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Honeybee Pollen Load: Phenolic Composition and Antimicrobial Activity and Antioxidant Capacity

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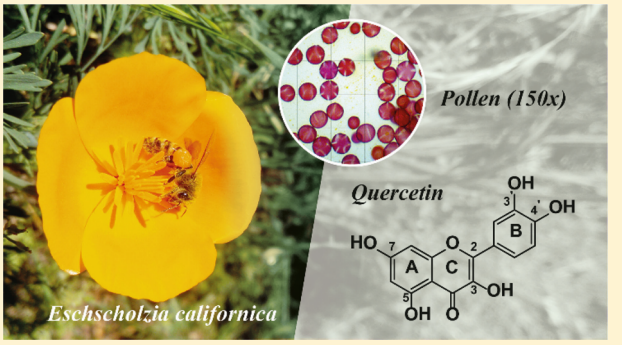
Supporting Information

ABSTRACT: Honeybee pollen loads result from the agglutination of pollen grains and salivary secretions of bees. The potential use of honeybee pollen as a food supplement greatly depends on its chemical composition, which varies depending on the botanical and geographical origin of the pollen grains. This study aimed to characterize the botanical origin, chemical composition, and antioxidant and antibacterial activities of honeybee pollen from the V Region of Chile. The introduced species *Brassica rapa* and *Eschscholzia californica* predominated in the bee pollen analyzed. The honeybee pollen extracts showed antioxidant and antibacterial properties, specifically against the pathogenic microorganism *Streptococcus pyogenes*. Quercetin and myricetin were found in all samples in large concentrations. The separation of pollen loads from a multifloral sample demonstrated that *E. californica* pollen loads are responsible for antibacterial activity. This sample also showed a high concentration of quercetin (304.8 mg/100 g of bee pollen). Based on the present results, honeybee pollen from the V Region of Chile has been found to exhibit antioxidant and antimicrobial activities. Furthermore, it is proposed to use quercetin as a quality indicator for honeybee pollen from this region of Chile. These results should help establish better quality control criteria for Chilean honeybee pollen and its potential use as a functional ingredient.



Pollen loads are produced by *Apis mellifera* L. as a result of collecting hundreds of pollen grains and using their salivary secretions to agglutinate them. These pollen loads are then transported to the hive on the legs of bees where they function as a source of proteins and other nutrients that help maintain the colony.[1,2] Honeybee pollen is collected by beekeepers by setting traps at the hive entrance so that pollen loads are retained at the moment the insects enter the hives. In each trip, a honeybee visits mainly one type of flower and the pollen loads present a characteristic color depending on the chemical composition of the plant pollen.[3] The chemical composition varies significantly according to the botanical and geographical origin of the pollen grains. It is possible to identify the botanical origin by morphological and structural analysis of the grains under a microscope[4−6] or by using flavonoid and phenolic profiles.[4] Research groups have outlined several potential bioactive roles for honeybee pollen including antioxidant, immunomodulatory, cardioprotective, and antimicrobial activities.[5,7−9] These activities are mainly attributed to phenolic compounds such as flavonoids.[10,11] Currently, these types of compounds are widely valued, and due to their medicinal properties, interest in bee pollen from the pharmaceutical, food, and cosmetic industries is ever increasing.[12,13] Many studies have been done in plants,[14−16] but few studies have been made on pollen loads produced by Chilean hives located in natural plant communities.[17,18] The Chilean flora has a high degree of endemism at 50%. Furthermore, the survival of some species, particularly the endemic species and the species restricted to the V Region, are threatened by indiscriminate extraction for medicinal uses. Beehives are usually located in a "matorral", which is a shrubby sclerophyllous type of vegetation that covers the slopes of the coastal range.[19] Dominant species such as *Quillaja saponaria* Molina (Quillajaceae), *Colliguaja odorifera* Molina (Euphorbiaceae), *Cryptocarya alba* (Molina) Looser (Lauraceae), and *Acacia caven* Molina (Fabaceae) are used by honeybees as pollen sources.

In order to evaluate some characteristics of honeybee pollen from the V Region of Chile, the objective of the present study was to investigate (i) the botanical origin; (ii) the phenolic profile; (iii) the antioxidant capacity; and (vi) the antimicrobial activity of the samples assayed against *Streptococcus*

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pyogenes, *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa*.

