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Analysis of content of phenolic acids in Lithuanian propolis using high-performance liquid chromatography technique

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Key words: *propolis; high-performance liquid chromatography; phytochemical compounds; phenolic acids.*

Summary. *The aim of the study was to analyze phenolic acids in Lithuanian propolis and to compare it with the composition of propolis in neighboring countries (Latvia and Poland) according to the predominant flora in the collecting places. The study was also aimed at the evaluation of the effect of the layer thickness (mm) of the harvested propolis on the quality of the raw material in determining the amount of phenolic acids.*

Materials and methods. The object of the study was propolis collected in Lithuania, Poland, and Latvia in late July of 2006 and 2007. The qualitative and quantitative analysis of phenolic acids was performed using the high-performance liquid chromatography technique (HPLC).

Results. The results of the study showed that the quantitative and qualitative composition of phenolic acids in propolis depended on the plants from which the bees in the area collected substances for the raw material of propolis. The predominant phenolic acids were determined to be ferulic and coumaric acids, and they may be among the main indicators of quality in the standardization of the raw material and preparations of propolis.

Conclusion. We created an HPLC-based analysis method for the identification and quantification of phenolic acids in propolis. The variety of phenolic acids in propolis depends on the vegetation predominating in the harvesting area. Studies have shown that the highest amount of phenolic acids is observed in propolis harvested in areas characterized by the predominance of deciduous trees and meadows. Results have also shown that ferulic and coumaric acids are the predominant phenolic acids in propolis. The thickness of the layer of the collected propolis in the hive also influences its chemical composition.

Introduction

Propolis is a resinous substance collected by honeybees from various plant sources. Propolis is collected in various countries – Greece, Turkey, Egypt, Croatia, Czech Republic, Bulgaria, Brasilia, Argentina, Chile, Russia, Taiwan, and Korea – and is analyzed using various methods of analysis, i.e. gas chromatography-mass spectrometry, high-performance liquid chromatography (HPLC), and capillary zone electrophoresis. Up to now, more than 200 chemical compounds have been identified in propolis (1). Furthermore, different compositions and amounts of the active substances are detected in separate samples of propolis (2). The findings of various studies confirm that chemical composition of propolis depends on trees and plants available to the bees, on the season in which it is collected, on the geographical area, and other factors (3, 4). The volatile substances (aromatic oils) determine the flavor of propolis, and the variety of flavor depends on the geographical area and assortment of plants (5). It is determined that the amount of wax depends on the locations in the hive where propolis was used – large amounts of wax are found in propolis used to cement the combs and to seal holes of the hive, and small amounts – in propolis used to cover the walls of the hive. Flavonoids comprise the major

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part of biologically active substances in propolis (6). Furthermore, propolis has been found to contain aromatic acids and carbonic acids with benzoic ring in the aliphatic chain (for example, prenilic derivates of cinnamic and coumaric acids), characterized by very potent antimicrobial activity (7) and effective action against *Bacillus subtilis* and *Pseudomonas aeruginosa*. These compounds are also known to relax muscles (8). Esters of phenolic acids such as ferulates and caffeates have antiviral, antibacterial, and anti-inflammatory activity (9) and inhibit the production of free radicals by as much as 86% (10). The antimicrobial and anti-inflammatory activity of European propolis is associated with the presence of flavonoids, flavones, and phenolic acids and their derivates (11). The antioxidant activity of propolis is mainly determined by phenolic compounds: kaempferol, galangin, caffeates, and free phenolic acids (12). Caffeic acid phenethyl esters have particularly potent antioxidant activity, being scavengers of oxygen free radicals and inhibiting xanthine oxidase (13). The chemical composition of propolis collected in Europe is being intensively investigated. In this region, bees collect substances for propolis from trees: poplars, birches, chestnuts, and alders (2, 14). The phenolic acids of Lithuanian propolis are still unknown. Thus, it is important to analyze the phenolic acids of Lithuanian propolis and to compare them with the phenolic acids of propolis collected in neighboring countries (Latvia and Poland) according to the predominant flora in the collecting places. It is also important to evaluate the effect of the layer thickness (mm) of the harvested propolis on the quality of the raw material in determining the amount of phenolic acids.

