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Quantitative analysis of the flavonoids in raw propolis from northern Croatia*

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Spectrometric analyses of flavonoids in twenty propolis samples, collected from ten different geographic localities in northern Croatia using two complementary methods, are reported. Flavones and flavonols were determined using aluminum chloride and expressed as quercetin equivalent while flavanones were determined using 2,4-dinitrophenylhydrazine and expressed as naringenin. Contents of flavones and flavonols were similar for most samples and ranged from 2 to 2.3%, except for one sample with a concentration of 1.3% and one sample in which it was not possible to detect flavones and flavonols. The content of flavanones in propolis samples is very variable. 55% of samples contained flavanones between 15 and 24% and 45% of samples between 4 and 14%. Total levels of flavonoids in raw propolis samples ranged between 5 and 26%; for the majority of samples (75%), the total level of flavonoids ranged between 15 and 25.9%. The high variability of flavanone concentration will affect the biological activity of propolis preparations.

Keywords: propolis, flavonoids, flavones, flavonols, flavanones, Croatia

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Propolis is a sticky, gummy, resinous substance collected by honeybees (*Apis mellifica* L.) from various plant sources. Bees collect propolis to seal holes in the hives, smooth out the internal walls and protect the entrance against intruders. The main plant sources of resin from cracks in trees and leaf buds in the northern hemisphere are poplar (*Populus* spp.), birch (*Betula* spp.), etc. (1).

Raw propolis is composed of approximately 50% resin (polyphenolic fraction), 30% wax, 10% essential oils, 5% pollen and 5% various organic and inorganic compounds (1, 2). Chemical composition of propolis is very complex. More than 200 compounds have been identified (1–3). Its biological activity depends on compounds from the polyphenolic

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nolic fraction, mainly flavonoids, followed by aromatic acids, phenolic acid esters, terpenes, lignans, *etc.* (1–5). These groups of compounds are reported to have bactericidal (6–8), fungicidal (9, 10), antiviral (11), antiprotozoal (8), antioxidant (12), anti-inflammatory (13) and immunomodulatory (14) activities.

Flavonoids, one of the main groups of phenolic compounds in propolis, are the key compounds for estimation of propolis quality. Flavonoids in propolis are aglycones (without the sugar component). These lipophilic flavonoids are chemically divided into subgroups of flavones, flavanones, flavonols, dihydroflavonols and chalcones (15, 16). The concentration of flavonoids in propolis depends on the geographic origin and ecosystem (plant sources) (17). It is known that flavonoid concentration will affect the biological activity of propolis (11).

Because of the phytogeographic dependence of the flavonoid content in raw propolis, our aim was to determine the concentration of flavonoids (flavones, flavonols and flavanones) in raw propolis samples collected from ten different localities in continental Croatia.

EXPERIMENTAL

Reagents and solvents

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

Instruments

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



Fig. 1. Localities for collecting propolis samples.

Propolis samples and extracts

Twenty samples of raw propolis were collected by scraping it off hive frames in September 2002. Geographic locations of hives: Đakovo (45° 18' N, 18° 24' E), Virovitica (45° 50' N, 17° 23' E), Čakovec (46° 23' N, 16° 26' E), Čulinec (45° 49' N, 16° 00' E), Osijek (45° 33' N, 18° 42' E), Slatina (45° 42' N, 17° 42' E), Sisak (45° 29' N, 16° 22' E), Gorjani (45° 24' N, 18° 23' E), Donji Miholjac (45° 36' N, 14° 39' E) (Fig. 1). Samples from one locality were taken from different bee-keepers.

The collected propolis samples were kept desiccated in the dark until analysis. The appearance, form, color and smell of collected raw propolis samples are described in Table I.

Propolis extract (PEE) was prepared as follows. One gram of raw propolis was extracted with 25 mL of 95% ethanol (*V/V*) for 24 h at 37 °C and the filtrate was adjusted to 25 mL with 80% ethanol (*V/V*).

Determination of flavones and flavonols

Flavones and flavonols in propolis were expressed as quercetine equivalent. Quercetine (Sigma, Germany) was used to make the calibration curve [standard solutions of 12.5, 25.0, 50.0, 80.0 and 100.0 $\mu\text{g mL}^{-1}$ in 80% ethanol (*V/V*)]. The standard solutions or PEE (0.5 mL) were 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. The 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. The coefficient of determination was $r^2 = 0.998$.

Determination of flavanones

Flavanones in propolis were expressed as (\pm)-naringenin equivalent. (\pm)-Naringenin (Sigma, Germany) was used to make the calibration curve (standard solution of 0.25, 0.30, 0.50, 1.00 and 2.00 mg mL^{-1} in methanol). One mL of standard solution or PEE was separately mixed with 2 mL of 1% 2,4-dinitrophenylhydrazine (*m/V*) and 2 mL of methanol at 50 °C for 50 min. After cooling at 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 \times 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. The coefficient of determination was $r^2 = 0.996$.

