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Flavonoid analysis and antimicrobial activity of commercially available propolis products

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Propolis ethanolic solutions are the most used propolis products on the market for the treatment of minor ulcers in the mouth, angina, thrush or skin infections. Since it is still an unofficial drug in pharmacy, we analyzed the contents of flavonoids in ten commercially available ethanolic solutions of propolis from the Croatian market using two complementary colorimetric methods. Antimicrobial activities, determined with the diffusion method, against six bacterial species (*Bacillus subtilis* NCTC 8236, *Staphylococcus aureus* ATCC 25923, *Streptococcus pyogenes* ATCC 12204, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 10536, *Pseudomonas aeruginosa* ATCC 27853, and one yeast-like fungus *Candida albicans* ATCC 10231 were compared. Results of flavonoids analysis suggested that the contents of flavones and flavonols in the products were uniform and ranged from 0.14 to 0.41%, but the content of flavanones varied greatly from 0.43 to 18.78%. Total flavonoid content, as the sum of two colorimetric methods, in propolis products was between 0.78 and 18.92%, and most products had the flavonoids content below 9%. All products with the total flavonoids content above 1% showed antimicrobial activity against the four Gram-positive bacterial species tested, and against *P. aeruginosa* and the yeast-like fungus *C. albicans*. Total flavonoids contents, expressed as the sum of two colorimetric methods, could be useful methods for estimating the flavonoid contents of propolis products. Our results indicate that the quality of commercially available propolis products requires verification.

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Propolis or bee-glue is a sticky substance collected in the hives by the honeybee (*Apis mellifica* L., Apidae) from various tree sources. In the northern hemisphere (Europe, North and South America and western Asia), the tree sources are: *Populus* spp., *Betula* spp., *Ul-*

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mus spp., *Quercus* spp., *Salix* spp., *Aesculus hippocastanum* L., *Picea* spp., *Fraxinus* spp., *etc*. (1, 2). These origins may account for the smell, color, constitution, and chemical composition of propolis. Chemical analyses have showed the presence of more than 300 different compounds, which can be separated in lipophilic flavonoid-aglycones (flavones, flavonols, flavanones, dihydroflavonols and chalcones), phenolic acids, aldehydes and polyphenolic derivates of cinnamic and benzoic acid, including caffeic acid esters and terpenes as the most abundant, and various other compounds including pollen, wax, vitamins, minerals, sugars *etc*. (3, 4).

Propolis was used for its healing properties by the Egyptians, Greeks, Romans, *etc*. (5). Nowadays, propolis is still an unofficial drug for pharmaceutical preparations, and without quality limits. Scientific research has revealed its very wide spectrum of effects, including antioxidant, antibacterial, antifungal, antiviral, anti-inflammatory, anti-oxidant, anti-tumor and immunomodulating properties. As an anti-inflammatory agent, propolis has been shown to inhibit the synthesis of prostaglandins, activate the thymus gland, aid the immune system by promoting phagocytic activity, stimulate cellular immunity, and augment healing effects in epithelial tissues (6–8). Antimicrobial activity of propolis and its preparations (especially ethanolic solutions) is very well known, and this activity is more potent against Gram-positive bacterial species than against Gram-negative bacterial species. Fungi, including yeast-like fungi from the genus *Candida* and filamentous dermatophytes are also sensitive to propolis ethanolic extracts (9, 10).

There are many products that contain propolis on the market, and these include ethanolic extracts (with or without other plant extracts, vitamins, honey, *etc*.), syrups, sprays, tablets and capsules. The products contain propolis of more or less unknown origin, and its biological effects are often questionable. Flavonoids, one of the main groups of phenolic compounds in propolis, are the key compounds for estimating of propolis quality, and therefore we analyzed the flavonoid contents of ten ethanolic solutions of propolis available on the Croatian market, as well as their antimicrobial activity against six bacterial species and one yeast-like fungus.

EXPERIMENTAL

Reagents and solvents

Ethanol, methanol, formamide, potassium acetate and potassium hydroxide used were of analytical grade and were purchased from Kemika (Croatia) and aluminium chloride hexahydrate and 2,4-dinitrophenylhydrazine used were of analytical grade, purchased from Merck (Germany).

Microbiological media (tryptic soy agar and Müller-Hinton agar) were purchased from Merck, Germany, and Sabouraud-2% (*m/V*) glucose agar from Biolife (Italy).

