The Composition, Active Components and Bacteriostatic Activity of Propolis in Dietetics

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The composition and bacteriostatic activities of fifteen propolis samples from various botanic and geographic origins were determined. Twenty-six phenolic components were identified by high-performance liquid chromatography with array photodiode detector. Acacetin and apigenin were most abundant. Pinocembrin, quercetin, rutin and vanillin were present in lesser quantities. Variance analysis shows significant differences ($P \leq 0.05$) in the contents of phenols, flavonoids and active components. The minimum inhibitory concentration of propolis is about 53 times higher than that reported for tetracycline against *Bacillus subtilis* and *Staphylococcus aureus*, and about 400 times higher against *Escherichia coli*.

KEY WORDS: Acacetin, apigenin, bacteriostatic and antioxidant activities, benzoic acid derivatives, cinnamic acid derivatives, color, composition, flavonoids, pinocembrin, polyphenolic components, propolis, quercetin, rutin, steroids.

Propolis is a sticky, gummy, resinous substance collected by bees from tree exudates and secretions (1,2). Bees significantly modify the original propolis composition to produce a cement-like substance that can be considered of both plant and animal origin. β-Glucosidase secreted by the bee during propolis collection and processing hydrolyzes flavonoid heterosides into aglicones, which improves pharmacological properties of the product.

The most important botanical sources for obtaining propolis are poplars (*Populus* spp.), birches (*Betula* spp.), willows (*Salix* spp.), chestnut tree (*Aesculus hippocastanum* L.), elms (*Ulmus* spp.), pine trees (*Pinus* spp.), oaks (*Quercus* spp.), spruces (*Picea* spp.) and ashes (*Fraxinus* spp.) (3,4). Propolis contains mainly resins and balsams (5), and phenolic aldehydes and polyphenolic components (derivatives of cinnamic and benzoic acids) account for half of its composition. Flavonoids (flavonoles, flavones and flavanones) contained in the propolis may be responsible for the pharmacological and antioxidant activities (6).

Presently, most propolis is consumed with bee honey and pollen. Products manufactured with propolis have increased bacteriostatic activity and improved pharmacological properties (7). Because of these biological properties, propolis is used in foods and in dietetics (8,9).

This paper presents and discusses the physicochemical results and bacteriostatic activities determined from propolis obtained from diverse botanical origins. Propolis minimum inhibitory concentrations are compared with tetracycline against *Bacillus subtilis*, *Staphylococcus aureus* and *Escherichia coli*. The active components are identified, and their use as quality parameters is suggested.

EXPERIMENTAL PROCEDURES

Materials. Fifteen propolis samples, obtained from various botanic plants from diverse geographical origins, were studied in their natural form, either as a powder or as an alcohol extract (Table 1). Samples were stored in the dark at room temperature. Samples were either homogenized or pulverized and analyzed in duplicate or triplicate.

TABLE 1

Propolis Samples

Sample number	Geographical origin	Presentation
1	Brazil	Natural
2	Uruguay	Alcohol extract
3	Uruguay	Powder
4	Uruguay	Powder
5	China	Natural
6	China	Natural
7	China	Natural
8	Uruguay	Alcohol extract
9	Uruguay	Powder
10	Uruguay	Alcohol extract
11	China	Natural
12	China	Natural
13	China	Natural
14	China	Natural
15	China	Natural

Analytical procedures. The water content of each propolis sample was determined by drying for 2 h to constant weight in a conventional kiln at 105°C. The wax content was determined by extracting with petroleum ether (40–60°C) in a Soxhlet extractor for 3 h. Mineral salt of each propolis sample was determined by incineration at 500–550°C. Heavy metals were determined by dissolution with 2 N HCl, and Cd, Cr and Pb were quantitated by atomic absorption spectrophotometry by the method of Barberá *et al.* (10). Resins and total balsams were extracted for 30 min with methanol at room temperature. Polyphenolic components (total phenols and polymerized phenols) were determined with a visible (VIS) spectrophotometric method (galangin and rutin used as external patterns) by the method of Marigo (11) and Lebreton *et al.* (12). Phenolic component profiles were determined by high-performance liquid chromatography (HPLC) with a photodiode array detector, in accordance with Villeneuve (13) and Amiot *et al.* (14) methods. Steroids were determined gravimetrically by digitonine-precipitation according to the method of the International Union of Pure and Applied Chemistry (15). Column-chromatographic separation and quantitation were conducted with a spectrophotometric method by Bourzeix *et al.* (16). Essential oils were determined by steam distillation according to the method of Godefroot *et al.* (17). Antioxidant power was determined by decoloration time(s) by using the potassium permanganate method of Vajonina and Dushkova (18). Carbohydrates were determined based on the analyses of their oxime trimethylsilyl derivatives by the gas chromatographic method of Serra Bonvehí and Bosch Callis (19).

