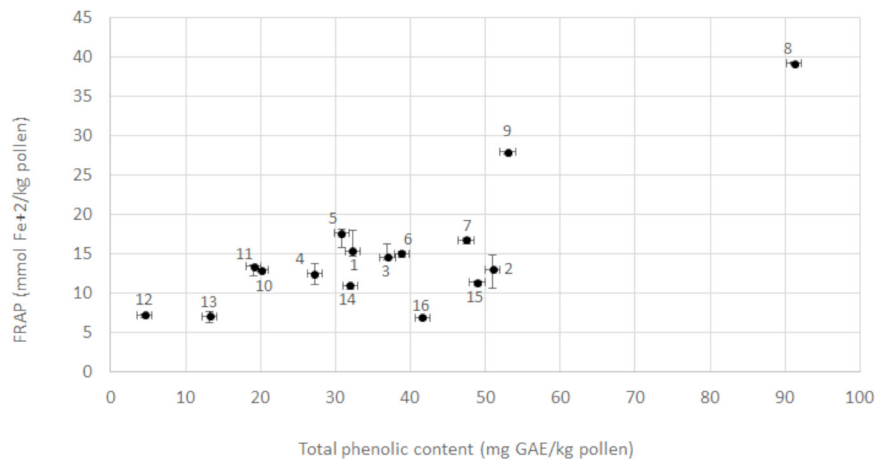


Fig. 2: Total phenolic content and antioxidant activity of samples (mean \pm SD; $n = 3$).

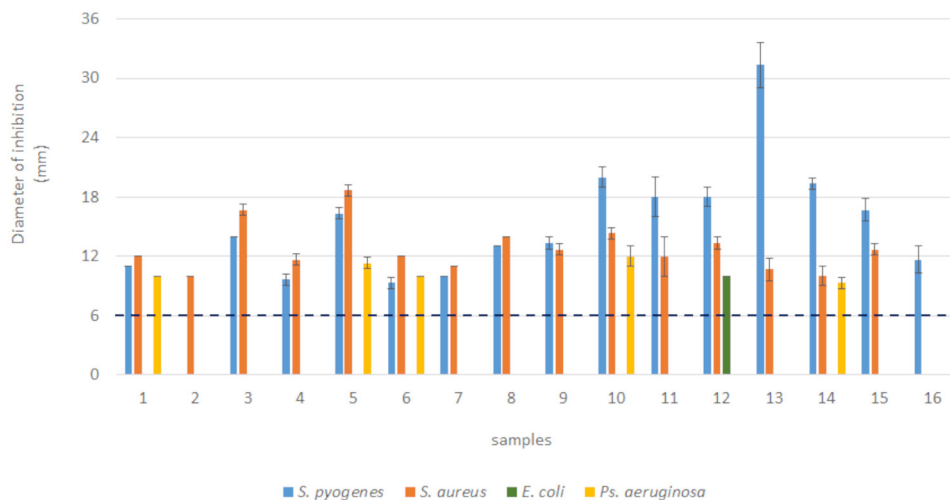


Fig. 3: Antibacterial activity showed by bee pollen extracts against *Streptococcus pyogenes*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli*. Dashed line indicate diameter of hole with bee pollen extracts (mean \pm SD; $n = 4$).

than 0.1 mmol Fe²⁺/g). This may be due to the fact that our extracts were obtained using water instead of ethanol or methanol (DO et al., 2014).

As expected from similar studies (ULUSOY and KOLAYLI, 2014; BORYCKA et al., 2016), there is a positive correlation coefficient between total phenolic content and FRAP: Pearson's correlation coefficient of 0.59 (Fig. 3). Thus, the antioxidant power showed by bee pollen samples can be attributed to their phenolic content. A positive correlation was also found between total phenolic content and *Prunus* sp. ($n=16$; $r=0.50$), *Trevoa quinquerteria* ($n=16$; $r=0.50$), *Oxalis* sp. ($n=16$; $r=0.79$), *Papilionaceae* ($n=16$; $r=0.79$) and *Sonchus* sp. ($n=16$; $r=0.79$), and between FRAP with *Prunus* sp. ($n=16$; $r=0.76$) and *Trevoa quinquerteria* ($n=16$; $r=0.76$). Furthermore a high dependence between total phenolic content and kaempferol ($n=16$; $r=0.78$) was observed. These results indicate that phenolic content of samples that includes bee pollen from *Prunus* sp., *Trevoa quinquerteria* or *Sonchus* sp. are result of kaempferol content.