Instruments

Measurements were carried out using a PU 8625 UV-Vis spectrophotometer diode--array (Philips, The Netherlands).

Determination of flavones and flavonols (11)

Flavones and flavonols in propolis were expressed as quercetine equivalent. Quercetine (Sigma, Germany) was used to make the calibration curve (standard solutions of 6.25, 12.5, 25.0, 50.0, 80.0 and 100.0 μg mL^{-1} in 80% ethanol *(V/V)*. 0.5 mL of a product (ethanolic solutions of propolis) was mixed with 1.5 mL 95% ethanol *(V/V)*, 0.1 mL 10% aluminum chloride *(m/V)*, 0.1 mL of 1 mol L^{-1} potassium acetate and 2.8 mL water. A volume of 10% (*m/V*) aluminum chloride was substituted by the same volume of distilled water in blank. After incubation at room temperature for 30 minutes, the absorbance of the reaction mixture was measured at 415 nm.

Determination of flavanones (11)

Flavanones in propolis were expressed as naringenin equivalent. Naringenin (Sigma, Germany) was used to make the calibration curve (standard solution of 0.125, 0.25, 0.30, 0.50, 1.00 and 2.00 mg mL^{-1} in methanol). One mL of a product (ethanolic solution of propolis) was separately mixed with 2 mL of 1% 2,4-dinitrophenylhydrazine *(m/V)* and 2 mL of methanol at 50 °C over a water-bath for 50 min. After cooling to room temperature, the solution was mixed with 5 mL of 1% potassium hydroxide *(m/V)* in 70% ethanol *(V/V)*. Then, 1 mL of the mixture was taken and centrifuged at 1000 *g* for 10 min and the supernatant was filtered through Whatman No. 1 filter paper. The filtrate was adjusted to 25 mL. The absorbance of the filtrate was measured at 495 nm.

Microorganisms tested

Bacterial species studies were from the *Collection of Microorganisms* of the Department of Microbiology, Faculty of Pharmacy and Biochemistry, and included *Bacillus subtilis* NCTC 8236, *Staphylococcus aureus* ATCC 25923, *Streptococcus pyogens* ATCC 12204, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 10536, *Pseudomonas aeruginosa* ATCC 27853, and *Candida albicans* ATCC 10231. Before the analysis, bacterial strains were cultured on tryptic soy agar under aerobic conditions for 24 h at 37 °C, with the exception of *Streptococcus pyogenes* ATCC 12204, which was cultivated on tryptic soy agar with addition of 5% sterile defibrinated sheep blood. The yeast-like fungal strain *Candida albicans* was cultivated on Sabouraud 2% (*m/V*)-glucose agar under aerobic conditions for 48 h at 37 °C.

Antibacterial activity

Antibacterial activity of commercial ethanolic extracts of propolis was estimated by the agar-diffusion method according to the *European Pharmacopoeia* (12). Briefly, inocula of bacterial cells and yeast-like fungal blastospores were prepared by washing one or two colonies using five milliliters of sterile phosphate buffer solution pH 7 per a concentration of cells or blastospores of approximately 10^6 mL^{-1}. Density of cells or blastospores was measured with McFarland's standard solution of freshly prepared barium sulfate in sterile water: density of mixture prepared of 0.1 mL of 1% $BaCl_2$ and 9.9 mL of 1% H_2SO_4 solution (13).

One milliliter of inoculum was added into 20 ± 2 mL of Müller-Hinton agar (for bacterial species) or Sabouraud-agar (for yeast-like fungi) at 50 ± 3 °C and mixed in Petri dishes (d = 9 cm). After drying the plates at room temperature for a maximum of 20 minutes, holes were made in the agar with sterile stainless steel cylinders (d = 6 mm). Undiluted products (40 μL) were added into the holes. Prior to 18 h incubation at 37 °C, inoculated Petri dishes were pre-incubated at 4 °C for 1 h.

Streptomycin sulphate was used as a reference antibacterial substance, and nistatin as a reference antifungal substance. 10 mg mL^{-1} of solution was prepared from the stock solution of streptomycin sulphate in sterile freshly distilled water (0.1%, *m/V*) with phosphate buffer solution pH 8 (12). Nistatin was prepared as 100 mg mL^{-1} solution in formamide, and diluted with phosphate buffer solution, pH 6 (14).