Bacteriostatic activities. The following bacteria were maintained and cultivated for bacteriostatic activity tests: (i) *B. subtilis CIP 155* was cultivated for 7 days at 37°C on Antibiotic Medium 32, which is the same as Medium

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1, except for the additional ingredient of 0.3 g $MnSO_4$ (20) (Difco 0243; Difco Laboratories, Detroit, MI). The final adjusted suspension was adjusted to 10% transmittance at 580 nm with a serum blank; (ii) *E. coli CECT 434* and *S. aureus CECT 435* were cultivated on Antibiotic Medium 1 (Difco 0263) for 24 h at 37°C. The final suspension was adjusted to 45% transmittance at 580 nm.

Growth measurement on solid medium. Five different concentrations of each sample were introduced on petri dishes containing Antibiotic Medium 1. Each petri dish was inoculated with one drop of the suspension of test microorganism. The pattern test was performed with tetracycline ethanolic solution (ethanol 40%, vol/vol). Dishes were incubated at 37°C for 20 ± 2 h (20,21).

RESULTS AND DISCUSSION

Table 2 shows the composition of propolis. Except for sample 1, water content does not surpass 3 g/100 g. Maximum water content reported in the literature is 4 g/100 g (22). The water content of propolis depends on the extraction technique applied. To determine whether or not the wax type coincided with beeswax, it was analyzed for hydrocarbons, monoesters, free acids and free alcohols by the method of Serra Bonvehi (23). The results indicate that wax from propolis coincides with beeswax (24).

Mineral salts ranged from 1.40 to 21.50 g/100 g. Maximum ash content reported in the literature is 15 g/100 g (22). Of the samples analyzed for metals, 67% were higher than 10 ppm for Cr and 32% were higher than 10 ppm for Pb. The highest Cd value detected was 2.60 ppm. The maximum Pb detected is 60 ppm (22).

Phenolic components were determined by spectrophotometric and HPLC methods (25) (Table 2). These components are the most representative part of the resin and balsam fraction. Component identification was performed according to an earlier method (26), which included array photodiode spectrum, chromatographic criteria and batochromic band shifts in the ultraviolet/VIS spectrum.

Significant amounts (>1 g/100 g) of phenolic components detected and identified are: (i) benzoic acid derivatives (gallic and protocatechuic acids); (ii) cinnamic acid derivatives (ferulic and *m*-cinnamic acids); (iii) benzaldehyde derivatives (vanillin); and (iv) flavonoids (rutin, quercetin, kaempherol, apigenin, acacetin, pinocembrin and tectocrysin) (Table 3). The quantitation of components not included in the above pattern was performed by using nearest-peak calibration graphs or by average value of the two neighboring peaks. All peaks showed r^2 values above 0.99. Total phenolic compounds range between 10.10 and 28.60 g/100 g (determined spectrophotometrically), and 73% of the samples showed contents no lower than 20 g/100 g (Table 2). The average variability value of the chromatographic method is 3.94 g/100 g, higher than the spectrophotometric method. Flavonoids are predominant in the phenolic fraction (Table 3). Both spectrophotometry and chromatography methods were used for quantitation. These methods show a large average difference (18 g/100 g) when applied to flavonoids. Verification of the spectrophotometric method was performed with two calibration graphs for galangin and rutin. No significant differences were found ($P \leq 0.05$) between the two calibrations. Precision of the two methods was evaluated by using 10 and 100 ppm of apigenin, one of the most abundant active components in propolis. Variation increases as the concentration of apigenin increases. The spectrophotometric method quantitates only 13% of the real apigenin. Because flavones and flavanones are so prominent in the propolis composition (Table 3), their presence can be detected by spectrophotometric method, and total flavonoids can be determined semiquantitatively. HPLC indicates that 80% of the samples contain at least 22 g flavonoids/100 g, with no less than eight different components. In most samples, at least fifteen components (flavones, flavonoles and flavanones) were identified. Acacetin and apigenin were the most abundant. Pinocembrin, quercetin and rutin appeared in smaller percentages (Table 3).

