CHEMIJA. 2010. Vol. 21. No. 2–4. P. 118–122

The influence of solvent on the quantity and antioxidant activity of ethanolic extracts of Lithuanian propolis

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Ethanolic extracts (60%, 70%, 80% and 96%) of propolis were analysed by the spectrophotometric and chromatographic methods for the quantification of bioactive compounds such as caffeic and ferulic acids, pinocembrin, pinostrobin, chrysin and the total content of polyphenols. The influence of ethanol concentration on the content of bioactive compounds and the antioxidant activity by DPPH$^{\bullet}$ inactivation was demonstrated. 60% ethanol extracted the highest amount of caffeic and ferulic acids, whereas 80% ethanol gave the highest yields of pinocembrin, pinostrobin and chrysin. The most potent solvent concentration was 80% for the evaluation of total polyphenols (9.57 ± 0.21 mg/mL GAE and 96% 32.7 ± 0.57 mg/mL PGE). All propolis extracts showed a high DPPH$^{\bullet}$ radical inactivation which ranged from 82.96 ± 1.12% to 75.81 ± 0.14%. Their antioxidant activity decreased with increasing ethanol concentration. There was a significant correlation between DPPH$^{\bullet}$ radical inactivation and the amounts of caffeic acid (r = 0.94) and ferulic acid (r = 0.96) in propolis extracts.

Key words: propolis, ethanolic extract, antioxidant activity

INTRODUCTION

Propolis (bee glue) is a sticky dark-coloured material collected by honeybees from plants and used to fill the gaps in and to seal parts of the hive. Propolis has been used extensively in folk medicine for ages, and there is substantial evidence indicating that propolis has antiseptic, antifungal, antibacterial, antiviral, anti-inflammatory, antioxidant, immunomodulatory and antitumour properties [1–3].

In general, propolis contains 50% of resin and balsam, 30% of wax, 10% of essential and aromatic oils, 5% of pollen, and 5% of other substances, such as aliphatic acids, esters, aromatic acids, fatty acids, carbohydrates, aldehydes, amino acids, ketones, chalcones, dihydrochalcones, terpenoids, vitamins (B1, B2, B6, C and E) and minerals (aluminium, antimony, calcium, cesium, copper, iron, lanthanum, manganese, mercury, nickel, silver, vanadium and zinc) [4].

The composition of propolis depends on the vegetation at the site of collection. The main compounds of four Anatolian propolis (Turkey) samples were flavonoids such as pinocem-

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brin, pinostrobin, isalpinin, pinobanksin, quercetin, naringenin, galangine and chrysin [5]. The main compounds found in Brazilian propolis are prenylated derivatives of *p*-coumaric acid and of acetophenone, diterpenes, lignans and flavonoids [6]. European propolis contains the typical "poplar bud" phenolics: flavonoid aglycones (flavones and flavanones), phenolic acids and their esters [7]. A lot of researches indicate that the main compounds of European (Bulgaria, Croatia, Italia, Switzerland) propolis are flavonoids (chrysin, pinocembrin, pinostrobin, galangin, naringin) and phenolic acids (caffeic, cinnamic, *p*-coumaric, ferulic, isoferulic) [8–10].

Investigations of the chemical composition of Lithuanian propolis were started in the recent decade. The main volatile compounds (terpenoids, aromatic and aliphatic acid esters) were identified in 80% ethanolic extracts of propolis by gas chromatography / mass spectrometry [11]. Propolis collected from different Lithuanian regions showed a significant variation in the concentration of total phenolic amounts (0.18–1.64 g/100 mL expressed in gallic acid equivalent (GAE)) [12]. Phenolic acids and phenylpropanoids (caffeic acid, chlorogenic acid, cinnamic acid, coumaric acid, ferulic acid, gallic acid, protocatechuic (3,4-dihydroxybenzoic) acid, rosmarinic acid, vanillic acid, and vanillin) were analyzed using high-performance liquid chromatography [13]. The amounts of phenolic acids were quantified in 80% ethanolic extracts of propolis collected from areas with different flora. The highest amounts of phenolic acids were determined in the propolis from areas with meadows and leafy forests [14]. The pharmacological properties of Lithuanian propolis were investigated for its antimicrobial activity, and it was related with the amount of phenolics [12, 15].

The differences in the chemical composition of propolis determine variations in the biological activity of propolis from different regions. Therefore, the evaluation, or standardization, of propolis and its preparations must be based on the content of groups of active compounds instead of individual components. This approach was confirmed by a higher correlation of biological activity with the percentage of total phenolic compounds than the quantification of individual components [8].