Materials and methods

The object of the study was propolis collected in Lithuania, Poland, and Latvia in late July of 2006 and 2007. In the study, we used 15 samples of propolis: samples No. 1–8 were harvested in 2006, and samples No. 9–15 – in 2007. Using the first technique, propolis was collected from beehive sites where it was used as a sealant. The layer thickness of the collected propolis was approximately 20 mm. Using the second technique, propolis was collected with the help of honeybees, using special propolis collectors hung in the hive. The major part of the collector was a linear wire mesh with a gap size not exceeding 4 mm. These gaps were sealed by bees using propolis, and the substance was subsequently collected.

The characteristics of the raw material are presented in Table 1.

Experimental fluid extracts were produced from propolis by maceration. Ethanol at the concentration

Table 1. **Characteristics of the vegetal raw material for propolis extracts in the harvesting sites**

Series	Location	Predominating plants in the region	The layer of the harvested propolis in the hive, mm
No. 1	South-west of Kėdainiai district, close to the Aluona brook, Lithuania	Rape fields, linden	20
No. 4 No. 11	South-west of Kėdainiai district, close to the Aluona brook, Lithuania	Rape fields, linden	4
No. 2 No. 9	Varėna district (Rusingė village), Lithuania	Buckwheat field, meadows, pine forest	4
No. 3 No. 10	Obelynė (Kamša forest), Kaunas region, Lithuania	Leafy forest, coniferous wood, raspberry-canes	4
No. 5 No. 12	Šilutė region, Lithuania	Meadows, leafy forest (alders, linden)	4
No. 6 No. 13	Vilkaviškis region (5 km from Vilkaviškis city), Lithuania	Leafy forest, meadows (quaking asps, birches, willows)	4
No. 7 No. 14	Riga region, Latvia	Meadows, leafy forest (alders, quaking asps)	4
No. 8 No. 15	Sejny county, Poland	Meadows, alders, pine forest	4

of 80% (*v/v*) was used for the extraction. All samples of propolis (30 g) were extracted for 5 days with 80% ethanol up to 100 mL at room temperature. After extraction, the propolis extract was filtered through a paper filter. Solutions were filtered through a membrane filter with a pore size of 0.22 μm (membrane filters for syringes, nylon membrane, diameter of 13 mm; Carl Roth GmbH, Karlsruhe, Germany).

Detection of phenolic acids using high-performance liquid chromatography.

Materials. Methanol for HPLC analysis was of HPLC grade and was purchased from Carl Roth GmbH (Karlsruhe, Germany). Distilled water used for the preparation of solvents was filtered through the Millipore HPLC grade water preparation cartridge with a pore size of 0.22 μm (Millipore, Bedford, USA). Standards of phenolic acids and phenylpropanoids were purchased from ChromaDex (Santa Ana, USA). HPLC analysis with UV/PDA detection was carried out using the Waters 2690 chromatography system model (Waters, Milford, USA), equipped with a Waters 2487 UV/Vis detector and Waters 996 PDA detector. For separation, a Hichrom column Hypersil H5ODS-150A 150×4.6 mm (Hichrom Ltd., Berkshire, UK) and an H5ODS-10C guard-cartridge were used. The data were collected and analyzed using a computer and the Waters Millennium 2000® chromatographic manager system (Waters Corporation, Milford, USA). Eluent A was methanol and eluent B was 0.5% (v/v) acetic acid in water. The elution profile was as follows: 0 min 10% A in B, 28 min 60% A in B, and 30 min 10% A in B. All gradients were linear. The flow rate was 1 mL/min, the column temperature was ambient, and the injection volume was 10 μL. UV detection was performed at 290 nm. The eluted components were identified based on the retention time by comparison with the retention time of the reference standard. The identity of constituents was also confirmed with a PDA detector by comparison with UV spectra of the reference standard in the wavelength range of 190–400 nm (15).

