Acta Pharm. 53 (2003) 275–285 Original research paper

Analysis of propolis from the continental and Adriatic regions of Croatia

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Thin-layer chromatography of ethanolic extract of propolis (EEP) from the continental and Adriatic regions of Croatia showed that 72.2% of propolis samples contain galangin, 88.8% of samples contain kaempferol, naringenin and apigenin and 66.6% of samples contain caffeic acid. Caffeic acid, pinocembrin, galangin, chrysin and naringenin were analyzed by HPLC. In all samples, pinocembrin was the dominant flavonoid. In samples from the Adriatic region, concentration of pinocembrin ranged from 0.03 to 6.14% ($\bar{x}$ = 2.87%) and in the continental region samples from 0 to 4.74% ($\bar{x}$ = 2.84%). Chrysin was found in all propolis samples in a concentration ranging from 0.22 to 5.32% ($\bar{x}$ = 1.86%) in the continental region samples and from 0.03 to 3.64% ($\bar{x}$ = 1.96%) in samples from the Adriatic region. Chrysin was followed by naringenin, ranging from 0 to 1.14% ($\bar{x}$ = 0.42%) in samples from the Adriatic region and from 0.22 to 2.41% ($\bar{x}$ = 0.60%) in the continental region samples. Concentration of caffeic acid ranged from 0 to 10.11% ($\bar{x}$ = 2.69%) in the Adriatic region samples and from 0.27 to 2.67% ($\bar{x}$ = 1.37%) in samples from the continental region of Croatia. Results of HPLC analyses suggest that propolis samples collected from various parts of Croatia do not differ markedly in contents of chrysin, pinocembrin naringenin and galangin but differ in the concentration of caffeic acid. All EEPs significantly inhibited the growth of *Bacillus subtilis* in comparison with the control (80% ethanol) ($p < 0.05$), showing inhibition zones of 16 ± 2 mm for samples from the continental region, and of 18 ± 3 mm for samples from the Adriatic region. There was no significant difference in antimicrobial activity of EEPs from the continental and Adriatic regions of Croatia, suggesting that bactericidal activity depends on synergism of all phenolic compounds.

Keywords: propolis, antibacterial activity, galangin, pinocembrin, chrysin, naringenin, flavonoids, TLC, HPLC

Received July 14, 2003
Accepted October 27, 2003

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Propolis or »bee glue« is a sticky, gummy, resinous product of honeybees *(Apis mellifica* L.) that is accumulated in hives. Bees collect propolis from various leaf buds and cracks in the bark of trees mainly for protection of hives as sealer, draught excluder, and embalming substance to cover carcasses from hive invaders and against bacterial and fungal infections (1). In the northern hemisphere (Europe, North and South America and western Asia), the tree sources are: *Populus* spp., *Betula* spp., *Ulmus* spp., *Quercus* spp., *Salix* spp., *Aesculus hippocastanum* L., *Picea* spp., *Fraxinus* spp, *etc.* (2). These origins may account for the smell, color, constitution, and chemical composition of propolis. Propolis is a very complex mixture and, in general, it is composed of 50% balsams and resins, 30% wax, 10% essential oils, 5% pollen and 5% of various other substances like sugars, vitamins, *etc*. (3). Bees modify propolis by β-glucodiases, enzymes from hypopharyngeal glands, during collection and processing. Results of this enzymatic modification are hydrolyzation of phenolic compounds like flavonoid heterosides to free flavonoid aglycones and sugars and enhancement of the pharmacological action of the resulting products. Chemically, flavonoid aglycones from propolis are flavones, flavonols, flavanones, dihydroflavonols and chalcones. Other phenolic compounds are phenolic aldehydes and polyphenolic derivates of cinnamic and benzoic acid, including caffeic acid esters, terpenes, β-steroids, sesquiterpenes, naphthalene and stilbene derivatives (4).

Ethanolic extracts of propolis and preparations containing propolis have a very long medical history in folk medicine (1, 3)**.** Propolis possesses pharmacological action, such as antibacterial, antifungal, antiviral, local-anesthetic, astringent, spasmolytic, anti--inflammatory, antiulcer, cytostatic, hypotensive, immunostimulatory activities (5–9).