The object of this study was ethanolic extracts due to their use for the production of many preparations containing propolis. The aim of the research was to evaluate the influence of ethanol concentration on extract biological compounds and the ability of different ethanolic concentration extracts of propolis to inactivate the 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) free radical.

EXPERIMENTAL

Raw material of propolis was collected in Southwest and West Lithuania where the predominant flora is leafy forests (*Alnus glutinosa*, *Tilia cordata*, *Populus nigra, Betula verrucosa, Betula pendula*) and meadows. The physical properties and chemical composition of collected propolis samples were similar [14], so all samples were mixed and used for extraction. 30% extracts of propolis (EEP) were produced by maceration at room temperature, using ethanol concentrations of 60%, 70%, 80% and 96% (v/v) [16]. Extraction time was 5 days. Liquid extracts were decanted and filtered after extraction.

High performance liquid chromatography and mass spectrometry enable to directly identify and qualitatively analyze the content of individual components in propolis [17]. Chromatographic analysis of propolis extracts was performed by Waters HPLC-DAD-MS, equipped with a Waters 1525 binary eluent pomp (Waters, Milford, MA); samples were injected using a Rheodyne 7725i injector (Rheodyne, Rohnert Park, CA), compounds were separated on a Synergy MAX-RP, 250 × 4.6 mm column (Phenomenex, Torrance, CA) and detected with Waters 996 PDA (Waters, Milford, MA). After the PDA detection, 0.2 mL/min from the total 1 mL/min flow was directed to a Waters-Micromass ZQ 2000 MS detector (Waters, Milford, MA), and the eluted compounds were analyzed using an ESI probe in negative and positive modes. Separation was carried out using a binary gradient, starting from 100% of solvent A (0% – B), then in 50 minutes increasing the concentration of solvent B to 100% and keeping it for 5 minutes. Afterwards the gradient in 3 minutes was returned to initial conditions, and then in 5 minutes the column was equilibrated. Solvent A was 5% CH_3COOH in ultra pure water (0.054 μS/cm), and solvent B was methanol. UV-Vis spectra were recorded in the range 210–400 nm, and the chromatogram was recorded at the max-plot. The parameters for MS were chosen as follows: capillary voltage 3.0 kV, cone voltage 25 V, source temperature 120 °C, desolvation temperature 250 °C, cone gas flow 80 L/h, desolvation gas flow 250 L/h.

The total amount of phenolic compounds was measured with a UNICAM Helios α UV spectrophotometer (Unicam, Cambridge, UK). The method is based on the colorimetric oxidation / reduction reaction using the Folin–Ciocalteau reagent with modifications and different standards: a) gallic acid [12] and b) mixture of pinocembrin and galangin at the 2 : 1 ratio [9]:

a) the standard calibration (0.02–170 mg/mL) curve was plotted using gallic acid dissolved in distilled water, 1 mL of the test solution, 5 mL of the Folin–Ciocalteu reagent (diluted 10 times with water) and 4 mL of sodium carbonate (75 g/L) were added. The sample was left for 30 min, and the absorbance at 765 nm was measured. The results were expressed as milligrams of gallic acid equivalent per millilitre of ethanolic extract (GAE mg/mL);

b) the standard calibration (0.02–0.326 mg/mL) curve was plotted using a pinocembrin and galangin mixture in the ratio 2 : 1 (w/w) by dissolving them in methanol; 1 mL of the test solution was transferred to a 50 mL volumetric flask containing 15 mL of distilled water and 4 mL of the Folin–Ciocalteu reagent, and 6 mL of a 20% sodium carbonate solution (w/v) was added. The volume was made up with distilled

Table. **Total content of phenolic compounds and the content of individual bioactive compounds in ethanolic extracts of propolis**

Ethanolic extract of propolis	Bioactive compounds[1], µg/mL					Total content of phenolic compounds[2], mg/mL	
	Caffeic acid	Ferulic acid	Pinocembrin	Pinostrobin	Chrysin	GAE	PGE
60%	6.20 ± 0.09	8.05 ± 0.15	0.74 ± 0.07	0.76 ± 0.02	1.85 ± 0.10	8.88 ± 0.26	16.52 ± 0.22
70%	4.06 ± 0.10	5.61 ± 0.11	2.56 ± 0.09	0.67 ± 0.02	2.43 ± 0.09	9.17 ± 0.26	29.19 ± 0.68
80%	3.38 ± 0.08	4.45 ± 0.09	2.63 ± 0.05	0.78 ± 0.02	2.72 ± 0.09	9.57 ± 0.21	27.44 ± 0.34
96%	3.24 ± 0.08	2.56 ± 0.10	1.55 ± 0.04	0.74 ± 0.03	2.31 ± 0.11	9.07 ± 0.61	32.70 ± 0.57

[1] HPLC-DAD-MS method.