RESULTS AND DISCUSSION

The potential beneficial value of phenolic compounds in preventing or decreasing oxidative damage and nitrosative stress caused by conditions such as cancer, cardiovascular diseases, atherosclerosis, neurological disorders, hypertension, and diabetes mellitus has been widely recognized in the last few decades.[20,21] Honeybee pollen contains a variety of chemical compounds, but phenolic compounds are considered the main bioactive constituents.[22] The botanical contribution, storage time, nutritional status of the plant, and environmental conditions during the pollen collection phase also influence the chemical composition.[23,24] Botanical origin describes the presence of different plant sources used by bees to produce bee pollen. This description enables their classification as native/non-native/mixed and monofloral/bifloral/multifloral bee pollen, according to the Chilean Norm (NCh 3255, 2011).[25] Botanical origin and classification of the bee pollen samples was performed using palynological analysis (Tables S1, S2, and S3, Supporting Information). Floral species found in the samples are closely related with the geographic location of hives. The analyzed samples were derived predominantly from non-native floral species and frequently from only one type of species. The majority of samples analyzed corresponded to non-native monofloral followed by non-native multifloral and non-native bifloral. Among the non-native samples analyzed, the plant species *Brassica rapa* and *Eschscholzia californica* predominated. Thus, *B. rapa* is the most abundant species and is a source that is appreciated by bees for pollen collection, while *E. californica* is a cosmopolitan species introduced from California that is distributed widely in this region. The predominant native species are *Colliguaja odorifera* (colliguay), *Cryptocarya alba* (peumo), and *Acacia caven* (espinillo).

Table 1 shows the average values of total phenol, flavonoids, FRAP, ORAC-FL, and antimicrobial activity against *S. pyogenes* (expressed as diameter of the growth inhibition zone) in bee pollen extracts (BPEs) from the V Region of Chile collected in 2016, 2017, and 2018. The highest total phenolic and flavonoid contents were from 2016 samples, and antioxidant capacity was greater in 2017 and 2018 samples. The analysis of these results indicated that total phenolic content [$F(2,27) = 5.44$; $p < 0.05$] and flavonoid content [$F(2,27) = 7.12$; $p < 0.05$] was significantly higher in the year 2016 compared to 2017 and 2018 (Table 1). On the other hand the antioxidant capacity, evaluated by FRAP [$F(2,27) = 8.22$; $p < 0.05$] and ORAC-FL [$F(2,27) = 7.68$; $p < 0.05$], was significant lower in 2016 than those of 2017 and 2018. Table 1 shows antimicrobial activity against *S. pyogenes* expressed as the diameter of the growth inhibition zone. No differences were found in the growth inhibition zone between bee pollens collected in 2016, 2017, and 2018. The average diameter of the growth inhibition zone for all samples analyzed was 14 mm. Also evaluated was antibacterial activity of bee pollen samples against *E. coli*, *P. aeruginosa*, and *S. aureus*, but the bee pollen extracts investigated did not produce inhibitory zones against these human pathogenic microorganisms.

A correlation was not found between the phenolic compounds present and antioxidant activity (ORAC-FL and FRAP) or antibacterial activity against *E. coli*. The correlation between phenolic content and antioxidant activity is controversial for bee pollen.[26] Furthermore, it has been suggested in other studies that specific phenolic composition is more relevant than total phenolic content.[26−28] It should be noted that the action of a natural antioxidant depends on several factors, such as the reaction mechanism, experimental conditions, and matrix heterogeneity.[23,27] It may be highlighted that of the 29 samples analyzed, *B. rapa* and *E. californica* predominated, which indicates the importance of these introduced species as melliferous plants. The samples with the highest total phenolic content were samples 7 from the year 2016 (*B. rapa* 36.09%/*Rubus ulmifolius* 33.33%) and 12 from the year 2017 (*E. californica* 33.91%/*B. rapa* 28.33%). The samples with the highest flavonoid content were 7 and 2 (*C. alba* 45.94%/*E. californica* 41.28%) (Tables S1 and S2, Supporting Information). The sample that showed the highest inhibition zone was a pollen extract collected in 2018 (27 ± 4 mm) that corresponded to a multifloral non-native sample composed of 41.45% *E. californica* and 34.21% *B. rapa* (sample 29) (Table S3, Supporting Information).

