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ANTIOXIDANT PROPERTIES AND POLYPHENOLS COMPOSITION OF SOME ROUMANIAN PROPOLIS SAMPLES

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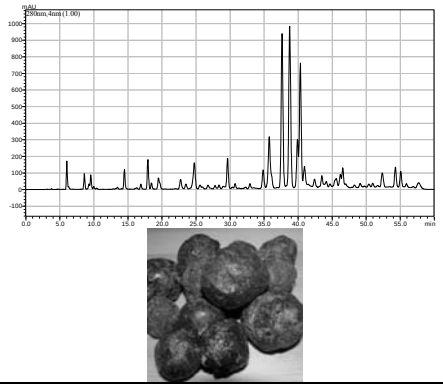
Different Roumanian propolis ethanol extracts were investigated regarding their content in total polyphenols, flavones, flavonols, flavanones and dihydroflavonols. The antioxidant activities were assessed using systems such as 1, 1-diphenyl-2-picrylhydrazyl, 2, 2`-azinobis (3-ethylbenzothiazoline-6-sulfonic acid), ferric reducing ability power and linoleic acid emulsion; the results were correlated with the concentrations in active compounds. A high performance liquid chromatography with diode array detection validated method was used for quantitation of 17 polyphenolic compounds in Roumanian propolis samples. The results confirmed that Roumanian propolis is an important sources of polyphenols and possesses a strong antioxidant activity.



INTRODUCTION

Propolis is created by bees to protect the hive, the whole colony, against pathogenic microorganisms.[1] The antimicrobial action was the first one from a long series of pharmacological effects demonstrated for propolis, its extracts or some of its individual compounds. The first information about propolis was found in ancient times and the pioneers who used propolis were Egyptians, Persians and Romans.[2]

Propolis is a resinous material produced by honeybees that collect different resinous exudates from the bud and bark of certain trees.[3] Main types of chemical substances found in propolis are waxes (30%), resins (50%), balsams, aromatic and ethereal oils (10%), pollen (5%) and other organic matter (5%).[4,5] The chemical diversity of propolis is given by the specificity of the local flora at the collection site and thus on the geographic and climatic characteristics of this site.[6] A recent study has shown that in temperate zones the propolis has mixed origins.[7] The main sources of bee glue from Europe and Asia are the resinous exudate of the buds of poplar trees, mainly the black poplar *Populus nigra,*[8] and birch trees mainly white birch *Betula pubescens*.[7] Another source of variability in the chemical composition and biological activity of propolis is represented by its harvesting period. The studies have shown that there may be differences in the composition of propolis from season to season,[9] and from one month to another.[10]

Due to strong pharmacological properties, non-toxicity and its particular chemical composition, the propolis was used frequently in traditional

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medicine. The biological or pharmacological activity of propolis was mainly associated with phenolic compounds such as flavonoids, phenolic acids and their esters.[11] For the propolis and its constituents, from different sites of the world, certain biological activities have been reported, such as: antimicrobial,[6,12,13] antiprotozoal,[14] antiviral,[15] antitumor,[16-18] immunomodulation,[19,20] anti-inflammatory,[21,22] antioxidant,[23-26] hepatoprotective,[13,27] etc.

Because of its chemical complexity, the complete characterization of propolis involves multiple quantitative and qualitative analyses. Techniques such as thin layer chromatography (TLC), gas (GC) and liquid chromatography (LC), capillary electrophoresis (CE) are used to quantify and identify the individual compounds in propolis.[6,28] Detection is routinely achieved by ultraviolet absorption, often involving a photodiode array detector. Coupled techniques, particularly mass spectrometry, are being used increasingly for routine work. Special attention was given to the analysis of polyphenols in propolis, which are responsible for the majority of its biological activities.

In this work, seven ethanolic extracts of propolis samples collected from different Roumanian zones were analyzed concerning their radical scavenger activities and correlated with their total contents in polyphenols and flavonoids. For the first time, 17 polyphenolic compounds with important biological properties were separated and quantified in propolis samples collected from different locations from Romania using a high performance liquid chromatography with diode array detection (HPLC-DAD) validated method.

MATERIALS AND METHODS

All reagents and solvents were analytical and HPLC grade and were purchased from Sigma-Aldrich Chemie (Steinheim, Germany). Ultrapure water was Mili Q grade (Millipore).