Inoculated plates were incubated for 18 h at 37 °C for bacterial species, and 48 h for *C. albicans*. Inhibition zones were expressed in mm as the diameters of clear zones around holes.

Statistical analysis

The results of spectrophotometric analyses as well as of antimicrobial activity were expressed as the mean obtained upon three independent analyses.

RESULTS AND DISCUSSION

The present study showed the flavonoid content determined by two independent colorimetric methods, one for determination of flavones and flavonols and the other for determination of flavanones, as reported by Chang *et al.* (11). Antimicrobial activities of ten commercially available products of ethanolic solutions of propolis were compared using four standard Gram-positive and two Gram-negative bacterial species, and one yeast-like fungus. The results are presented in Table I.

The propolis content in products varied from 7 to 25%, as declared by the producers. Samples of propolis products varied in their flavonoids content, especially when the content of flavanones was analyzed. The levels of flavones and flavonols were uniform, and ranged from 0.14 to 0.41%. Most of the products analyzed had the level of flavones and flavonols around 0.36%. The content of flavanones varied greatly, from 0.43 to 18.78%. Two products had the lowest content of flavanones, below 1% (products with codes 4 and 9). Two products had contents of flavanones higher that 10% (products with code 5 and 1), and most of the other products had a similar flavanones content, between 6.45 and 8.62%. Total contents of flavonoids in the ethanolic solutions of propolis products were expressed as the sum of two complementary methods for the determination of flavones, flavonols and flavanones, and the results showed that most products had flavonoids contents below 9%.

Tremendous variations in total flavonoid contents of commercial propolis products are similar to the results of described by Chang *et al.* (11). Great differences between total flavonoid contents of samples of ethanolic solutions of propolis products indicate that the quality of commercial products requires verification. This variation is probably due

Table I. Flavonoid content and antimicrobial activity of propolis products

Product code	Content of propolis in products (%)[a]	Flavonoid content (%)			Inhibition zones (mm)						
		Flavones and flavonols[b]	Flavanones[c]	Total[d]	*Bacillus subtilis* NCTC 8236	*Staphylococcus aureus* ATCC 25923	*Streptococcus pyogenes* ATCC 12204	*Enterococcus faecalis* ATCC 29212	*Escherichia coli* ATCC 10536	*Pseudomonas aeruginosa* ATCC 27853	*Candida albicans* ATCC 10231
2	7	0.36	8.61	8.97	12.5	17.0	9.0	9.5	ND	8.0	10.5
3	10	0.34	8.13	8.47	16.0	15.0	9.0	8.0	ND	8.0	12.5
4	10	0.35	0.43	0.78	13.5	11.5	ND	ND	ND	8.5	10.0
6	12	0.41	8.54	8.95	17.0	12.0	9.0	8.0	ND	11.0	11.0
7	12	0.37	6.45	6.82	15.0	14.0	8.0	10.0	ND	12.5	11.5
8	12	0.37	8.62	8.99	13.5	12.0	9.5	12.0	ND	8.5	12.5
5	13	0.36	10.30	10.66	13.5	10.5	9.0	8.0	ND	8.8	10.5
9	15	0.36	0.69	1.05	14.0	15.5	9.0	11.0	ND	8.0	11.0
1	25	0.14	18.78	18.92	11.5	10.0	8.5	8.0	ND	7.5	10.5
10	25	0.36	7.70	8.06	19.0	14.5	11.5	12.5	ND	12.0	13.5
				Streptomycin sulphate[e]	18.0	23.0	ND	ND	20	23.0	ND
				Nistatin[f]	ND	ND	ND	ND	ND	ND	26.0

[a] As declared on the products.
[b] Expressed as quercetin equivalent.
[c] Expressed as naringenin equivalent.
[d] Sum of the flavones, flavonols and flavanones content.
[e] Reference antibacterial substance.
[f] Reference antifungal substance.
ND – Not detected.

to different contents of flavonoids in raw propolis samples. Our previous studies (15–18) showed that the flavonoid content, as well as flavonoids (galangin, pinocembrin, chrysine, quercetine) vary even in raw propolis samples collected from the same geographical area. Our findings are similar to those of other researchers (19), who found differences in all other classes of propolis compounds (such as aromatic acids, phenolic acid and its esters, diterpenic acids, *etc*.).