The minimum inhibitory concentration (MIC) of BPE for *S. pyogenes* is shown in Figure 1. The MIC values were determined as the lowest concentrations of the extracts that inhibited the growth of the microorganism tested. The MIC values of all bee pollen loads studied ranged from 0.78 to 6.25 mg/mL. It was observed that bee pollen from three different years showed the same average diameter of inhibition for *S.*

Table 1. Average Values of Total Phenol, Flavonoids, FRAP, ORAC-FL, and Antimicrobial Activity against *Streptococcus pyogenes* (Expressed as the Diameter of the Growth Inhibition Zone) in Bee Pollen Extracts from the V Region of Chile Collected in 2016, 2017, and 2018[a]

sample	2016	2017	2018
	total phenols (mg GAE/100 g bee pollen)		
mean	1074[b]	840	813
v_{max}	1424	1249	1035
v_{min}	698	504	580
	flavonoids (mg QE/100 g bee pollen)		
mean	250[b]	156	189
v_{max}	375	232	273
v_{min}	180	98	127
	FRAP (μmol TE/g bee pollen)		
mean	66[b]	93	86
v_{max}	81	116	107
v_{min}	42	60	56
	ORAC-FL (μmol TE/g bee pollen)		
mean	213[b]	324	337
v_{max}	291	477	421
v_{min}	167	178	231
	antimicrobial activity *S. pyogenes* (mm)		
mean	14	14	14
v_{max}	17	17	27
v_{min}	13	10	11

[a]Values represent the mean for 29 samples (each sample was analyzed by three independent experiments performed in triplicate; v_{max} = maximum value; v_{min} = minimum value. [b]$p < 0.05$ compared to other groups (years) (Tukey's multiple-range test). The total phenol results are expressed as mg gallic acid equivalents (GAE)/100 g fresh bee pollen; flavonoids are expressed as mg quercetin equivalents (QE)/100 g fresh bee pollen; TRAP as μmol Trolox equivalents (TE)/g fresh bee pollen, and ORAC-FL as μmol Trolox equivalents/g fresh bee pollen; antimicrobial activity is expressed as the diameter of the growth inhibition zone. The standard deviations of these results were lower than 5%.

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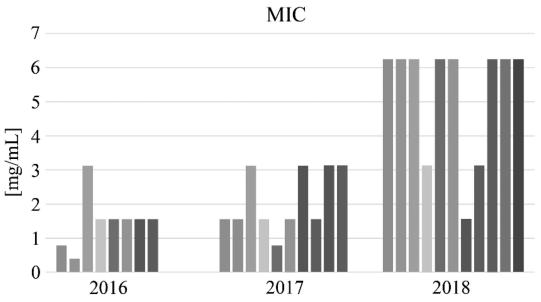

Figure 1. Minimum inhibitory concentrations (MIC) of honeybee pollen collected in 2016, 2017, and 2018 against *Streptococcus pyogenes*. Results are given in mg/mL. The standard deviation of these results was lower than 5%. The MIC for penicillin was 0.04 mg/mL.

pyogenes, but not the same MIC values. Nonetheless, all of them were capable of controlling the growth of the *S. pyogenes* bacteria. It has been reported that some flavonoids (quercetin, myricetin, galangin, apigenin) and hydroxycinnamic acids present in honeybee pollen have quite potent antimicrobial properties against some pathogenic bacteria. The effects of phenolic compounds on bacteria are connected with the disruption of their metabolism. The mechanism consists of forming complexes with bacterial cell walls, causing structural and functional damage.[29−32] It has also been observed that Gram-positive bacteria were more susceptible to pollen action than Gram-negative bacteria.[28]

To gain more insights into the chemical characterization of the bee pollen samples investigated, the concentrations of the most representative phenols (cinnamic acids, flavonols, flavone, and flavanone) were determined; the results are depicted in Tables 2 and 3. Five compounds were found in all pollen samples, four phenolic compounds (syringic acid, *p*-coumaric acid, myricetin, and quercetin) and abscisic acid. Cinnamic acid, apigenin, and rhamnetin were also present in most samples in considerable amounts. Sinapic acid, ferulic acid, catechin, kaempferol, and rhamnetin were also present in some BPEs in variable amounts. The extracts showed a considerable content of quercetin (average 82.7 mg/100 g fresh bee pollen) and myricetin (average 169.4 mg/100 g fresh bee pollen) in their compositions. Analysis of the free flavonoid aglycones is considered an important parameter for defining the quality of bee pollen.[33] Most pollen flavonoids exist in the form of glycosides, especially *O*-glycosides. In the case of honeybee-collected pollen, hypopharyngeal gland secretions from the honeybee along with the presence of α/β glucosidase hydrolytic enzymes accompany the pollen pellets, which cause partial enzymatic hydrolysis of glycosides