Preparation of extracts

The propolis samples were collected from different regions of Roumania: S1-Arad (South-West); S2- Caras-Severin (South-West); S3-Ialomita (South); S4-Dambovita (South); S5-Teleorman (South); S6-Harghita (Central) and S7-Olt (South). Hand collected propolis were cut in a small pieces, grounded to powder by hand in a porcelain mortar and extracted with 80 % ethanol (1:10 w/v) at 25^{o}C for 10 days in darkness. Afterwards, the waxes were removed by centrifugation. The samples were brought to a final volume of 100 mL and concentrated in a rotary evaporator to obtain the crude extract in paste form (EEP).

The total phenolic content

Total polyphenols of EEPs were obtained using the Folin-Ciocalteu reagent solution.[29,30] Methanolic solutions of propolis extracts (500 μg mL^{-1}) or standard solutions of caffeic acid or methanol as blank (0.2 mL) were transferred to a 10 mL volumetric flask, containing 1.5 mL water, 0.8 mL Folin–Ciocalteu reagent and 1.2 mL 20% sodium carbonate solution (w/v). The volume was adjusted to 10 mL with ultrapure water. The absorbance was measured at 760 nm after 2 h of incubation at room temperature. Total phenolics content was estimated using calibration curve of standard caffeic acid: y=0.0134x - 0.06, R^2=0.9993 in concentration range 10-70 μg mL^{-1}.

The flavone and flavonol content

This was acquired using the method described by Popova *et al.*[30] employing $AlCl_3$ to form a yellow complex, which was measured spectrophotometrically at 425 nm. To 0.2 mL of methanolic solutions of EEP propolis extracts (2 mg mL^{-1}) or standard solutions of galangin or methanol as blank were added 4 mL methanol and 0.2 mL 5% $AlCl_3$ (w/v) and the volume was adjusted to 10 mL with water in a volumetric flask. After 30 min, the absorbance was measured at 425 nm. Total flavone and flavonol content was estimated using the calibration curve of standard galangin: y = 0.025x - 0.0267, R^2=0.9993, in concentration range 5-50 μg mL^{-1}.

The flavanone and dihydroflavonol content was determined with the procedure described by Nagy and Grancai,[31] employing 2,4 dinitrophenylhydrazine (DNP) reaction with ketones and aldehydes to form dinitrophenylhydrazones. At 1 mL of methanolic solutions of propolis extracts (2 mg mL^{-1}) were added 2 mL of DNP solution (1 g DNP in 2mL 96% sulfuric acid, diluted to 100 mL with methanol),

and the mixture was heated 50 min at 50°C, cooled at room temperature, and adjusted to 10 mL with 10% KOH in methanol (w/v). 0.5 mL of final solution was diluted to 25 mL with methanol and the absorbance was measured at 494 nm.[30] The same procedure was applied for blank (1 mL methanol) and standard pinocembrin solutions. Total flavanone and dihydroflavonol contents were estimated using the calibration curve of standard pinocembrin: y = 0.0011x - 0.0385, R^2=0.999, in concentration range 60-840 μg mL^{-1}.

Free radical scavenging activity on DPPH

The free radical scavenging capacity of samples was tested by its ability to bleach the stable 1,1-diphenyl-2-picryl-hydrazyl radical (DPPH).[32] One ml of $DPPH^{\bullet}$ solution (25 mM in MeOH) was added to 0.1 mL of methanolic solutions of EEP (final concentration 40 μg mL^{-1}) and 1.9 mL MeOH.[33] The absorbance decreasing was recorded for 6 min at 517 nm and compared to a blank which contain 0.1 mL of MeOH instead of sample. The antioxidant activity was expressed as mmol Trolox g^{-1} EEP (y = 346069x + 0.0129; R^2 = 0.9988).