Statistical analysis was performed using statistical software package Statistica 5.5. The data are presented as mean ± SD (standard deviation), SE (standard errors), and %CV (coefficient of variation). Statistical analysis was performed using Student's *t* test, and $P<0.05$ was used as the level of significance. All samples were prepared in triplicate.

Results and discussion

The following active substances were identified in ethanol solutions of propolis: gallic, caffeic, coumaric, ferulic, rosmarinic, and cinnamic acids.

All propolis extracts were found to contain caffeic acid that is one of the main active substances in the evaluation of the chemical composition of European propolis (2, 11, 13). During the analysis, higher amounts of cinnamic and caffeic acids (Table 2) were detected in propolis extract No. 7 produced from raw material collected in areas with predominant deciduous trees and meadows, whereas the lowest amounts of these acids were found in propolis extract No. 3 produced from raw material harvested from areas where not only deciduous, but also coniferous trees predominated, without the predominance of meadows. The amount of gallic acid found in the examined samples of European propolis was very low, and only insignificantly influenced its chemical composition. Small amounts of gallic acid having no influence on the chemical composition were also found in another apian product – honey (16). Additionally, gallic acid was not detected in propolis extracts No. 6 and No. 7 (Table 2). For the first time, rosmarinic acid was detected in ethanol extracts of propolis (Table 2). Due to its potent antioxidant properties, rosmarinic acid may also influence the biological effect of propolis (17).

Study results demonstrated the comparable amounts of phenolic acids in extracts No. 2 and No. 8 (Table 2). As it is seen in Table 1, raw material of propolis for extract No. 2 was collected in Lithuania, and for extract No. 8 – in Poland. Although the regions are different, dominant plants (pine forest and meadows) are the same. This confirms literature data (2, 3, 14) that chemical composition of propolis particularly depends on flora in the places where propolis was collected. Furthermore, the amounts of active substances in extract No. 7 were similar to the respective amounts in extract No. 5. Raw material for both extracts was collected in places with similar flora (leafy forest and meadows). The findings of the study coincide with literature data indicating that bees collect honey mostly from deciduous trees (2, 14, 18).

The present study showed that the predominant phenolic acids were coumaric and ferulic acids in tested extracts of propolis (Table 2). A significant difference was found in the amounts of ferulic ($P=0.010$) and coumaric acids ($P=0.014$) in different samples of propolis (Table 2). Table 2 shows that the concentration of coumaric acid was highest in extracts No. 5, No. 6, and No. 7 as compared to other extracts. All three extracts were produced from propolis collected from areas that were characterized by the predominance of deciduous trees and meadows. The lowest

concentration of coumaric acid was found in propolis extract No. 3, where the raw material was collected from the area with predominant raspberry-cane and tame meadows. The highest amount of ferulic acid was found in propolis extracts No. 4, No. 5, and No. 7. The results of the study showed that only in propolis extract No. 6, there was a significant difference in the amounts of coumaric (P=0.026) and ferulic (P=0.010) acids compared to other extracts (Table 2). These findings were confirmed by studies performed in 2007 in the same areas. The findings presented in Fig. also showed that the amount of coumaric acid was the highest in propolis extract samples No. 12, No. 13, and No. 14 that were collected from the same areas as samples No. 5, No. 6, and No. 7 (Table 1). The amount of ferulic acid was also the highest in propolis extract samples No. 11, No. 12, and No. 14 (Fig.) that were produced from raw material harvested in 2006 from the same areas as in samples No. 4, No. 5, and No. 7 (Table 1).

We found that the amount of ferulic and coumaric acids depended not only on the plants from which propolis was collected, but also on the quality of collection process of the raw material.

The comparison of the results of the examination of propolis (No. 1 and No. 4) collected in the same geographical region but using different collection techniques showed that the amounts of ferulic (P=0.038) and coumaric (P=0.031) acids were significantly higher in sample No. 4 that was harvested using special propolis collectors, thus ensuring not greater than 4-mm layer of the raw material in the hive (Table 2).