Statistical analysis

The results were expressed as mean \pm SD obtained upon three independent analyses. Statistical significance of the differences was evaluated using Student's *t*-test for samples from Đakovo and Virovitica.

Table 1. Physical characteristics and flavonoid content in raw propolis samples from continental Croatia

| Sample No. | Locality | Appearance and form | Colour and smell | Flavonoids | | | |
|------------|----------------|---------------------|-------------------------------|---|--------------------|-----------------------------|-------------------------|
| | | | | Flavones and flavonols ^{a,b,c} | $\bar{x} \pm SD^d$ | Flavanones ^{b,c} | $\bar{x} \pm SD^d$ |
| 2 | | Waxy | Brown, not aromatic | 2.2 ± 0.3 | 2.2 ± 0.1 | 20.7 ± 0.1 ^A | 16.2 ± 4.3 ^G |
| 6 | | Dry, waxy | Light brown, not aromatic | 2.2 ± 0.1 | | 15.1 ± 0.2 ^{AB} | |
| 7 | | Waxy | Light brown, not aromatic | 2.3 ± 0.4 | | 13.8 ± 0.2 ^{ABC} | |
| 9 | Đakovo | Waxy | Light brown, aromatic | 2.2 ± 0.4 | | 10.3 ± 0.1 ^{ABC,D} | |
| 10 | | Solid | Dark brown, not aromatic | 2.2 ± 0.1 | | 20.6 ± 0.1 ^{B,C,D} | |
| 13 | | Solid | Light brown, not aromatic | 2.2 ± 0.2 | | 20.3 ± 0.1 ^{B,C,D} | |
| 17 | | Waxy | Dark brown, aromatic | 2.3 ± 0.2 | | 12.4 ± 0.1 ^{ABC,D} | |
| 15 | Gorjani | Waxy, solid | Brown, aromatic | 2.2 ± 0.2 | | 23.8 ± 0.1 | |
| 1 | Osijek | Gummy, waxy | Light brown, aromatic | 2.1 ± 0.1 | | 22.2 ± 0.1 | |
| 16 | Donji Miholjac | Waxy | Brown, aromatic | 2.3 ± 0.1 | | 13.1 ± 0.1 | |
| 4 | | Waxy, solid | Light brown, not aromatic | 2.2 ± 0.3 | 2.1 ± 0.1 | 19.4 ± 0.2 ^E | 15.1 ± 3.2 ^I |
| 11 | Virovitica | Very solid | Light brown, not aromatic | 2.0 ± 0.4 | | 11.6 ± 0.1 ^{EF} | |
| 12 | | Solid, waxy | Light brown, not aromatic | 2.1 ± 0.1 | | 14.4 ± 0.1 ^{EF} | |
| 5 | Čakovec | Dry | Dark brown, not aromatic | 2.3 ± 0.2 | | 20.6 ± 0.1 | |
| 14 | | Solid | Light brown, not aromatic | 2.3 ± 0.4 | | 17.8 ± 0.1 | |
| 18 | Čulinec | Waxy | Brown, aromatic | 2.3 ± 0.2 | | 17.9 ± 0.1 | |
| 19 | | Powdered | Yellowish-brown, aromatic | 1.3 ± 0.3 | | 3.9 ± 0.3 | |
| 8 | Sisak | Solid | Dark brown, not aromatic | 2.0 ± 0.2 | | 13.1 ± 0.2 | |
| 3 | Slatina | Dry, solid | Brown, aromatic | 2.1 ± 0.2 | | 17.8 ± 0.2 | |
| 20 | Čabar | Solid | Yellowish-brown, not aromatic | ND | | 10.6 ± 0.1 | |

^a Expressed as quercetin equivalent. ^b Expressed as naringenin equivalent.^c Mean ± SD of data from the same locality.^d Grand mean ± SD ($n = 3$).ND – Not detected. A–F – Each letter denotes significant differences ($p < 0.001$) between the respective samples.G – Significant difference ($p < 0.05$).

RESULTS AND DISCUSSION

Twenty samples of raw propolis were collected from hives situated on 10 different locations in continental Croatia. The samples studied varied in consistency, colour and smell (Table I). Geographic position of the hives (eastern and western parts of continental Croatia) did not influence the physical characteristics of propolis. Appearance and form did not influence the colour and smell of propolis samples. Only one propolis sample, powdered (No. 19) differed markedly from other samples. Samples of propolis from different hives but from the same locality differed to the same extent as samples from different localities. For example, four samples from Đakovo did not differ in the appearance and form of wax (samples 2, 7, 9, and 17) but they differed in colour and smell. Samples from the same locality with a solid form (samples 10 and 13) have a different color but the same (not aromatic) smell.

Flavonoids were 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.* (18). The results are shown in Table I.

In the seven propolis samples from Đakovo, the level of flavones and flavonols varied from 2.2 to 2.3% (average 2.2%), the content of flavanones varied between 10.3 and 20.7% (average 16.2%). The level of total flavonoids in Đakovo propolis samples varied between 12.4 and 20.7%.