Scavenging activity of ABTS radical cation

The 2, 2`-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) radical cation ($ABTS^{\bullet+}$) scavenging activity was measured according to the method described by Erel, with some modifications.[34] The ABTS radical cation was produced by reaction between ABTS stock solution (7mM in water) and 2.45 mM potassium persulfate; the obtained stock solution was preserved in the dark, at room temperature, for at least 12 h to allow the completion of free radicals generation. Next, 2.5 mL of this $ABTS^{\bullet+}$ solution was added to 0.1 mL of methanolic solution of EEP (final concentration 40μg mL^{-1}) and 0.4 mL distilled water; the absorbance decrease was recorded for 6 min at 734 nm. Control sample contained 0.1 mL of MeOH instead of propolis sample. The antioxidant activity was expressed as mmol Trolox g^{-1} EEP (y = 8920.9x + 0.1393 R^2 = 0.9988).

Ferric reducing antioxidant power (FRAP)

The FRAP values were estimated according to the procedure described by Benzie and Strain.[35] The change of absorbance at 593 nm for blue coloured ferrous-tripyridyltriazine (Fe^{3+}-TPTZ) complex in diluted ethanolic extracts of propolis samples was compared with those containing Trolox in known concentrations. Briefly, 1.5 ml of working, warmed at 37°C FRAP reagent (10 volumes of 300 mM acetate buffer, pH 3.6, with 1 volume of 10 mM 2,4,6-tripyridyl-S-triazine in 40 mM HCl, with 1 volume of 20 mM $FeCl_3$) was mixed with 50 μl of methanolic solutions of EEP (final concentration 40 μg mL^{-1}), or standard solutions. The test was performed at 37°C and the 0–4 min reaction time window was used. Standard curve was obtained using different concentrations of Trolox: y = 2.3599 x + 0.0211, R^2 = 0.999. The FRAP value of each sample was expressed in mmol Trolox g^{-1} EEP.

Inhibition of linoleic acid oxidation

The antioxidant activity was evaluated in a linoleic acid (LA) oxidation system.[36] 0.083 mL of sample solution and 0.208 mL of 0.2M sodium phosphate buffer (pH 7.0) were mixed with 0.208 mL of 2.5% (w/v) linoleic acid in ethanol. The peroxidation was initiated by the addition of 20.8 μL of 0.1M AAPH (2,2'-azobis-2-methyl-propanimidamide dihydrochloride) and carried out at 37°C for 100, 200 and 300 min in the dark. The thiocyanate method was used to measure the degree of oxidation by reading the absorbance at 500 nm after colouring with $FeCl_2$ and ammonium thiocyanate. The control was achieved using linoleic acid in the absence of sample solution. Trolox was used as positive control. The lipid peroxidation inhibition (LPI) % was calculated with the formula: LPI (%) = [1-(OD at 500 nm in presence of sample/OD at 500 nm in absence of sample)] x100.

The EEP samples were assessed at a final concentration of 40 μg ml^{-1}, and Trolox at 20 μg ml^{-1} was used as the reference sample. Jasco spectro-photometer V-600 was used for all spectrometric analysis. All the measurements were performed in triplicates.

HPLC-DAD analysis of polyphenols

The assessment of phenolic compounds was performed using a liquid chromatographic system (Shimadzu, Japan) with a diode array detector (DAD). Separation was achieved on a 250 mm x 4.6 mm, 5 μm particle, Fortis C18 (Fortis Technologies Ltd., Cheshire, United Kingdom)

column at 35°C. The mobile phase consisted from a gradient based on water and phosphoric acid at pH 2.5 (solvent A) and acetonitrile (solvent B). The samples were eluted as follows: Step *1*: 30% to 50% B in 0.00-28.00 min; Step *2*: 50% to 62% B in 28.00-45.00 min; Step *3*: 62% to 70% B in 45.00-50.00; Step *4*: 70% to 30% B in 50.00-55.00 min; Step *5*: 30% B in 55.00-60.00 min. Flow rates were initially 0.8 mL min^{-1}, changed to 0.6 mL min^{-1} from 20.00 min to 27.00 min, and then returned to 0.8mL min^{-1}. The injection volume was 20 µL. UV spectra were recorded between 280-400 nm at a rate of 0.8 spectrum s^{-1} and 4.0 nm resolution. Data were acquired and handled by LC Workstation LabSolution/LCsolution software (Shimadzu, Japan). The assignment of peaks in propolis samples was performed using the authentic standards: rutin, caffeic acid, caffeic acid phenethyl ester (CAPE), p-coumaric acid, ferulic acid, cinnamic acid, chrysin, daidzein, luteolin, naringenin, rhamnetin, kaempferol, galangin, myricetin, pinostrobin, pinocembrin, quercetin (all standards purity ≥ 98.0%).