Table 2. Phenolic Acids of Honeybee Pollen Extracts (BPEs) as Determined by HPLC-DAD[a]

	mg/100 g bee pollen					
sample	syringic acid	cumaric acid	sinapic acid	ferulic acid	cinnamic acid	abscisic acid
			2016			
1	28.8 ± 0.30	2.21 ± 0.09	0	0	0.52 ± 0.12	16.06 ± 0.12
2	15.39 ± 5.03	1.31 ± 0.05	16.55 ± 2.63	3.93 ± 0.89	0.39 ± 0.04	9.29 ± 0.20
3	21.90 ± 0.36	3.25 ± 0.21	0	0	0	22.82 ± 0.15
4	11.20 ± 0.20	1.61 ± 0.05	10.93 ± 0.08	9.97 ± 0.15	56.17 ± 0.50	15.98 ± 0.24
5	19.65 ± 0.63	2.62 ± 0.09	4.63 ± 0.79	2.01 ± 0.87	19.38 ± 0.08	24.14 ± 0.28
6	17.34 ± 0.10	1.15 ± 0.03	0	0	28.37 ± 0.13	27.49 ± 0.55
7	14.73 ± 4.06	0.84 ± 0.18	0	9.84 ± 0.15	11.48 ± 3.33	17.18 ± 0.19
8	12.81 ± 0.06	1.94 ± 0.05	10.24 ± 0.96	12.91 ± 0.61	41.81 ± 0.24	23.08 ± 0.38
			2017			
9	4.95 ± 0.12	1.15 ± 0.3	0	11.23 ± 0.66	27.71 ± 0.04	14.58 ± 0.31
10	16.88 ± 0.11	4.21 ± 0.58	0	0	5.97 ± 0.05	7.89 ± 0.28
11	10.01 ± 0.13	1.06 ± 0.22	0	0	0	7.93 ± 0.13
12	4.30 ± 0.06	0.96 ± 0.26	0	7.53 ± 1.87	7.63 ± 0.07	5.73 ± 0.38
13	0.17 ± 0.02	1.15 ± 0.8	0	4.98 ± 0.30	0	11.06 ± 0.20
14	31.54 ± 0.06	7.63 ± 0.57	0	0	14.85 ± 0.05	13.88 ± 0.26
15	2.04 ± 1.09	0.19 ± 0.05	0	4.63 ± 0.66	0	27.13 ± 0.33
16	12.98 ± 0.10	2.07 ± 0.40	0	0	27.11 ± 0.13	9.34 ± 0.08
17	0.64 ± 0.05	5.06 ± 2.14	126.82 ± 0.17	0	13.38 ± 0.39	14.48 ± 0.74
18	0.89 ± 0.08	3.06 ± 0.05	87.67 ± 0.63	15.01 ± 1.41	174.98 ± 0.45	97.44 ± 0.61
			2018			
19	12.02 ± 0.15	2.21 ± 0.03	0	0	0	28.59 ± 0.20
20	17.83 ± 0.52	2.38 ± 0.08	0	14.01 ± 0.28	26.78 ± 0.057	19.38 ± 0.08
21	5.74 ± 0.26	1.37 ± 0.19	0	6.05 ± 0.39	0	5.90 ± 0.08
22	8.49 ± 0.06	1.69 ± 0.09	0	0	0	13.92 ± 0.20
23	8.15 ± 0.06	1.26 ± 0.19	0	10.05 ± 1.30	20.20 ± 0.43	11.45 ± 0.08
24	20.87 ± 0.21	6.48 ± 1.87	1.41 ± 0.08	0	27.36 ± 0.17	21.80 ± 0.46
25	2.64 ± 0.11	1.53 ± 0.66	0	0	19.60 ± 0.05	22.55 ± 0.08
26	11.46 ± 2.55	0.93 ± 0.31	0	0	29.5 ± 0.I5	18.14 ± 0.40
27	4.06 ± 0.07	0.54 ± 0.05	0	0	0	10.26 ± 1.00
28	28.78 ± 0.07	1.93 ± 0.06	0	5.54 ± 0.54	9.45 ± 0.05	9.03 ± 0.65
29	24.53 ± 0.15	4.79 ± 0.09	31.02 ± 0.23	10.09 ± 0.10	0	25.42 ± 0.20

[a]Data are expressed as mg/100 g fresh bee pollen for BPE, and the values represent the means ± SD (n = 3).

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Table 3. Flavonoids of Honeybee Pollen Extracts (BPEs) as Determined by HPLC-DAD[a]