[2] Spectrophotometric method.

water to 50 mL. The sample was left for 2 h, and the absorbance at 760 nm was measured. The results were expressed as milligrams of pinocembrin and galangin equivalent per millilitre of ethanolic extract (PGE mg/mL).

Antioxidant activity was determined by the 2,2-diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$) free radical inactivation method. Ethanolic extracts of propolis (50 µL) were mixed with 6×10^{-5} M methanolic DPPH$^{\bullet}$ solution (2.00 mL) in a 1 cm path length disposable cuvette. The decrease in absorption at 515 nm was recorded after an incubation period of 16 min at 25 °C. The absorbance was read on a UNICAM Helios α UV spectrophotometer (Unicam, Cambridge, UK).

The inhibition percentage of the 2,2-diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$) free radical was calculated according to the formula

$$DPPH^{\bullet}_{(\%\ inhibition)} = [(A_{blank} - A_{sample}) / A_{blank} \times 100,$$

where A_{blank} is the absorbance of the blank solution and A_{sample} is the absorbance of a sample and 2,2-diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$) free radical solution after 16 min [18].

The data are presented as the mean ± standard deviation (SD). Statistical analysis was performed using Student's t-test, the correlation matrix test, and $P < 0.05$ was used as the level of significance.

RESULTS AND DISCUSSION

The spectrophotometrical analysis was started using the most popular standard – gallic acid. The highest amount of total phenolic compounds was extracted with 80% ethanol (9.57 ± 0.21 mg/mL) and the lowest with 60% ethanol (8.88 ± 0.26 mg/mL) expressed in the gallic acid equivalent (GAE). The previous analysis of 70% ethanolic extract Lithuanian propolis showed a wide range of quantity variations. The content of total phenolic compounds varied from 1.8 mg/mL to 16.4 mg/mL expressed in gallic acid equivalent (GAE), and this variation depended on the locality where the raw material was collected [12].

The latest chemical analysis of Lithuanian propolis has demonstrated that gallic acid is a minor, if any, compound in propolis samples [14] as is typical of the "poplar" type. Therefore, the other validated – spectrophotometrical – method was used for the analysis when the amount of total phenolic compounds was expressed as a pinocembrin and galangin mixture in the ratio 2 : 1 (w/w). The highest amount of total phenolic compounds was extracted with 96% ethanol (32.70 ± 0.57) and the lowest with 60% ethanol (16.52 ± 0.22 mg/mL), expressed in the pinocembrin and galangin equivalent (PGE). Data in propolis ethanolic solutions, expressed in pinocembrin and galangin equivalent, are comparable with data of other studies: the range of 22.4–26.0 mg/mL of total phenolic compounds was found in 70% ethanolic extract of Bulgarian propolis, 23.8–24.4 mg/mL – of Italian propolis and 19.0–23.9 mg/mL – of Switss propolis [9], while in Lithuanian propolis it varied within 29.19 ± 0.68 mg/mL. The total amounts of phenolic compounds evaluated using gallic acid and a mixture of pinocembrin and galangin standards are presented in Table.

In order to confirm the effect of the solvent (ethanol) of different concentrations on the extraction of biologically active compounds from propolis, a high performance liquid chromatography and mass spectrometry analysis was performed. Bioactive compounds were identified by comparison to standards with regard to retention time, UV-Vis, and mass spectra. The identified compounds were quantified on the basis of their MS chromatograms recorded in a negative mode; however, pinostrobin showed a higher response in the positive ionization mode, and therefore a positive ionization MS chromatogram was used to quantify pinostrobin. A typical chromatogram of a propolis extract is presented in Fig. 1.