All propolis products exert bactericidal activity against bacterial species *Bacillus subtilis* and *Staphylococcus aureus* and fungicidal activity against the yeast-like fungus *Candida albicans*. One product (code 4) was not active against *Streptococcus pyogenes*, the causative agent of angina. The same propolis product had no bactericidal activity against *Enterococcus faecalis*, while all other propolis products were active. No activity was noticed against *Escherichia coli*. Contrary to *E. coli*, all propolis products showed bactericidal activity against *Pseudomonas aeruginosa*.

The largest inhibition zones were noticed against *B. subtilis* (from 11.5 to 19 mm) and against *S. aureus* (from 10 to 17 mm). Smaller inhibition zones were recorded for the propolis products against *S. pyogenes* (from 8 to 11.5 mm), against *E. faecalis* (from 8 and 12.5 mm), and also against *P. aeruginosa*. Strong fungicidal activity was shown by all propolis products against *C. albicans*, with the inhibition zones similar to *S. aureus* (from 10 to 13.5 mm).

The results suggest that even the content of flavonoids in propolis products varied greatly, antimicrobial activity was expressed against the Gram-positive pathogenic bacterial strains *Staphylococcus aureus*, *Streptococcus pyogenes* and *Enterococcus faecalis*, and the yeast-like fungus *Candida albicans.* Only one propolis ethanolic solution, with the total flavonoid content below 1%, showed no bactericidal activity against *S. pyogenes* and *E. faecalis*, probably because low content of bactericidal flavonoids. All products expressed bactericidal activity against *Pseudomonas aeruginosa* but not against *Escherichia coli*, members of Gram-negative bacterial species.

CONCLUSIONS

Our results show very balanced concentrations of flavones and flavonols, but a high variability of flavanones concentrations in the ten commercially available ethanolic solutions of propolis. Our investigation has shown that application of two individual and complementary methods (flavones and flavonols together with flavanones) offers a simple method for estimation of flavonoids as the key compounds for evaluating the quality of propolis products, especially ethanolic solutions. Even the content of flavonoids varied greatly, antimicrobial activity against *B. subtilis*, *S. aureus*, *S. pyogenes*, *E. faecalis* and *C. albicans* was noticed in samples where the flavonoid content was higher than 1%.

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S A Ž E T A K

Analiza flavonoida i antimikrobna aktivnost komercijalno dostupnih proizvoda od propolisa

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Etanolne iscprine propolisa najčešće su korišteni proizvodi od propolisa na tržištu za liječenje manjih vrijedova u usnoj šupljini, angine, mlječca i kožnih infekcija. Zbog toga što je propolis još uvijek neoficijelna droga u ljekarništvu, ispitali smo sadržaj flavonoida u deset komercijalno dostupnih etanolnih iscrpina propolisa na hrvatskom tržištu koristeći dvije komplementarne spektrofotometrijske metode. Antimikrobna aktivnost uspoređena je primjenom difuzionog testa na šest bakterijskih vrsta – *Bacillus subtilis* NCTC 8236, *Staphylococcus aureus* ATCC 25923, *Streptococcus pyogens* ATCC 12204, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 10536, *Pseudomonas aeruginosa* ATCC 27853 i jednu kvascima sličnu gljivičnu vrstu – *Candida albicans* ATCC 10231. Rezultati sadržaja flavonoida pokazuju da je sadržaj flavona i flavonola u proizvodima ujednačen (od 0,14 do 0,41%), međutim sadržaj flavanona jako varira između 0,43 i 18,78%. Ukupni sadržaj flavonoida prikazan kao zbroj dviju komplementarnih metoda u proizvodima od propolisa je između 0,78 i 18,92%, a većina proizvoda sadrže flavonoide ispod 9%. Svi proizvodi sa sadržajem ukupnih flavonoida višim od 1% pokazuju antimikrobnu aktivnost protiv četiri gram-pozitivne bakterijske vrste, na vrstu *Pseudomonas aeruginosa* i kvascima sličnu gljivičnu vrstu *Candida albicans*. Ukupni sadržaj flavonoida prikazan kao zbroj dviju spektrofotometrijskih metoda koristan je podatak za procjenu sadržaja flavonoida u proizvodima od propolisa. Naši rezultati ukazuju na potrebu analize kakvoće proizvoda od propolisa.

Ključne riječi: propolis, proizvodi, flavonoidi, antimikrobno djelovanje, spektrofotometrija

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