	mg/100 g bee pollen						
sample	catechin	myricetin	quercetin	apigenin	kaempferol	narigenin	rhamnetin
				2016			
1	8.75 ± 0.23	49.56 ± 0.18	94.50 ± 1.66	52.25 ± 0.24	0	0	72.82 ± 0.48
2	4.21 ± 0.15	20.99 ± 1.52	53.50 ± 0.27	4.14 ± 0.20	0	0	175.89 ± 0.64
3	0	51.31 ± 0.24	76.06 ± 1.45	11.02 ± 0.47	0	0	173.98 ± 11.12
4	44.9 ± 0.3	78.40 ± 0.31	0	1.80 ± 0.41	0	0	24.30 ± 0.51
5	0	58.25 ± 0.24	82.86 ± 0.98	28.85 ± 0.16	26.33 ± 1.08	3.09 ± 1.86	14.92 ± 0.81
6	0	115.61 ± 3.2	51.83 ± 0.28	14.45 ± 0.0	0	0	71.70 ± 2.11
7	0	65.83 ± 14.05	21.71 ± 2.01	31.86 ± 7.71	14.07 ± 3.56	0	0
8	0	59.05 ± 0.36	73.19 ± 0.86	30.74 ± 0.56	14.98 ± 1.79	0	0
				2017			
9	0	246.59 ± 0.51	115.71 ± 0.61	17.82 ± 0.27	0	0	15.16 ± 2.39
10	8.61 ± 0.08	115.56 ± 0.24	57.73 ± 0.40	1.04 ± 0.15	0	0	56.64 ± 4.70
11	2.23 ± 0.93	465.61 ± 0.16	5.74 ± 0.21	2.48 ± 0.78	0	0	0
12	0	224.06 ± 0.55	24.58 ± 0.46	12.29 ± 0.27	0	0	8.8 ± 0.4
13	0	637.74 ± 2.13	94.30 ± 0.46	1.04 ± 0.31	0	0	0
14	15.87 ± 0.22	22.32 ± 2.19	128.75 ± 1.21	3.74 ± 0.39	0	0	67.76 ± 0.95
15	0	680.60 ± 1.59	111.38 ± 0.57	0.81 ± 0.14	0	0	15.62 ± 0.11
16	0	303.99 ± 0.64	105.28 ± 1.60	126.20 ± 0.34	0	0	56.78 ± 2.49
17	408.8	6.47 ± 0.24	21.66 ± 0.17	0	9.66 ± 1.51	3.49 ± 0.31	0
18	0	6.25 ± 0.18	110.77 ± 0.52	0	0	43.38 ± 0.79	18.24 ± 5.92
				2018			
19	0	38.80 ± 0.42	22.97 ± 0.54	12.15 ± 1.41	0	4.49 ± 1.53	15.65 ± 0.88
20	0	70.92 ± 0.57	137.11 ± 1.06	64.13 ± 0.36	0	0	15.50 ± 0.83
21	0	316.92 ± 0.57	75.36 ± 0.63	47.39 ± 0.41	0	0	0
22	6.29 ± 0.08	363.89 ± 0.40	120.64 ± 0.31	51.71 ± 0.14	0	0	44.01 ± 3.1
23	0	301.98 ± 0.57	99.53 ± 0.86	5.8 ± 0.23	0	0	9.07 ± 2.97
24	0	43.78 ± 0.46	134.24 ± 3.03	135.20 ± 0.39	28.72 ± 1.44	0	10.23 ± 1.46
25	1.64 ± 0.55	354.45 ± 0.89	121.14 ± 1.13	28.89 ± 0.48	0	0	26.54 ± 1.97
26	0	52.16 ± 0.16	145.98 ± 1.18	4.59 ± 0.81	24.95 ± 2.28	0	3.53 ± 0.09
27	2.31 ± 0.37	133.10 ± 1.39	84.22 ± 20.77	16.83 ± 0.82	0	3.95 ± 1.31	275.63 ± 12.62
28	0	49.30 ± 0.22	175.22 ± 0.96	57.59 ± 0.28	37.78 ± 0.61	0	4.96 ± 1.08
29	8.61 ± 0.17	14.04 ± 0.33	133.68 ± 1.10	55.63 ± 0.36	0	0	53.66 ± 0.09

[a]Data are expressed as mg/100 g fresh bee pollen for BPE, and the values represent the means ± SD (n = 3).

to the free state aglycone.[10,33−35] The presence of rutin was not identified in any Chilean sample studied, which suggests the hydrolysis of this glucoside to its aglycone had occurred. Accordingly, quercetin was found in large concentrations. Thus, the presence of quercetin and myricetin flavonoids may be considered as good markers for determining the quality of Chilean honeybee pollen.