The amounts of bioactive compounds tested by the HPLC-DAD-MS method are presented in Table. The highest amounts of phenolic acids (caffeic and ferulic) were determined in the 60% ethanolic extract of propolis, and they decreased by half when ethanol concentration was increased to 96%. The amounts of phenolic acids quantified in the 80% ethanolic extract of Lithuanian propolis by the HPLC method demonstrated also a higher content of ferulic acid as compared with caffeic acid [14]. The highest content of pinocembrin, pinostrobin and chrysin was found in 70–80% ethanolic extracts and the lowest in the 60% ethanolic extracts of propolis. These results are comparable with data of spectrophotometrical analysis which showed that higher concentrations of ethanol extract contained higher amounts of bioactive compounds.

Propolis is one of the the richest sources of polyphenols which are generally known as strong antioxidants. To compare different concentrations of ethanolic extracts of propolis, the disappearance of coloured stable 2,2-diphenyl-1-picrylhy-

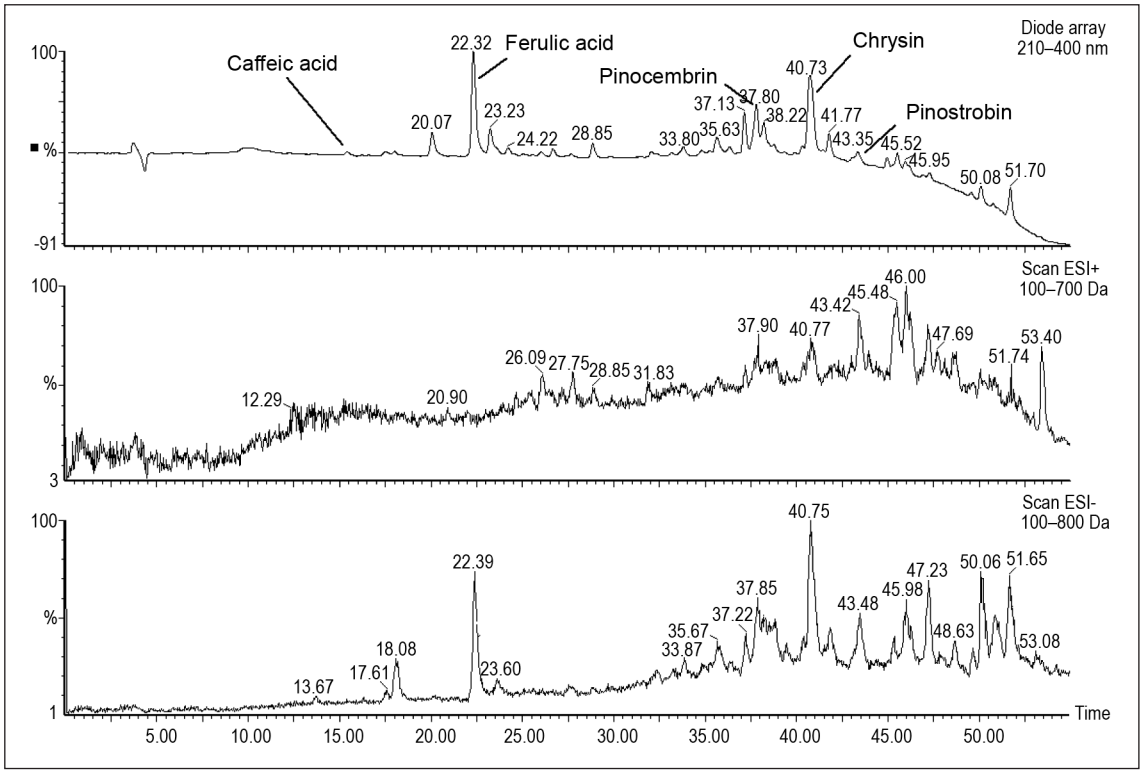

Fig. 1. HPLC-DAD-MS chromatogram of propolis extract (96% ethanol)

drazyl (DPPH•) free radical was measured. The results (presented in Fig. 2) showed that the antioxidant activity of ethanolic extracts of propolis depended on the concentration of the solvent. The highest antioxidant activity (82.96 ± 1.12%) was determined for the 60% ethanolic extract and the lowest (75.81 ± 0.14%) for the 96% extract. It was obvious that the decreasing antioxidant activity correlated with the decreasing content of ferulic and caffeic acids in the extracts when the concentration of ethanol increased. The obtained linear correlation coefficient was r = 0.96354 for ferulic acid and r = 0.9408 for caffeic acid. Comparing the total content of phenolic compounds, a higher correlation was observed between the pinocembrin and galangin mixture (r = –0.8427) than between the gallic acid equivalent r = –0.6151 and antioxidant activity.