RESULTS AND DISCUSSION

Total polyphenols and flavonoids

Data previously reported have shown that the polyphenol content of EEP from different geographical origins varies greatly. For example, the EEP samples from China,[37] and Europe,[38] were estimated as having the polyphenol content ranging between 31.2 and 299 mg g^{-1} of EEP. The concentrations of total phenolics in the Roumanian ethanolic propolis extracts are high, ranging from 189.49 ± 0.23 to 242.55 ± 0.41 mg caffeic acid g^{-1} of EEP (Table 1). The highest level of phenolics was found in sample S4 (South of Romania) while the lowest was in sample S6 (Central region). Regarding the total flavones and flavonols, all the samples had concentrations up to 90 mg galangin g^{-1} of EEP. The highest amount was found in the samples S3 and S4 (South regions). The results are similar with others obtained on different European propolis samples.[38,39]

Total flavanones and dihydroflavonols contents of ethanolic extracts of propolis samples varied from 25.41 ± 0.21 to 43.57 ± 0.15 mg pinocembrin g^{-1} of EEP (Table 1) with the lowest amount for S6 (Central) and the highest for S3 and S4 (South) propolis samples. Using colorimetric analysis, the different propolis extracts (aqueous, ethanolic, methanolic) from various geographical origins (Argentina, China, Australia, Hungary, Greece, and India) were found to possess similar levels of total phenols (31.2–299 mg g^{-1}) and total flavonoids (2.5–176 mg g^{-1}).[26,37,38,40,41] The study of Lagouri *et al.*[26] on methanolic and aqueous propolis extracts from two different zones from Greece proved similar region diversity in the content of total polyphenols and total flavonoids.

Table 1

Total polyphenol and flavonoid contents of propolis extracts

Propolis	Collecting site	Phenolics[a,b] mg g^{-1} of EEP	Flavones and flavonols[a,c] mg g^{-1} of EEP	Flavanones and dihydroflavonols[a,d] mg g^{-1} of EEP
S1	Arad	216.18 ± 0.15	107.06 ± 0.18	32.12 ± 0.21
S2	Caras-Severin	211.74 ± 0.21	125.23 ± 0.15	36.64 ± 0.22
S3	Ialomita	216.51 ± 0.40	139.32 ± 0.24	43.57 ± 0.15
S4	Dambovita	242.55 ± 0.41	128.52 ± 0.17	37.90 ± 0.20
S5	Teleorman	228.93 ± 0.12	115.04 ± 0.22	33.35 ± 0.19
S6	Harghita	189.49 ± 0.23	90.43 ± 0.21	25.41 ± 0.21
S7	Olt	216.16 ± 0.14	131.84 ± 0.16	36.96 ± 0.30

[a] Values are means ±SD(n=3). [b]As caffeic acid (CA). [c]As galangin. [d]As pinocembrin.

Table 2

Antioxidant activities of propolis samples

Propolis	Collecting site	DPPH[a] (mMTrolox g^{-1} EEP)	ABTS[a] (mMTrolox g^{-1} EEP)	FRAP[a] (mMTrolox g^{-1} EEP)
S1	Arad	3.85 ± 0.01	10.30 ± 0.09	4.17 ± 0.01
S2	Caras-Severin Severin	3.87 ± 0.02	9.08 ± 0.12	4.08 ± 0.02
S3	Ialomita	3.76 ± 0.02	9.27± 0.28	3.92 ± 0.01
S4	Dambovita	4.73 ± 0.01	12.16 ± 0.20	5.48 ± 0.09
S5	Teleorman	4.92 ± 0.01	11.34 ± 0.02	5.03 ± 0.01
S6	Harghita	3.30 ± 0.01	7.98 ± 0.21	4.33 ± 0.01
S7	Olt	3.92 ± 0.01	8.81 ± 0.26	4.24 ± 0.07

[a] Values are expressed as means ± standard deviation of three independent experiments.