To further understand the influence of botanical origin on the antibacterial properties of bee pollen, the predominant pollen loads of sample 29 were separated using color as an indicator. This sample showed the highest inhibition zone against *S. pyogenes* (27 ± 4 mm) and corresponds to a multifloral non-native sample composed of 41.45% *E. californica* and 34.21% *B. rapa*. An agar-well diffusion analysis was performed using *E. californica* and *B. rapa* bee pollen extracts obtained in the same conditions as previously discussed in the Experimental Section. The results showed that the *E. californica* bee pollen loads are 100% responsible for the antibacterial effect against *S. pyogenes* (27 ± 3 mm) (Figure S1, Supporting Information). The total phenolic content determined by FC assay in *E. californica* bee pollen extract (524 ± 13 mg equivalents GAE)/100 g bee pollen) was almost 4 times higher than *B. rapa* (132 ± 11 mg equivalents GAE)/100 g bee pollen), and the flavonoid content was 3 times higher (187 ± 14 mg equivalents QE/100 g bee pollen and 62 ± 5 mg equivalents QE/100 g bee pollen, respectively, than *E. californica* and *B. rapa*). HPLC-DAD analysis of these extracts showed that *E. californica* bee pollen extract contained 304.80 mg of quercetin/100 g of bee pollen and 47.36 mg of myricetin/100 g of bee pollen, whereas *B. rapa* extract contained 2.78 mg/100 g and 5.3 mg/100 g, respectively. These results indicate that the antibacterial activity could be related to the abundant presence of these flavonoids in the *E. californica* pollen.

In summary, the present study has determined the antioxidant and antibacterial properties, specifically against *S. pyogenes*, of honeybee pollen produced in the V Region of Chile during the years 2016, 2017, and 2018. The chemical composition and biological properties of honeybee pollen are particularly dependent on plant origin, together with other factors such as climatic conditions, soil type, beekeeper activities, and the different processes or storage treatments in commercial production.[23] Is important to point out that the pollen studied in the present investigation was frozen fresh, vacuum packed, and stored at −20 °C. These conditions helped maintain the biological properties found. Currently, there are only a few countries such as Brazil, Argentina, Switzerland, Spain, and Mexico that have established official quality standards and that have recognized honeybee pollen as a food product or food additive.[36,37] These minimal require-

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ments for honeybee pollen are related to water content, the temperature used in the drying process, and other nutritional factors such as protein or vitamin content. Chemical analyses such as the FC method for determining phenolic compounds and aluminum chloride for quantifying flavonoids are used as parameters to guarantee the quality for some honeybee products, such as propolis.[38] It is considered appropriate to guarantee the biological effects related to the antioxidant or antimicrobial potential and that simple analytical tests such as FRAP or antimicrobial tests should be incorporated when determining quality requirements. Functional ingredients are playing an ever more important role in health maintenance. However, better quality control standards and criteria need to be established before incorporating these honeybee products in food products. On the basis of the present results, free flavonoid aglycons seem to be related to the quality of the honeybee pollen, and minimum values of these compounds could be used as objective specifications for acceptance of the product. Specifically, we suggest using quercetin as an indicator for beekeeping pollen for this region of Chile.

EXPERIMENTAL SECTION

General Experimental Procedures. 6-Hydroxy-2,5,8-tetramethylchroman-2-carboxylic acid (Trolox), fluorescein disodium salt (FL), 2,2′-azo-bis(2-amidinopropane) dihydrochloride (AAPH), 2,4,6-tri(2-pyridyl)-*s*-triazine (TPTZ), ferric chloride, and all standards of compounds studied were purchased from Sigma-Aldrich (St. Louis, MO, USA). Folin-Ciocalteu's phenol reagent, aluminum chloride, disodium hydrogen phosphate dihydrate, and sodium phosphate monobasic reagent were supplied by Merck (Darmstadt, Germany). All solvents were High-Performance Liquid Chromatography (HPLC) grade. Water was purified in a Milli-Q system (Synergy, Millipore, Darmstadt, Germany).

Honeybee Pollen Samples. Twenty-nine samples from the V Region of Chile (GPS coordinates 33°3′47″ S, 71°38′22″ W) were provided as vacuum packed when fresh and were frozen at −20 °C by associated beekeepers. The samples were collected during the dry seasons of 2016, 2017, and 2018. The determination of botanical origin was performed using the palynological analysis method described in Chilean Regulation (NCh3255, 2011).[25] Five grams of each type of bee pollen corbiculae were separated by color, and each fraction was weighed. After this, one corbicula of each type of bee pollen sample was crushed with alcohol to disperse the pollen grains. Several drops of red calberla were used to stain the grains to allow observation under a light microscope. To determine botanical origin, specific literature[39,40] and the botanical bee pollen catalog at the Laboratory of Botany (Department of Plant Sciences, Faculty of Agronomy and Forest Engineering, Pontificia Universidad Católica de Chile, Santiago, Chile) were consulted.

Honeybee Pollen Extracts. One gram of fresh honeybee pollen was mixed with 10 mL of analytical grade absolute ethanol and ultrasonicated (Elmasonic S 10 H ELMA) for 10 min. The mixture was centrifuged at 3130*g* for 5 min and filtered using Whatman No.1 paper. This procedure was repeated in triplicate for each sample, and the collected extracts were combined to a final volume of 50 mL (1 g/50 mL). BPEs were stored at −80 °C in the dark until use.