Among the most potent microbicidal compounds in propolis are flavanone pinocembrin (5,7-dihydroxyflavanone) (10) and its 3-OH analogue flavonol galangin (3,5,7-trihydroxyflavon) (11–13). Caffeic acid (3,4-dihydroxycinnamic acid) and its esters, volatile fractions with phenols and/or terpenoids (14) and chrysin (5,7-dihydroxyflavone) possess notable antimicrobial activities as well (1). It is still not known whether antibacterial and antifungal activities of ethanolic extracts of propolis depend on the concentration of galangin, pinocembrin and caffeic acid derivates or on synergism of these or other compounds (15, 16).

Propolis compounds have been analyzed by thin-layer chromatography (TLC), gas--chromatography-mass spectrometry (GC-MS), high performance liquid chromatography (HPLC) and micellar electrokinetic capillary chromatography (MEKC) (17, 18). Bankova *et al*. (19) analyzed by HPLC flavonoids from propolis collected in South Bulgaria. Pinocembrin was the main flavonoid. Bonvehí *et al.* (20) analyzed 15 samples of propolis from South America and China. Pinocembrin was present in smaller quantities than acacetin and apigenin, indicating that the geographic position of hives and plants around the have a major influence upon the chemical composition of propolis. García-Viguera (21) found by HPLC analysis that flavanon pinocembrin and the flavones chrysin and galangin are major constituents of propolis collected in Canada. High concentrations of these flavonoids indicate that, like in European propolis, the main plant sources of this propolis are poplars (*Populus* spp.) (22).

Because of the diverse physiological activities of propolis, its composition is of great importance, especially for the standardization of propolis as raw material used in different pharmaceutical products and for further investigations of its activity, notably antimicrobial activity.

The aim of this research was to estimate the concentration of flavonoid aglycones (naringenin, chrysin, pinocembrin, galangin) and caffeic acid from propolis samples collected in two regions in Croatia and to compare the antibacterial activity of propolis ethanolic extracts against *Bacillus subtilis.* Some other flavones, flavanones and flavonones, as well as ferrulic acid and *p*-coumaric acid, one expected in porolis. Because of the lack of literature data about their bactericidal activity, they were not the subject of this investigation (16).

EXPERIMENTAL

Reagents and solvents

Diphenylboric acid aminoethyl ester, polyethylene glycol 4000, galangin, chrysin, naringenin, caffeic acid and streptomycin sulphate were purchased from Sigma (Germany) and pinocembrin was purchased from Extrasynthese (France). Acetonitrile and acetic acid for HPLC analysis were of HPLC grade and were purchased from Fluka (Switzerland). All other solvents and reagents were of analytical grade and were purchased from Kemika, Croatia.

Microbiological media (tryptic soy agar and Müller-Hinton agar) were purchased from Merck, Germany.

Propolis samples

Eleven samples of raw propolis from the continental part of Croatia (around the city of Osijek) and seven samples from the Adriatic part of Croatia (the island of Hvar and Dalmatinska Zagora, around the city of Imotski) were collected in August or September of 2001 and 2002 from hives. Until analysis, samples of raw propolis were kept at room temperature in the dark. Propolis (10 g) was crushed into small pieces in a mortar and mixed vigorously with 34.85 mL of 80% (*V/V*) ethanol during 48 h at 37 ± 1 °C (23). After extraction, the ethanolic extract of propolis was filtered through Whatman No. 4 paper. To obtain 25% (*m/V*) ethanolic extract of propolis (EEP), the filtrate was adjusted to 40.0 mL with 80% (*V/V*) ethanol and kept at ± 4 °C until analysis.

Thin-layer chromatography

TLC analyses of EEP were performed on silica gel plates 60 G_{254} (Merck, Germany) by the method of Arvouet-Grand *et al.* (24).

EEP samples were diluted with 96% ethanol (1 + 4) and 5-μL aliquots were applied to the plates with a micro syringe (Hamilton, Switzerland). Two mobile phases were used, containing different concentrations of toluene, ethyl-acetate and formic acid: A (5:4:1, *V/V/V*) and B (36:12:5, *V/V/V*). The TLC chamber was saturated with the mobile phase at least 1 hour before analysis. After developing, plates were dried in the air and flavonoids and phenolic acids were visualized under ultraviolet light (365 nm) after spraying with 1% (*m/V*) methanolic solution of diphenylboric acid aminoethyl ester, followed by 5% (*V/V*) ethanolic solution of polyethylene glycol 4000.