Antibacterial activity

Antibacterial assays showed that bee pollen is more active against Gram-positive (i.e., *S. aureus* and *S. pyogenes*) than Gram-negative bacteria (i.e., *P. aeruginosa* and *E. coli*) (Fig. 3). Fifteen samples

inhibited *S. pyogenes*, fifteen samples inhibited *S. aureus*, five samples inhibited *P. aeruginosa* and only one showed inhibition against *E. coli*. These results show that Gram-positive bacteria are better controlled by the bee pollen than Gram-negative bacteria, which showed more resistance. This tendency was also reported in another study from our research group (CABRERA and MONTENEGRO, 2013). Gram-negative bacteria have a double cell wall, composed by lipopolysaccharides and proteins, which hinders the antibacterial action of bee pollen thus *E. coli* and *P. aeruginosa* because are more resistant therefore were less controlled (TAFUR et al., 2008).

Fifteen out of sixteen samples showed control against *Streptococcus pyogenes*. The range of inhibition was observed between 9.3 and 31.3 mm, similar to the range of between 9 and 28 mm reported in the literature (ABOUDA et al., 2011; CABRERA and MONTENEGRO, 2013). The highest diameter of inhibition was observed in sample 13 (31.3 mm), higher than tetracycline and closer to ampicillin. This sample was composed by *Convolvulus arvensis* 43.2% / *Galega officinalis* 38.6% / *Brassica* sp. 16%. There are no reports indicating that *Galega officinalis* and *Convolvulus arvensis* bee pollen have antibacterial activity against *S. pyogenes*. CABRERA and MONTENEGRO (2013) report one sample that contains 30% of *Brassica* sp. bee pollen which controlled *S. pyogenes*.

There is a positive correlation between *Chenopodiaceae* ($r=0.65$;

n=16) and *Convolvulus* sp. ($r=0.58$; n=16) with the inhibition activity against *S. pyogenes*, and a negative correlation with *Eschscholzia californica* ($r=-0.58$; n=16) and *Olea europaea* ($r=-0.57$; n=16). These correlations would indicate that some species enhance the antibacterial activity of bee pollen while others decrease it when are present. These species could contain phenolic/flavonoid compounds that would not necessarily be poor antibacterials since they could compete for sites of action with phenolic/flavonoid compounds from other species or results in antagonistic effects (KUMAR and PANDEY, 2013; MANDALARI et al., 2010; PALAFOX-CARLOS et al., 2012). It is also possible that samples containing bee pollen from *Chenopodiaceae* and *Convolvulus* sp. contain compounds that inhibit the bacterial growth of *S. pyogenes*, however do not exist in the literature reports on this. Since *Eschscholzia californica*, *Olea europaea* and *Chenopodiaceae* correlates with cinnamic acid ($r=0.54$, 0.54 and 0.56 , respectively) it suggest a relation with antibacterial exerted against *S. pyogenes*. However 15 samples inhibited *S. pyogenes* and only 4 samples have cinnamic acid. Therefore no correlation between botanical origin and antibacterial activity against *S. pyogenes*.

Fifteen out of sixteen samples showed growth inhibition against *Staphylococcus aureus*. Sample 5 showed the highest inhibition diameter with 18.7 mm, similar to half of the ampicillin and tetracycline diameters. *Medicago sativa* (58.0%) and *Galega officinalis* (39.6%) predominate in sample 5. There is no data indicating that the bee pollen obtained from these species has antibacterial activity. However, there are many studies indicating that some of these plants have antibacterial activity against *S. aureus*, which has been attributed to certain flavonoids, saponins and peptides (RODRIGUES et al., 2013; KARAKAS et al., 2012; ERTURK, 2010).

There is a positive correlation between antibacterial activity of bee pollen samples against *S. aureus* and *Medicago sativa* (n=16; $r=0.50$) and a negative correlation with *Malvaceae* (n=16; $r=-0.72$), *Medicago* sp. (n=16; $r=-0.81$) and *Mirtaceae* (n=16; $r=-0.81$). The antibacterial activity in this case is mainly a result of chlorogenic/caffeic and p-coumaric acids since correlation were found between these compound and bee pollen from these species (see section above: Identification and quantification of flavonoids and phenolic acids). The samples with a higher concentration of p-coumaric acid than chlorogenic/caffeic acids (i.e., samples 2, 3, 5, 10, 16) showed a higher antibacterial activity.