Table 2. **The quantitative identification of phenolic acids in propolis extracts**

Phenolic acid	Sample of propolis extract Mean (μg/mL) ± SD*							
	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6	No. 7	No. 8
Gallic acid	9.7±0.10	7.86±0.07	6.59±0.07	10.8±0.09	44.22±0.36	–	–	7.22±0.06
Caffeic acid	115.45±0.55	141.11±0.35	67.97±0.07	160.60±0.41	219.30±0.5	165.99±0.42	327.31±0.47	123.23±0.40
Coumaric acid	508.38±0.61	1025.51±0.33	623.59±0.45	1600.60±0.36	2932.31±0.23	2784.64±0.42	3075.15±0.37	1089.41±0.21
Ferulic acid	461.46±0.26	1085.18±0.32	526.94±0.06	1470.13±0.24	2370.11±0.31	939.87±0.22	2377.54±0.30	1104.26±0.22
Cinnamic acid	49.49±0.04	60.23±0.04	43.62±0.02	80.21±0.07	164.37±0.05	104.07±0.10	611.97±0.11	52.26±0.04
Rosmarinic acid	22.05±0.03	21.79±0.01	21.75±0.01	21.48±0.02	20.35±0.02	22.28±0.03	19.42±0.08	22.11±0.03
Total amount of phenolic acids	3349.48	2341.68	1290.46	3343.77	5750.66	4016.85	6411.39	2398.49

*Values are expressed as a mean value of triplicate analyses for each sample ± standard deviation (SD).
Note: the characteristics of raw material used in the production of propolis extracts are presented in Table 1.

Conclusions

We designed an analysis method by applying high-performance liquid chromatography for the identification and quantification of phenolic acids in propolis. The variety of phenolic acids in propolis depends on the vegetation predominating in the harvesting sites of raw material. Studies have shown that the highest amounts of phenolic acids are found in propolis harvested in areas characterized by predominant deciduous trees, and the predominant phenolic acids in propolis are ferulic acid and coumaric acid. The predominant phenolic acids that were detected – ferulic and coumaric acids – may be among the major indicators of quality in the standardization of propolis raw material and propolis preparations. Experimental studies have shown that the thickness of the layer of the harvested propolis in the hive also affects its chemical composition. Higher amounts of phenolic acids are detected in propolis samples harvested using special