Flavonoids in the EEP were identified using the standards galangin, chrysin, naringenin and caffeic acid dissolved in ethanol (96%, *V/V*) to give 0.05 mg mL^{-1} solutions. Aliquots (5 μL) of these solutions were applied to the plates.

High performance liquid chromatography

HPLC analysis was performed by the method of Castellotti and Bendazzoli (25). Defermination of flavonoids and caffeic acid was performed by HPLC on a reversed--phase column (Lichrochart RP-18, 12.5 × 0.4 cm, particle size 5 μm, Perkin Elmer, USA), using a binary LC pump (Perkin Elmer) and a mobile phase made of acetonitrile/acetic acid, 10% (36:75) (A) and acetonitrile/acetic acid, 10% (45:55) (B) with final pH = 2.64. Elution was performed at a flow rate of 1 mL min^{-1} (volume applied 10 μL) using a linear gradient starting with mobile phase A and changing after 30 min to mobile phase B. Detection was performed with a photodiode array detector (Perkin Elmer). Chromatograms were recorded at 255 nm.

Samples were made by dilution of EEP 1:50 with methanol. Flavonoids in the EEP were identified and determined using the standards galangin, pinocembrin, chrysin, naringenin and caffeic acid dissolved in ethanol (96%, *V/V*) to give 0.05 mg mL^{-1} solutions.

Total polyphenols were expressed as galangin (25). Galangin pinocembrin, chrysin, naringenin and caffeic acid were expressed as percentage of raw propolis.

Antibacterial activity

Antibacterial activity of ethanolic extracts of propolis was estimated by the diffusion method according to the *European Pharmacopoeia* (26). The assay was performed on the Gram-positive bacterial strain *Bacillus subtilis* NCTC 8236, which was cultivated on tryptic soy agar under aerobic conditions for 18 h at 37 ± 1 °C. Inoculum was prepared by washing the one or two colonies using five milliliters of sterile physiological saline to a concentration of bacterial cells of approximately 10^6.

Density of bacterial cells was measured with McFarland's standard solution of freshly prepared barium sulfate in sterile water: density of 0.01% mL $BaCl_2$ in 1% H_2SO_4 solution equals approximately 3×10^8 bacterial cells mL^{-1} (27).

One milliliter of inoculum was added into 20 ± 2 mL of Müller-Hinton agar at 48–50 °C and mixed in Petri dishes (d = 9 cm). After drying the plates at room temperature for 20 minutes, holes were made in agar with sterile stainless steel cylinders (d = 6 mm). EEP (40 μL) was added into the holes. Prior to 18-h incubation at 37 °C, inoculated Petri dishes were incubated at 4 °C for 1 h. The same procedure was done with 80% ethanol as a negative control.

Streptomycin sulphate was used as a reference antibacterial substance. Solution of 10 mg mL^{-1} was prepared from the stock solution of streptomycin sulphate in sterile distilled water (0.1%, *m/V*) with phosphate buffer solution pH 8 (26).

Statistical analysis

The zones of inhibition data were related to solvent control (80% ethanol) and were compared by the non-parametric Kruskal-Wallis test. The level of significance was $p < 0.05$.

RESULTS AND DISCCUSION

Nine different phenolic compounds from EEP could be separated by TLC using mobile phase B (Table I). Mobile phase B (toluene/ethyl-acetate/formic acid, 36:12:5, by volume) enabled better separation of phenolic compounds (with the clearest spots) than mobile phase A. TLC of EEP showed that 72.2% of propolis samples contained bactericidal flavonol galangin, 88.8% contained kaempferol, naringenin and apigenin and 66.6% samples contained caffeic acid (Table I). Sample No. 25 from the continental region of Croatia had only one phenolic compound, whereas sample No. 20 from the Adriatic region had no phenolic compounds.