The bacterial growth inhibition exerted by bee pollen extracts against *P. aeruginosa* was less effective than the other bacteria assayed. Only 5 out of sixteen samples showed positive results. The sample composed by *Medicago sativa* 58.0% / *Galega officinalis* 39.6% / *Hypochaeris-Taraxacum* 2.4% (sample 5) showed the highest diameter of inhibition (11.3 mm). This result was similar to tetracycline and higher than ampicillin that did not have inhibition against *P. aeruginosa*. The inhibition diameters observed were found to be similar to that reported by ABOUDA et al. (2011). There is a positive correlation between antibacterial inhibition showed against *P. aeruginosa* and *Hypochaeris-Taraxacum* sp. ($r=0.52$; n=16). Since a moderate dependence were found between *Hypochaeris-Taraxacum* sp. and ferulic and sinapic acids, it suggests a relation of these compounds with antibacterial effect exerted against *S. pyogenes*. However, only two out of five samples that inhibited *S. pyogenes* have ferulic and sinapic acids in their composition. Therefore no correlation between botanical origin and antibacterial activity against *P. aeruginosa*.

Escherichia coli was controlled only by one sample (sample 12) that formed an inhibitory diameter of 10.0 mm. However, zones of inhibition were reported in the literature ranging from 15 to 40 mm (KHIDER et al., 2013). This difference could be explained by the different botanical origin of bee pollen sample compared with KHIDER et al. (2013) and the extractant used (i.e., methanol/hexane vs. water). The botanical origin of sample 12 was composed

mainly by *Galega officinalis* 74.5% / *Brassica* sp. 19.6%, which have shown inhibition against Gram-negative bacteria, so they should be further investigated for the responsible compounds of their activity (ERTURK, 2010). Inhibition activity against *E. coli* showed a positive and moderate dependence the presence of bee pollen from *Galega officinalis* ($r=0.52$; n=16). However, the antibacterial activity observed cannot be attributed to *Galega officinalis* since is also presented in other samples that no showed this control against *E. coli*.

Conclusions

We reported for the first time the relationship between botanical origins of bee-pollen from Chile and their phenolic, protein and carotenoid content, and antioxidant/antibacterial activities. It was demonstrated that several plant species contribute these parameters, mainly *Brassica* sp. and *Galega officinalis*. Less frequent species such as fruit and endemic species as *Medicago sativa*, *Prunus* sp., *Trevoa quinquenervia*, *Prunus* sp., and *Convolvulus arvensis* contribute to differences between composition and antioxidant/antibacterial activities of bee pollen samples. In addition species present in lower concentrations also help to accentuate these differences. Samples with high protein content are composed by bee pollen from *Prunus* sp., *Trevoa quinquenervia*, *Medicago sativa* and *Galega officinalis*. Samples with high carotenoid content are composed by bee pollen from *Galega officinalis*, *Asteraceae*, *Raphanus* sp. The samples with higher content of polyphenols are composed by *Medicago*, *Brassica* sp. and *Galega officinalis* bee pollen. Six phenolic acids (p-coumaric, chlorogenic / caffeic, ferulic, sinapic and cinnamic acids) and two flavonoids (kaempferol and luteolin) were identified in the samples. The highest content of phenolics was presented in samples composed by *Prunus* sp. and *Brassica* sp. bee pollen. While those with higher antioxidant power (FRAP) presented *Prunus* sp. and *Trevoa quinquenervia* bee pollen. Samples composed by *Convolvulus arvensis*, *Galega officinalis* and *Brassica* sp. showed inhibitory activity against *S. pyogenes*; those which contain *Medicago sativa* and *Galega officinalis* bee pollen inhibited *S. aureus* and *P. aeruginosa*; *E. coli* was controlled by samples with *Galega officinalis* and *Brassica* sp. bee pollen. Botanical origin analysis of bee pollen permits correlation with some chemical, antioxidant and antibacterial properties, suggesting new resources of bioactive compounds. Further studies with monofloral bee pollen loads are needed in order to provide more accurate correlations between botanical origin and composition and antioxidant/antibacterial activities. Likewise, a more accurate determination of the phenolic/flavonoid and other active compounds that make up the extracts is needed since they play a crucial role in the bioactivity of bee pollen.

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Conflict of interest disclosure

The authors declare that there is no conflict of interest regarding the publication of this paper.

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