Total Phenolic Determination. Total phenolic analyses were carried out on a Cytation 5 multimode microplate reader from BioTek Instruments, Inc. (Winooski, VT, USA), using 96-well polystyrene microplates. Then, 125 μL of Folin-Ciocalteu reagent, 25 μL of diluted BPE (1:100), and 100 μL of Na_2CO_3 at 7.5% were added to each cell. The samples were incubated for 30 min at 37 °C in a microplate reader and measured at 765 nm.[41] Quantification was done by linear regression from a calibration curve constructed from gallic acid (10 to 180 mg/L). Results were expressed as milligrams of gallic acid equivalents (GAE) per 100 g of bee pollen (mg GAE/100 g). Values are reported as means ± standard deviations (SD) of three independent determinations.

Flavonoid Determination. Methanol (105 μL) was added to each of the 96-well polystyrene microplates, followed by 20 μL of diluted BPE (1:20) and 125 μL of 2% $AlCl_3$ solution. After 60 min at room temperature, the absorbance was measured at 420 nm using a Cytation 5 multimode microplate reader from BioTek Instruments, Inc. Total flavonoid content was calculated as milligrams of quercetin equivalents (QE) per 100 g of bee pollen (mg QE/100 g) from a calibration curve (20 to 180 mg/L). Values are reported as means ± SD of three independent determinations.

Ferric Reducing Antioxidant Potential (FRAP). The ferric reducing power of the pollen extracts was determined as previously described by Furlan.[42] The working FRAP solution was prepared daily by mixing 10 parts of acetate buffer (0.3 M pH 3.6), one part of 10 mM TPTZ (Sigma), and one part of 20 mM ferric chloride. Aliquots of 270 μL of FRAP solution were mixed with 30 μL of diluted BPE (1:100). The reaction mixtures were incubated for 30 min at 37 °C, and the absorbance was measured at 594 nm using a Cytation 5 multimode microplate reader from BioTek Instruments, Inc. As positive controls, an ethanol solution and Trolox (5−30 μM) were used. The results are expressed as μmol Trolox equivalents per g of bee pollen (μmol TE/g). Values are reported as means ± SD of three independent determinations.

ORAC (Oxygen Radical Absorbance Capacity). The antioxidant capacity of BPEs was measured by using the ORAC-fluorescein (ORAC-FL) assay conducted on the basis of a report by Ou et al.[43] and adapted to the use of a fluorescent microplate reader (Cytation 5 from BioTek Instruments Inc.). The fluorescein consumption was assessed by the decrease in fluorescence intensity of the sample (excitation 493 nm; emission 515 nm). AAPH was used as the peroxyl ion generator and μM Trolox as a standard (2−10 μM). The results are expressed as μmol Trolox equivalents per 100 g of bee pollen (μmol TE/100 g). Values are reported as means ± SD of three independent determinations.

HPLC-DAD Analysis. BPE samples were analyzed with a Hitachi Chromaster 5000 series HPLC instrument equipped with an autosampler and a photodiode array detector (Hitachi, Tokyo, Japan). The HPLC system was controlled by a Chromaster system manager V1.2. The BPEs were separated using a mobile phase mixture of (A) methanol, (B) acetonitrile, and (C) 0.1% aqueous formic acid. The composition of the mobile phase mixture varied by employing the following HPLC stepwise gradient program: 0−10 min 20% B, 80% C; 10.1−40 min 7.5% A, 25% B, 67.5% C; 40.1−50 min 15% A, 25% B, 60% C; 50.1−65 min 15% A, 45% B, 40% C, and returned to starting conditions during the following 15 min. The column used was a 250 × 4.6 mm i.d. Purospher STAR RP-18 end-capped with a guard column of the same type. Other chromatographic conditions were the following: a flow rate of 0.8 mL/min and oven column set at 35 °C. The absorbance of 10 μL of eluate was monitored with a diode array detector (DAD) set in the 210−550 nm range, and the chromatograms were integrated for all standards and BPE samples at 290 nm. Phenolic compound detection was performed by comparison of the UV−vis spectrum and retention times exhibited by the standards. For quantification, a multistandard mixture was performed in equal concentrations of each phenol (range 5−250 μM of each component) to obtain calibration curves of all standards studied. Detection limits of standards oscillated between 2 and 133 μg/g in honeybee pollen. No differences were detected in the retention times of the standards alone or in combination in the multistandard matrix. All analyses were performed in triplicate for standards and BPE samples.