Table I. TLC analysis of ethanolic extracts of propolis from Croatia

R_F	Color of fluorescence (365 nm)	Continental region samples											Adriatic region samples						
		5499	5274	5274b	23	18	26	29	35	28	31	25	20	21	22	5544	5582	5581	5538
0.89[f]	light green	+	+	+	+	+	+	+	+	+	–	–	–	+	+	+	–	+	+
0.82[f]	dark brown	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+	+
0.73[a]	greenish blue	+	+	+	+	+	+	+	+	–	–	–	–	+	+	+	–	+	+
0.68[f]	dark brown	+	+	+	+	+	+	+	+	+	+	_	–	+	+	+	–	+	+
0.63[f]	greenish blue	+	+	+	+	+	+	+	+	+	+	–	–	+	+	+	+	+	+
0.59[b]	yellow	+	+	+	+	+	+	+	+	+	+	–	–	+	+	+	+	+	+
0.56[c]	yellowish brown	+	+	+	+	+	+	+	+	+	+	–	–	+	+	+	+	+	+
0.51[d]	yellowish brown	+	+	+	+	+	+	+	+	+	+	–	–	+	+	+	+	+	+
0.43[e]	blue	+	+	+	+	+	–	+	+	–	–	–	–	+	+	+	–	+	+

Key to identification: [a] galangin, [b] kaempferol, [c] naringenin, [d] apigenin, [e] caffeic acid, [f] not identified components. Notable fluorescence (+), no fluorescence (-)

Quantitative analyses of ethanolic extracts of propolis are presented in Tables II and III. Fig. 1 represents a typical HPLC chromatogram of EEP.

HPLC analyses showed that flavanon pinocembrin was the dominant flavonoid found in four propolis samples from the Adriatic part of Croatia (from 0.03 to 6.14%, $\overline{x}$ = 2.87%) and in nine samples of propolis from the continental region (from 0 to 4.74%, $\overline{x}$ = 2.84%). It was followed by flavon chrysin from 0.03 to 3.64% ($\overline{x}$ = 1.96%) in samples from the Adriatic region of Croatia and from 0.22 to 5.32% ($\overline{x}$ = 1.86%) in samples from the continental region.

Concentration of galangin varied greatly in propolis samples: from those in which galangin was not detected (samples No. 28, 31 and 25 from the continental region of Croatia) to other samples in which concentration of galangin ranged from 0.42 to 2.12% ($\overline{x}$ = 0.84%) (Table II). Galangin was detected in all investigated samples of propolis from the

Table II. HPLC analysis of ethanolic extracts of propolis from the continental region of Croatia

Component (%)	Sample code											$\overline{x}$
	5499	5274	5274b	23	18	26	29	35	28	31	25	
Caffeic acid	2.67	1.34	2.48	1.32	2.09	0.59	1.02	0.78	1.78	0.69	0.27	1.37
Naringenin	0.32	0.32	0.63	0.28	2.41	0.31	0.22	0.25	1.02	0.34	0.46	0.60
Chrysin	1.33	0.98	1.34	2.06	2.45	2.02	1.34	1.66	1.76	5.32	0.22	1.86
Pinocembrin	2.41	1.53	2.66	3.98	3.06	4.74	2.92	3.32	2.71	3.96	ND	2.84
Galangin	0.49	0.42	0.69	1.40	2.12	1.52	1.28	1.28	ND	ND	ND	0.84
Total flavonoids	4.56	3.25	5.31	7.71	10.05	8.58	5.80	6.50	5.48	6.88	0.68	5.90
Total polyphenols	7.30	4.63	7.79	12.34	14.78	16.03	7.69	10.14	11.36	11.82	2.66	9.69

ND – not detected

Table III. HPLC analysis of ethanolic extracts of propolis from the Adriatic region of Croatia

Component (%)	Sample code							$\overline{x}$
	20	21	22	5544	5582	5581	5538	
Caffeic acid	ND	1.17	1.71	2.82	0.12	10.11	2.91	2.69
Naringenin	ND	0.26	0.43	0.54	0.07	1.14	0.50	0.42
Chrysin	0.03	2.79	2.38	3.64	0.28	2.24	2.36	1.96
Pinocembrin	0.06	2.48	5.46	3.33	0.03	6.14	2.58	2.87
Galangin	0.02	1.66	3.07	1.44	0.01	1.22	0.68	1.16
Total flavonoids	0.11	7.18	1.13	8.95	0.14	10.74	6.12	4.91
Total polyphenols	0.11	8.74	16.79	12.60	0.13	15.72	9.03	9.02