Three propolis samples from Virovitica contained between 2.0 and 2.2% (average 2.1%) of flavones and flavonols, and between 11.6 and 19.4% (average 15.1%) of flavanones. The level of total flavonoids varied from 13.6 to 21.6%.

Two samples from Čakovec contained equal shares of flavones and flavonols (2.3%) but different levels of flavanones (from 17.8 to 20.6%).

Two samples from Čulinec differed greatly in both flavones and flavonols (1.3 and 2.3%), and flavanones (17.9 and 3.9%).

Single samples from Osijek, Slatina, Sisak, Gorjani, Donji Miholjac and Čabar did not differ in the content of flavones and flavonols (2.0 to 2.3%), but the level of flavanones varied between 10.6 and 23.8%. The content of flavonoids in these samples varied from 10.6 to 25.9%.

Determination of flavones, flavonols and flavanones in raw propolis samples showed that 16 out of 20 samples (80%) contained more than 2% of flavones and flavonols (from 2.1 to 2.3%, average 2.2%). Only three samples contained less than 2% of flavones and flavonols (from 1.3 to 2%, average 1.8%). We could not detect any flavones and flavonols in sample No. 20 from Čabar.

The content of flavanones in the propolis samples studied varied greatly (4 to 24%, average 16%). Six propolis samples (30%) contained more than 20% of flavanones (from 20.6 to 23.8%, average 21.4%) whereas the majority of the samples studied (13 from 20, or 65%) had 10 to 20% of flavanones. Only one sample (sample No. 19), which also had a low concentration of flavones and flavonols (1.3%), had a low concentration of flavanones (3.9%).

Comparison of the concentrations of flavones and flavonols in propolis samples from the same locality and between different localities showed that there were no signi-

ficant differences, except for sample No. 19 from Čulinec and sample No. 20 from Čabar. However, significant differences ($p < 0.001$) of flavanone concentration were found within and between the localities of Đakovo and Virovitica.

With regard to the geographic position, samples from the eastern part of continental Croatia and samples from the western part had comparable concentrations of flavones, flavonols and flavanones. The exceptions were the samples from the western part, namely from Čabar and one sample from Čulinec, with low concentrations of flavonoids.

The total level of flavonoids in the raw propolis samples studied varied between 5% and 26%, with an average of 19%. For the majority of propolis samples (75%), the total level of flavonoids was between 15 and 26%.

Our results show uniformity of the concentrations of flavones and flavonols but great differences in flavanone concentrations. High variability of the flavonoid concentration led us to conclude that this will affect the biological activity.

CONCLUSIONS

Our results show very balanced concentrations of flavones and flavonols, but a high variability of flavanones concentrations in the propolis samples collected in the continental region of Croatia. Significant differences in the concentration of flavanones between localities and within the same locality were observed. However, no influence of the geographic position of hives was found in the eastern and western parts of continental Croatia.

Differences in specific groups of flavonoids will affect biological activities and our future research will be focused on investigating the influence of concentration of the flavones, flavonols and flavanones on the bactericidal activity, especially against Gram-positive bacterial species.

Our investigation has shown that application of two individual and complementary methods (flavones and flavonols together with flavanones) makes a simple method for estimation of flavonoids as the key compounds for evaluating the quality of raw propolis. These spectrophotometric methods could be the first step for the estimation of raw propolis quality. The same method might be applicable for the determination of flavonoids in propolis preparations as well.

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S A Ž E T A K

Kvantitativna analiza flavonoida u sirovom propolisu sjeverne Hrvatske

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Udio flavonoida u 20 uzoraka propolisa sakupljenih iz košnica s 10 različitih lokaliteta kontinentalne Hrvatske određen je spektrofotometrijskim postupcima. Flavoni i flavonoli prezentirani su kao ekvivalenti kvercetina i određeni uporabom aluminijevog klorida, a flavanoni su prezentirani kao ekvivalent naringenina i određeni uporabom 2,4-dinitrofenilhidrazina. Količina flavona i flavonola je vrlo slična u većini ispitivanih uzoraka i kreće se između 2,0 i 2,3%, osim jednog uzorka s koncentracijom od 1,3% i jednog uzorka u kojem flavone i flavonole nije bilo moguće otkriti. Količina flavanona u uzorcima propolisa je vrlo varijabilna. 55% uzoraka sadrži flavanone između 15 i 24%, a 45% uzoraka sadrži flavanone u koncentraciji između 4 i 14%. Ukupan udio flavonoida u uzorcima sirovog propolisa kreće se između 5 i 26%. U većini uzoraka (75%) dokazan je udio ukupnih flavonoida između 15 i 26%. Visoke razlike u koncentraciji flavanona utječu na biološku aktivnost preparata s propolisom.

Ključne riječi: propolis, flavonoidi, flavoni, flavonoli, flavanoni

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