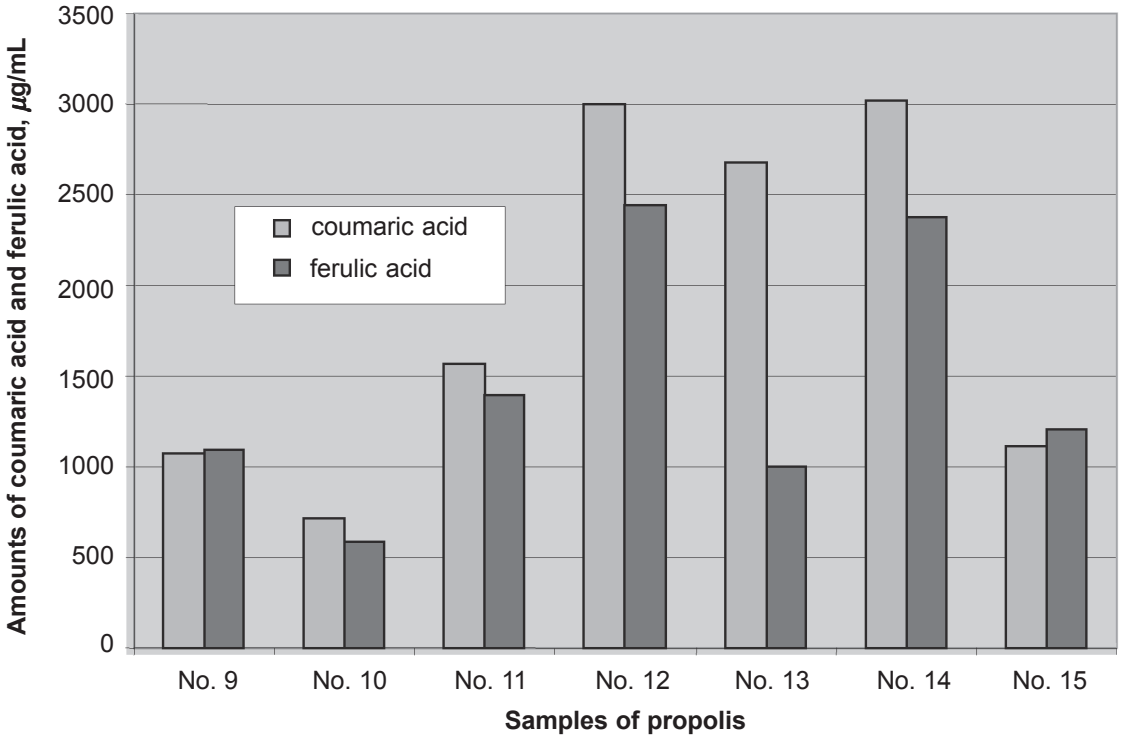

Fig. **The amounts of ferulic (mean=1882.47, SD=989.46, %CV=52.56) and coumaric acids (mean=1440.65, SD=705.57, %CV=48.98) in the studied propolis extracts**

propolis collectors and controlling the layer thickness of the harvested propolis. The production of pharmaceutical preparations necessitates the regulation of the propolis harvesting technique and conditions ensuring high quality of the harvesting.

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Fenolinių rūgščių kiekio lietuviškame propolyje analizė pritaikant efektyviosios skysčių chromatografijos metodą

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Raktažodžiai: propolis, efektyvioji skysčių chromatografija, fitocheminiai junginiai, fenolinės rūgštys.

Santrauka. *Tyrimo tikslas.* Ištirti lietuviško propolio fenolines rūgštis ir palyginti su kaimyninių šalių (Latvijos ir Lenkijos) propolio sudėtimi, atsižvelgiant į propolio rinkimo vietose dominuojančią augmeniją. Taip pat įvertinti surenkamo propolio sluoksnio dydžio (mm) įtaką žaliavos kokybei, nustatant fenolinių rūgščių kiekį.

Medžiagos ir metodai. Propolis surinktas Lietuvoje, Lenkijoje ir Latvijoje 2006 ir 2007 m. liepos mėnesį. Fenolinių rūgščių kokybinė ir kiekybinė analizė atlikta pritaikant efektyviosios skysčių chromatografijos metodą (ESCh).

Rezultatai. Tyrimų duomenimis, kokybinė ir kiekybinė fenolinių rūgščių sudėtis propolyje priklauso nuo to, kokie augalai dominuoja vietovėse, kuriose bitės renka medžiagas propolio žaliavai. Be to, didesnis kiekis veikliųjų medžiagų nustatyta propolyje, surinktame vietovėse, kuriose dominuoja lapuočiai medžiai. Nustatyta,

kad dominuojančios fenolinės rūgštys yra ferulinė rūgštis ir kumaro rūgštis. Jos galėtų būti kokybės rodikliu žaliavos standartizavimui, gaminant propolio preparatus.

Išvados. Sukurta analizės metodika, pritaikant efektyviosios skysčių chromatografijos metodą, fenolinių rūgščių identifikavimui ir kiekio nustatymui propolyje. Fenolinių rūgščių įvairovė propolyje priklauso nuo žaliavos surinkimo vietovėje dominuojančios augmenijos. Tyrimais įrodyta, kad didžiausias fenolinių rūgščių kiekis propolyje, surinktame vietovėse, kuriose dominuoja lapuočiai medžiai bei pievos. Tyrimais įrodyta, kad dominuojančios fenolinės rūgštys propolyje yra ferulinė rūgštis ir kumaro rūgštis. Eksperimentiniai tyrimai parodė, kad surenkamo propolio sluoksnio storis bičių avilyje taip pat turi įtakos jo cheminei sudėčiai.

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