ND – not detected

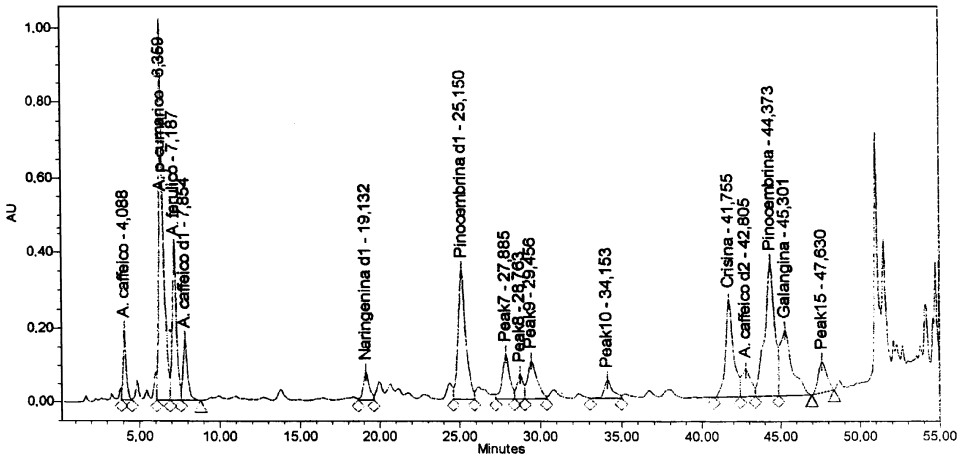

Fig. 1. HPLC of propolis ethanolic extract (sample No. 22).

Adriatic region with concentrations ranging from 0.01 to 3.07% ($\bar{x}$ = 1.16%). Naringenin was detected in all investigated propolis samples from the continental region (from 0.22 to 2.41%, $\bar{x}$ = 0.60%), whereas it was not detected in one sample from the Adriatic region but ranged from 0.07 to 1.14% in the others ($\bar{x}$ = 0.42%).

Concentration of total flavonoids in propolis from the continental part of Croatia varied from the lowest level of 0.68% in sample No. 25 to the highest level of 10.05% in sample No. 18. The concentration varied greatly also in the samples from the Adriatic region of Croatia: from the lowest level of 0.11% in sample No. 20 to the highest level of 10.74%. in sample No. 5581. Both groups of propolis samples contained similar average concentrations of flavonoids: 5.89% in samples from the continental region and 4.91% in samples from the Adriatic region of Croatia.

Concentration of caffeic acid varied greatly in investigated propolis samples and was detected in all samples from the continental part of Croatia: its concentration ranged from 0.27 to 2.67% ($\bar{x}$ = 1.37%). In propolis samples from the Adriatic part its concentrations ranged from 0 to 10.11% ($\bar{x}$ = 2.69%). However, caffeic acid and naringenin could not be detected in sample No. 20; this is the sample with the lowest concentration of total flavonoids and polyphenols (Table III).

It is interesting to note that the samples of propolis No. 20 and 5582 from the Adriatic region contained less than 1% of total flavonoids and bellow 0.1% of galangin, pinocembrin and naringenin. Despite the low concentrations of galangin and pinocembrin, their inhibition zones ranged between 13 and 14 mm. Pinocembrin and galangin were not detected in sample No. 25 from the continental region of Croatia, but EEP showed antimicrobial activity against *Bacillus subtilis* with an inhibition zone of 13 mm. This sample contained the lowest concentration of caffeic acid (0.27%) and chrysin (0.22%). Some higher inhibition zones, ranging from 17 mm to 21 mm, noticed in the EEP of samples No. 22, 5544, 5581 and 5538. Concentration of pinocembrin in these samples was higher than 2% and ranged from 2.58 to 6.14% ($\bar{x}$ = 4.38%) as well as the concentration of

chrysin (from 2.23 to 3.64%, $\bar{x}$ = 2.66%) The highest inhibition zones were found for samples No. 5581 and 5538, containing different concentrations of flavonoids and caffeic acid. Percentage of total polyphenols also varied in these two samples. Equal inhibitory activity of samples No. 5581, 5538 and sample No. 20 against *Bacillus subtilis* could be the consequence of some other compounds with antimicrobial activity, for example, caffeic acid esters or essential oil components (3).