Variance analysis shows a significant difference ($P \leq 0.05$) in the total contents of phenols, flavonoids and active components. All samples show steroid values below 70 mg/100 g (Table 2). Essential oil contents ranged between 0.58 and 1.30 g/100 g (Table 2) and agreed with literature values (27).

The oxidation rate was expressed as the time(s) to decolor 0.1 N $KMnO_4$ solution in aqueous acid medium (Table 2). In impure propolis, containing high wax percentages, the contents of its active substances decrease with

TABLE 2

Propolis Composition (g/100 g)

Sample number	Wax	Resins and balsams	Moisture	Ash	Phenols[a]	Phenols[b]	Flavonoids[a]	Steroids[c]	Essential oils	Decoloration time (s)
1	15.60	47.60	22.60	3.10	10.10	13.10	3.00	15.00	0.58	80
2	—	80.00	—	1.70	19.90	26.70	6.40	53.50	1.20	7
3	2.50	72.20	2.50	21.50	18.70	18.80	5.50	57.20	1.30	9.33
4	2.40	82.50	2.10	13.20	20.00	22.90	6.60	49.60	0.98	6.00
5	30.60	60.70	2.30	5.10	23.20	28.90	5.30	65.70	1.06	13.67
6	24.80	69.60	2.20	3.50	24.40	31.20	5.10	63.20	1.10	8.67
7	18.70	71.00	2.50	3.90	25.00	29.40	4.10	59.70	0.68	4.67
11	23.40	67.70	2.30	3.70	24.80	29.60	5.50	68.20	0.93	5.33
12	26.60	67.30	2.00	4.40	22.20	25.80	5.80	61.30	0.88	5.33
13	8.00	87.40	2.60	1.40	25.40	28.90	4.60	57.20	1.27	5.33
14	18.80	77.30	2.80	2.80	26.40	27.60	3.90	61.50	0.77	5.33
15	21.50	72.60	2.80	2.60	28.60	33.10	5.70	63.70	1.08	5.33

[a]Spectrophotometry.
[b]High-performance liquid chromatography.
[c]mg/100 g.

TABLE 3

Phenolic Components (high-performance liquid chromatography) (g/100 g)

Component	Sample number														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Gallic acid	—	—	—	—	—	—	—	—	—	—	—	—	—	0.03	—
3,4-Dihydroxybenzoic	—	—	—	—	—	0.10	0.04	—	—	—	0.03	0.03	0.06	—	—
Caffeic acid	—	—	—	—	0.23	0.29	—	—	—	—	—	—	0.07	0.17	0.21
Vanillin	1.35	0.14	0.06	—	1.36	1.70	1.16	0.29	0.88	—	1.65	1.41	2.37	1.95	2.49
Ferulic acid	0.40	0.40	0.24	0.38	1.16	0.96	1.66	0.16	0.75	0.45	1.00	0.78	0.46	0.99	1.25
Sinapic acid	—	0.06	—	—	0.33	0.31	0.39	—	0.26	0.18	0.22	0.31	0.14	0.36	0.95
m-Coumaric acid	0.19	0.10	0.16	0.60	0.78	0.58	0.02	0.38	0.03	0.79	0.58	0.63	0.78	0.97	—
Naringin	0.29	—	0.16	—	—	—	—	—	—	—	—	—	1.35	—	—
Rutin	0.42	1.55	0.65	1.39	3.18	3.79	3.44	0.48	1.71	1.04	3.70	3.09	3.17	4.09	3.84
4-Hydroxybenzoic	1.48	0.96	0.19	—	0.18	0.46	0.12	0.05	0.23	1.03	0.08	0.19	0.14	0.09	0.73
o-Cinnamic acid	0.01	0.32	0.17	0.25	0.26	0.23	0.11	0.09	0.12	0.15	0.29	0.28	0.12	0.15	0.08
Quercetin	0.87	1.79	1.05	0.94	1.35	1.19	1.43	1.30	1.35	1.33	0.97	0.97	1.10	0.95	1.25
Hesperitin	0.08	0.04	0.22	—	—	0.13	0.21	—	—	—	0.11	0.14	0.12	—	—
Pinobanskin	0.17	0.20	0.17	—	0.21	0.15	0.12	0.16	0.18	0.22	0.16	0.04	0.09	0.10	0.22
Kaempherol	0.05	2.02	1.14	2.13	0.67	0.56	1.28	0.30	0.73	1.38	0.37	0.64	0.28	0.15	0.79
Apigenina	1.04	5.32	3.17	5.86	4.58	5.37	6.70	2.79	3.85	3.47	6.69	3.95	5.74	5.52	4.29
Apigenin[a]	—	2.41	1.81	—	2.62	2.49	2.48	1.91	1.75	1.48	2.63	1.66	—	1.34	1.94
Galangin	0.16	0.33	0.24	0.99	0.73	0.98	0.69	0.25	0.45	0.31	0.70	0.76	1.98	0.86	0.76
Chrysin	0.49	0.07	—	—	0.01	—	—	0.11	0.06	0.12	0.11	0.01	—	0.09	0.02
Acacetin	0.57	6.86	6.66	7.49	8.47	8.71	6.17	7.14	7.35	6.75	6.94	7.29	8.86	6.97	7.58
Not identified	0.88	0.07	0.21	—	0.19	0.39	0.22	0.18	0.10	0.08	0.18	0.28	0.76	0.05	0.14
Pinocembrin	3.46	1.73	0.74	0.94	1.38	1.68	1.37	1.23	1.16	1.66	1.45	1.52	0.90	1.68	1.73
Pinostrobin	—	0.19	—	—	0.26	0.09	0.14	0.09	0.12	0.09	0.07	0.18	0.05	0.08	0.26
Tectochrysin	0.48	1.57	1.41	1.95	0.84	0.36	0.52	1.37	1.08	1.56	1.00	0.87	0.08	0.84	0.69
Not identified	0.27	0.15	0.06	0.06	0.35	0.41	0.13	0.08	0.20	0.10	0.40	0.59	0.30	0.18	0.32
Rhamnetin	0.63	0.41	0.26	0.35	0.19	0.21	—	0.33	0.21	0.42	—	0.12	0.14	—	1.72
Total	13.10	26.70	18.80	22.90	28.90	31.20	29.40	18.70	23.10	22.10	29.60	25.80	28.90	27.60	33.10