Collection of the Chromatographic Peaks That Arise from the Analysis of BPEs. The peaks that could not be identified by coelution experiments using commercial standards in the HPLC-DAD analysis were collected using an Agilent 1200 series (Agilent Technologies, Santa Clara, CA, USA) HPLC instrument equipped with an autosampler, photodiode array detector, and automated fraction collector. The elution of the BPE samples was performed using the same stepwise gradient elution system described above for the HPLC-DAD analysis. The HPLC system was controlled by

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J. Nat. Prod. XXXX, XXX, XXX−XXX

ChemStation software (Agilent). The chemical identity of the molecules present in the re-collected peaks was elucidated by an ESIMS/MS procedure, as described below.

HPLC-ESIMS/MS. The re-collected peaks were analyzed using ESIMS/MS. MS/MS spectra were acquired using a triple quadrupole spectrometer employing electrospray ionization (ESIMS/MS). HPLC-ESIMS/MS analysis of BPEs was conducted under the same chromatographic conditions as in the HPLC-DAD analysis and was performed on an Ekspert UltraLC 100 coupled to a Triple Quad 4500 tandem mass spectrometer equipped with a turbo-spray ionization source (AB Sciex, Ontario, Canada). The mass spectrometer was operated in the negative-ionization mode, and data were acquired in the multiple reaction-monitoring mode. The source temperature was 650 °C, and the ESI voltage and entrance potential were set at −4.5 kV and −10 V, respectively. The gas flow rates were as follows: ion spray gas, 50 psi; heater gas, 40 psi; curtain gas, 20 psi; and collision gas, 7 psi. The decluttering potential, collision energy, and collision exit potential for each compound were each adjusted in tandem with the MS stage to determine the optimum parameters at which the maximum amount of signal information could be obtained. In addition, a scanning rate of 150 ms was used. The ESIMS/MS system was controlled by Analyst 1.6.2 software (AB Sciex, Darmstadt, Germany).

Antibacterial Activity. The antibacterial activity of BPE was evaluated by the growth inhibition diameter against *Escherichia coli* ATCC-25922, *Staphylococcus aureus* ATCC-25923, *Pseudomonas aeruginosa* ATCC-27853, and *Streptococcus pyogenes* I.S.P. 364-00 (supplied by the Chilean Public Health Institutes). Diameter of growth inhibition was determined using the standard of CLSI (2006): bacterial strains were inoculated on Mueller Hinton agar for 24 h at 37 °C. After that time, colonies were selected and diluted in saline solution to a concentration of 0.5 McFarland (1.5×108, Becton Dickinsson Company, USA). Strain colonies were swabbed on the agar culture, and holes of 6 mm in diameter were made. A 100 μL amount of the pollen extract (1:20; ETOH 50%) was then deposited in each hole. Cultures were incubated between 18 and 24 h at 37 °C. Each inhibition growth diameter was measured and compared with tetracycline, ampicillin, penicillin, and chloramphenicol as antibiotic controls.

Broth Microdilution Method. Minimum inhibitory concentration against *S. pyogenes* was determined using a standard microdilution technique. Concentrations of bacteria were determined by using 0.5 McFarland diluted in 5 mL of physiological serum, and from this solution 10 μL was extracted and diluted in 10 mL of physiological serum. This allowed a concentration of approximately 10^3 UFC. Penicillin and chloramphenicol controls were used. One of these contained bee pollen extract and growth medium, and other control contained bacteria. MIC values were taken as the lowest concentration that produced no visible bacterial growth (no turbidity) when compared with controls after 24 h of incubation at 37 °C.

Statistical Analysis. Data were analyzed by one-way analysis of variance (ANOVA) followed by the Duncan multiple range test when the F value was significant. Linear regression analysis was also used to test dose-dependent effects. All analyses were performed using Origin Pro 8 software. A value of $p < 0.05$ was considered to be significant.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.8b00945.

Botanical origin and classification of samples as native/non-native/mixed and monofloral/bifloral/multifloral bee pollen according to the Chilean Norm (NCh 3255, 2011); agar well diffusion assay for the determination of antibacterial activity against *S. pyogenes* of bee pollen extract of *E. californica* and *B. rapa*; multistandard chromatogram for pure compounds used in the HPLC-DAD identification; HPLC-ESIMS selected ion chromatogram and registered ESIMS spectra (PDF)

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Notes

The authors declare no competing financial interest.

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■ DEDICATION

Dedicated to Dr. Rachel Mata, National Autonomous University of Mexico, Mexico City, Mexico, and Dr. Barbara N. Timmermann, University of Kansas, for their pioneering work on bioactive natural products.

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