The results of antimicrobial activity of ethanolic extracts of propolis from Croatia against Gram-positive bacteria *Bacillus subtilis* are presented in Table IV. All EEP showed a significantly different ($p < 0.05$) antibacterial activity compared to the solvent control (80% ethanol). All EEP from the continental part showed inhibition zones between 13 and 18 mm ($\bar{x} \pm$ SD = 16 ± 2 mm, n = 11). EEP of samples from the Adriatic part showed inhibition zones in the range between 13 and 21 mm ($\bar{x} \pm$ SD = 18 ± 3 mm, n = 7). Although concentrations of galangin, pinocembrin and caffeic acid in extracts of propolis from the continental and Adriatic parts of Croatia varied greatly, their antimicrobial activities were not found significantly different.

Table IV. Antimicrobial activity of ethanolic extracts of propolis from Croatia against Bacillus subtilis[a]

	Sample code	Inhibition zone (mm)
Samples from the continental region	5499	17[a]
	5274	15[a]
	5274b	17[a]
	23	17[a]
	18	18[a]
	26	16[a]
	29	16[a]
	35	14[a]
	28	15[a]
	31	16[a]
	25	13[a]
$\bar{x} \pm$ SD		16 ± 2
Ssamples from the Adriatic region	20	14[a]
	21	17[a]
	22	17[a]
	5544	20[a]
	5582	13[a]
	5581	21[a]
	5538	21[a]
$\bar{x} \pm$ SD		18 ± 3
Streptomycin (10 mg mL^{-1}) $\bar{x} \pm$ SD		21 ± 1

[a] Significantly different ($p < 0.05$) from 80% ethanol as a negative control.

It sholud be pointed out that EEPs of samples No. 5544, 5581 and 5538 (from the Adriatic region) showed an antibacterial activity (inhibiton zones of 21 ± 1 mm) as high as streptomycin.

Other samples from the Adriatic region showed lower inhibition zones ranging from 13 to 17 mm. Samples of EEP from the continental region of Croatia showed lower inhibition zones than streptomycin, with the highest of 18 mm and the lowest of 13 mm.

CONCLUSIONS

Almost equal or simalar average concentrations of pinocembrin, chrysin, galangin and naringenin were found in propolis samples frrom both the Adriatic and continental regions. However, a markly higher concentration of caffeic acid was found in samples from the Adriatic regions than in samples from the continental region. Aberrations in the concentration of particular flavonoids and caffeic acid are primarily governed by the environment from which honeybees collect propolis and the mode of collecting propolis (wax, impurities). Herbal material, including plant species state of the flora in the field, and contamination may affect the composition of propolis. Nevertheless, all samples showed antimicrobial activity against *Bacillus subtilis* but no significant differences in bactericidal activity between samples from the continental and Adriatic regions of Croatia were found. These results indicate that the antimicrobial activity of EEP against Gram-positive bacterial strain *Bacillus subtilis* does not depend upon the concentration of particular flavonoids but on the synergistic effect of all phenolic compounds. Future researches should be focused on establishing the possible synergistic antimicrobial activity of individual flavonoids and phenolic acids. Also, the relation between the chemical structures of flavonoids and phenolic acids and their antimicrobial activity on different bacterial species and yeasts should be investigated.

Acknowledgements. – The authors wish to thank Asja Smolčić-Bubalo and Castelotti Giovanni (Specchiasol, Italy) for providing HPLC analyses.