[a]Apigenin derivative.

the $KMnO_4$ decoloration rate. An absorbance variation at 460 nm is detected, ranging from reddish brown (sample 3) to yellowish brown (sample 13). The change in color is measured by the OD_{460}/OD_{525} ratio, which increases with brownish color and indicates the degree of degradation. Acceptability increases with the color ratio.

Sugars detected were arabinose, fructose, glucose, sucrose and maltose. Significant amounts of sucrose, glucose and fructose were found. No significant differences ($P \leq 0.05$) were found between pre- and post-hydrolysis samples, when hydrolysis was performed according to Garrido *et al.* (28). Thus, propolis sugars come mainly from bee-secreted β-glucosidase enzymatic hydrolysis products.

The bacteriostatic activity of propolis alcohol extract (40%, vol/vol) is higher against *S. aureus* than *B. subtilis* and much less than against *E. coli*, which coincides with Kedzia's results (29). The most active propolis samples against *B. subtilis* and *S. aureus* show a minimum inhibitory concentration (MIC) around 80 μg/mL (Table 4). Samples are classified against *B. subtilis* and *S. aureus* within the same activity order: 67% of the samples inhibit both microorganisms starting at 100 μg/mL. Propolis samples do not present the same activity order against *E. coli* (Table 4) because 60% of the samples' MIC values ranged between 800 and 900 μg/mL as opposed to a tetracycline MIC value of 1.50 μg/mL against *B. subtilis* and *S. aureus* and 2 μg/mL against *E. coli*. The propolis optimum MIC value is 53 times higher than that reported for tetracycline against *B. subtilis* and *S. aureus* and about 400 times that against *E. coli*.

TABLE 4

Propolis Minimum Inhibitory Concentration (μg/mL)

Sample number	*Bacillus subtilis*	*Staphylococcus aureus*	*Escherichia coli*
1	>100	>100	>900
2	200	100	>900
3	>100	>100	>900
4	90	90	900
5	>100	>100	900
6	100	90	800
7	>100	>100	900
11	100	100	900
12	80	80	800
13	90	90	800
14	90	90	800
15	100	100	900

Data were processed, and correlations between MIC and the various active propolis components were studied for individual component's effects in bacteriostatic activity. No individual propolis compound surpassed Pearson-Lee values ($P \leq 0.05$). The observed increase in bacteriostatic activities is probably due to a synergic effect of all flavonoids and other phenolic components. Only galangin showed some indication of bacteriostatic activity behavior.

The minimum presence of propolis in other food products should be assessed according to their antioxidant

power and bacteriostatic activity. The latter was determined by MIC against pathogenic microorganisms. Propolis contents in honey and bee pollen, according to the results obtained in a previous study (25), is estimated to be at least 10%.

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