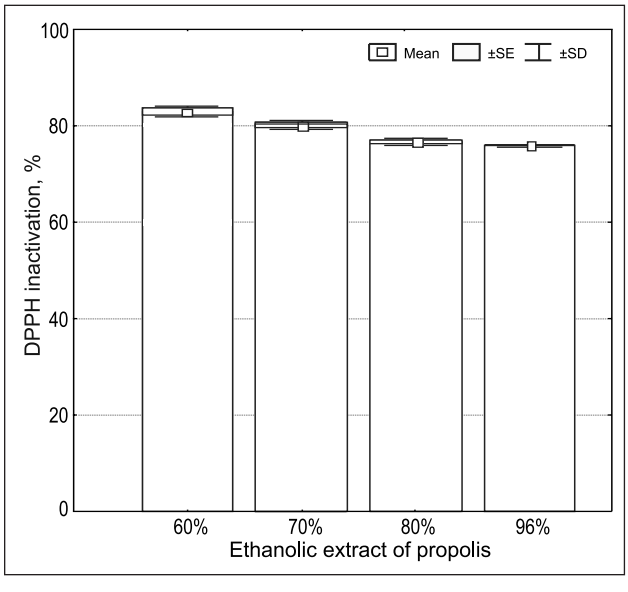

Fig. 2. 2,2-diphenyl-1-picrylhydrazyl (DPPH•) free radical inactivation percentage by 60%, 70%, 80% and 96% ethanolic extracts of propolis

CONCLUSIONS

Most preparations are based on ethanolic extracts of propolis, and the solvent influences the composition of the extract. The ethanol concentration above 70% ensures a higher group content of bioactive compounds; therefore, it can be recommended for the preparation of ethanolic extracts of propolis.

The antioxidant activity of the ethanolic extracts of propolis was investigated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH•) free radical inactivation method which showed a relatively high antioxidant activity of the test samples. For this reason, preparations produced from this natural raw material may be used as an effective means of prevention and as a valuable additional treatment for diseases caused by free radicals.

The correlation analysis has shown that the antioxidant activity of propolis extracts has the highest correlation with the content of individual compounds – ferulic and caffeic acids – under solvent-influenced test conditions. The results confirm that phenolic acids can be markers for the standardization of raw propolis and its preparations.

ACKNOWLEDGEMENTS

The study was performed within the "Eureka" program "Creation of a Multifunctional Cream from Natural Products" supported by the International Agency for Research and Technology.

Received 1 December 2009
Accepted 1 February 2010

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TIRPIKLIO ĮTAKA LIETUVIŠKO PROPOLIO ETANOLINIŲ EKSTRAKTŲ KIEKYBEI IR ANTIOKSIDACINIAM AKTYVUMUI

S a n t r a u k a

Etanoliniai propolio ekstraktai (60 %, 70 %, 80 % ir 96 %) buvo tiriami spektrofotometriniu ir chromatografiniu metodais siekiant nustatyti individualių bioaktyvių junginių kiekius: kavos ir ferulo rūgšties, pinocembrino, pinostrobino, chrisino bei bendrą polifenolių kiekį. Buvo nustatyta etanolio koncentracijos įtaka bioaktyvių junginių kiekiui ir antioksidaciniam aktyvumui inaktyvinant DPPH$^{\bullet}$ laisvuosius radikalus. 60 % etanolis išekstrahavo didžiausią kiekį kavos ir ferulo rūgščių, 80 % etanolis – pinocembrino, pinostrobino, chrisino. Vertinant bendrą polifenolių kiekį, veiksmingiausia tirpiklio koncentracija buvo 80 % pagal galo rūgšties ekvivalentus 9,57 ± 0,21 mg/mL ir 96 % pagal pinocembrino ir galangino mišinio ekvivalentus 32,7 ± 0,57 mg/mL. Visi tirti propolio ekstraktai pasižymėjo dideliu DPPH$^{\bullet}$ radikalų inaktyvinimu, kuris kito nuo 82,96 ± 1,12 % iki 75,81 ± 0,14 %. Antioksidacinis aktyvumas mažėjo, kai etanolio koncentracija didėjo. Reikšminga koreliacija buvo nustatyta tarp DPPH$^{\bullet}$ radikalų inaktyvinimo ir kavos rūgšties (r = 0,94) bei ferulo rūgšties (r = 0,96) kiekių propolio ekstraktuose.