REFERENCES

1. G. A. Burdock, Review of the biological properties and toxicity of bee propolis (propolis), *Food Chem. Toxicol.* **36** (1998) 347–363.
2. S. English, W. Greenway and F. R. Whatley, Analysis of phenolics in the bud exudates of *Populus deltoides, P. fremontii, P. sargentii* and *P. wislizenii* by GC-MS, *Phytochem.* **31** (1992) 1255–1260.
3. V. S. Bankova, S. L. de Castro and M. C. Marucci, Propolis: recent advances in chemistry and plant origin, *Apidologie* **31** (2000) 3–15.
4. M. C. Marcucci, F. Ferreres, C. García-Viguera, V. S. Bankova, S. L. De Castro, A. P. Dantas, P. H. M. Valente and N. Paulino, Phenolic compounds from Brazilian propolis with pharmacological activities, *J. Ethnopharmacol.* **74** (2001) 105–112.
5. T. Miyake and T. Shibamoto, Antioxidative activities of natural compounds found in plants, *J. Agric. Food Chem.* **45** (1997) 1819–1822.
6. Y. K. Park, M. H. Koo, J. S. Abreu, M. Ikegaki, J. A. Cury and P. L. Rosalen, Antimicrobial activity of propolis on oral microorganisms, *Current Microbiol.* **36** (1998) 24–28.

7. H. Tapiero, K. D. Tew, G. N. Ba and G. Mathé, Polyphenols: do they play a role in the prevention of human pathologies? *Biomed. Pharmacother.* **56** (2002) 200–207.
8. F. Borrelli, A. A. Izzo, G. Di Carlo, P. Maffia, A. Russo, F. M. Maiello, F. Capasso and N. Mascolo, Effect of a propolis extract and caffeic acid phenethyl ester on formation of aberrant crypt foci and tumors in the rat colon, *Fitoterapia* **73** (Suppl. 1) (2002) S38–S43.
9. F. A. Santos, E. M. A. Bastos, M. Uzeda, M. A. R. Carvalho, L. M. Farias, E. S. A. Moreira and F. C. Braga, Antibacterial activity of Brazilian propolis and fractions against oral anaerobic bacteria, *J. Ethnopharmacol*. **80** (2002) 1–7.
10. P. J. Houghton, T. Z. Woldemariam, W. Davey, A. Baser and C. Lau, Quantitation of the pinocembrin content of propolis by densitometry and high performance liquid chromatography, *Phytochem. Anal.* **6** (1995) 207–210.
11. S. Pepeljnjak, I. Jalšenjak and D. Maysinger, Flavonoid content in propolis extracts and growth inhibition of *Bacillus subtilis*, *Pharmazie* **40** (1985) 122–123.
12. N. B. Takaisi and H. Schilcher, Electron microscopy and microcalorimetric investigations of the possible mechanism of the antibacterial action of a defined propolis provenance, *Planta Med*. **60** (1994) 222–227.
13. A. J. Afoloyan and J. J. M. Meyer, The antimicrobial activity of 3,5,7-trihydroxyflavone isolated from the shoots of *Helichrysum aureonitens*, *J. Ethnopharmacol.* **57** (1997) 177–181.
14. V. Bankova, R. Christov, A. Kujumgiev, M. C. Marucci and S. Popov, Chemical composition and antibacterial activity of Brazilian propolis, *Z. Naturforsch.* **50c** (1995) 167–172.
15. A. Kujumgiev, V. Bankova, A. Ignatova and S. Popov, Antibacterial activity of propolis, some of its components and their analogs, *Pharmazie* **48** (1993) 785–786.
16. H. Arima, H. Ashida and G. Danno, Rutin-enhanced antibacterial activities of flavonoids against *Bacillus cereus* and *Salmonella enteritidis*, *Biosci. Biotechnol. Biochem*. **66** (2002) 1009–1014.
17. K. R. Markham, K. A. Mitchell, A. L. Wilkins, J. A. Daldy and Y. Lu, HPLC and GC-MS identification of the major organic constituents in New Zealand propolis, *Phytochem.* **42** (1996) 205–211.
18. J. D. Fontana, M. Passos, M. H. R dos Santos, C. K. Fontana, B. H. Oliveira, L. Schause, R. Pontarolo, M. A. Barbirato, M. A. Ruggiero and F. M. Lanças, Profiling propolis flavonoids by means of micellar electrokinetic capillary chromatography, capillary gas chromatography and bactericidal action, *Chromatographia* **52** (2000) 147–151.
19. V. S. Bankova, S. S. Popov and N. L. Marekov, High-perfomance liquid chromatography analysis of flavonoids from propolis, *J. Chromatog.* **242** (1982) 135–143.
20. J. S. Bonvehí, F. V. Coll and R. E. Jordà, The composition, active components and bacteriostatic activity of propolis in dietetics, *JAOCS* **71** (1994) 529–532.
21. C. García-Viguera, F. Ferreres and F. A. Tomás-Barberán, Study of Canadian propolis by GC-MS and HPLC, *Z. Naturforsch.* **48c** (1993) 731–735.
22. W. Greenway, C. G. Davidson, T. Scaysbrook, J. May and F. R. Whatley, *Z. Naturforsch.* **45c** (1990) 594–598.
23. Y. K. Park and M. Ikegaki, Preparation of water and ethanolic extracts of propolis and evaluation of the preparations, *Biosci. Biotechnol. Biochem.* **62** (1998) 2230–2232.
24. A. Arvouet-Grand, B. Vennat, A. Pourrat and P. Legret, Standardisation d'un extrait de propolis et identification des principaux constituents, *J. Pharm. Belg.* **49** (1994) 462–468.
25. G. Castellotti and P. Bendazzoli, Determinazione della frazione flavonoidica e degli acidi fenolici nella propoli, *Erbori. Doma.* **3** (1997) 102–105.
26. *European Pharmacopoeia*, 4th ed., Council of Europe, Strasbourg 2001, pp. 93–99.
27. J. F. MacFaddin, *Biochemical Tests for Identification of Medical Bacteria*, 3rd ed., Lippincott Williams & Wilkins, Philadelphia 2000, pp. 825–826.

S A Ž E T A K

Analiza propolisa kontinentalnog i jadranskog područja Hrvatske

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Metodom tankoslojne kromatografije etanolnog ekstrakta propolisa (EEP) kontinentalnog i jadranskog područja Hrvatske dokazano je da 72.2% uzoraka propolisa sadržava galangin, 88.8% kemferol, naringenin i apigenin i 66.6% kavenu kiselinu. Kavena kiselina, pinocembrin, galangin, krizin i naringenin određeni su HPLC-om. U svim uzorcima najzastupljeniji flavonoid bio je pinocembrin. U uzorcima propolisa iz jadranskog područja koncentracija pinocembrina bila je od 0.03 do 6.14% ($\bar{x}$ = 2.87%), a u uzorcima iz kontinentalnog dijela od 0 do 4.74% ($\bar{x}$ = 2.84%). Krizin je dokazan u svim uzorcima, i to u koncentraciji od 0.22 do 5.32% ($\bar{x}$ = 1.86%) u kontinentalnim uzorcima i od 0.03 do 3.64% ($\bar{x}$ = 1.96%) u uzorcima iz priobalne regije. Za njim slijedi naringenin od 0 do 1.14% ($\bar{x}$ = 0.42%) u uzorcima iz jadranskog područja i od 0.22 do 2.41% ($\bar{x}$ = 0.60%) u uzorcima kontinentalne Hrvatske. Koncentracija kavene kiseline je u uzorcima propolisa jadranskog područja od 0 do 10.11% ($\bar{x}$ = 2.69%), a kontinentalnog područja od 0.27 do 2.67% ($\bar{x}$ = 1.37%). Rezultati HPLCa ukazuju da se uzorci propolisa sakupljeni iz različitih dijelova Hrvatske ne razlikuju značajno u sadržaju krizina, pinocembrina, naringenina i galangina, ali se razlikuju u koncentraciji kavene kiseline. U usporedbi s kontrolom (etanol 80%, *V/V*) svi su EEP značajno inhibirali rast bakterijske vrste *Bacillus subtilis* ($p < 0.05$), pokazujući zone inhibicije od 16 ± 2 mm za uzorke iz kontinentalnog područja te 18 ± 3 mm za uzorke jadranskog područja. Budući da nema značajne razlike u antimikrobnoj aktivnosti EEP iz kontinentalne i jadranske Hrvatske, pretpostavlja se kako se antimikrobna aktivnost zasniva na sinergizmu ukupnih fenolnih sastavnica.

Ključne riječi: propolis, antibakterijsko djelovanje, galangin, pinocembrin, krizin, naringenin, flavonoidi, TLC, HPLC

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