Free radical scavenger activity

It is known that more than 300 compounds have been identified in propolis samples.[4] The measurements of antioxidant activity cannot be evaluated satisfactorily using a single antioxidant procedure when the antioxidants are complex mixtures and too many variables influencing the results. Different test procedures are required to evaluate such complex antioxidant activities.[42] Four *in vitro* assays (DPPH, ABTS, FRAP and linoleic acid peroxidation assays) were used to assess the potential antioxidant activity of the ethanolic propolis extracts. The in vitro antioxidant activities were assessed using three methods that involved only a single electron transfer (SET) reaction (DPPH, ABTS and FRAP), and one method involving a hydrogen atom transfer (HAT) mechanism. The inhibition of the AAPH-induced linoleic acid (LA) peroxidation is considered a more physiological and food-relevant test.[43] The results obtained for the EEPs using the DPPH, ABTS and FRAP methods for radical scavenger activities are summarized in Table 2.

The DPPH assay is frequently used to assess the antioxidant activities of natural extracts and compounds which act as free radical scavengers. The antioxidant activity values determined by DPPH assay for individual varieties of propolis extracts decreased in the order: S5 > S4 > S7 (South regions), while the lowest values were for S1, S2 and S6, but the variation between samples is low, the sample from Central Roumania (Harghita, S6) presenting the lowest antioxidant activity. The relationship between DPPH radical scavenging activity of various EEP and total polyphenol content was calculated, and a positive correlation between them was observed (correlation coefficient r = 0.779, data not shown).

Radical scavenging activity of EEP samples in ABTS reaction varied from 7.98 ± 0.2, to 12.16 ± 0.20 mM Trolox g^{-1} EEP. The highest antioxidant activities were recorded for samples S4 and S5 (South Roumania), the opposite being recorded for S6 (Central) and S7 (South). A sample possessing ABTS free radical scavenging activity indicates that its mechanism of action was as a hydrogen donor and ended the oxidation process by converting the free radicals to more stable products.[44] A positive correlation coefficient between polyphenol content and scavenger activity was calculated for the values obtained with ABTS method (correlation coefficient r = 0.705, data not shown). Even the value of this correlation coefficient was lower than that obtained for correlation of total polyphenol and DPPH the results indicate that all EEP samples were more effective in ABTS reaction than in DPPH reaction.

FRAP assay was used to measure the antioxidant capacity from a wide range of biological samples.[45] This assay is different from the DPPH, ABTS and linoleic acid assays because there are no free radicals or oxidants used in such a protocol.[46] It seems that the antioxidant capacity of an antioxidant against a free radical does not necessarily match its ability to reduce $Fe^{3+}$to Fe^{2+}.[47] The ferric reducing antioxidant power (FRAP) of propolis samples showed the same trend observed

in the previous DPPH and ABTS assays and in correlation with other study.[39] Samples S4 and S5 showed significantly higher activities than S3 and S2. In this assay, sample S4 (South Roumania) showed the highest antioxidant activity value (5.48 ± 0.09 mMTrolox g^{-1} EEP). A positive correlation was found between phenolic contents of EEP samples and the FRAP values (correlation coefficient r = 0.796).

The results obtained for the EEP samples by the method based on the inhibition of the AAPH-induced linoleic acid (LA) peroxidation are presented in the Fig. 1. The samples presented high and similar activities and the highest capacity to prevent linoleic acid peroxidation was shown by sample S5 (66.20 %) followed closely by S4, S6 and S1 (64.79%, 60.86%, 60.56). This results were correlated with phenolic contents of EEP samples and a high positive value was noticed (correlation coefficient r = 0.965). Recently, Boufadi *et al.*[48] evaluated the antioxidant effect of Algerian propolis extracts using methods based on DPPH and lipid peroxidation scavenging, and the both methods presented similar results with our data. These results reveal that propolis extracts could induce protection for lipid peroxidation.

In Table 3, the correlation coefficients between the results obtained from the four methods used to assess the antioxidant activity of EEP samples are shown. The best correlation were obtained between the values resulted from DPPH and FRAP methods, r = 0.958, and between DPPH and ABTS methods, r = 0.938.

HPLC-DAD

Seventeen phenolic compounds were identified in Roumanian EEP samples and each component was quantitatively analyzed from the calibration curve of the HPLC chromatogram using authentic compounds (Figure 2). The selectivity of the method was demonstrated by comparison of UV-spectra and retention time. The method was previous developed and validated in our laboratory,[49] and some performance characteristics are presented in Table 4.

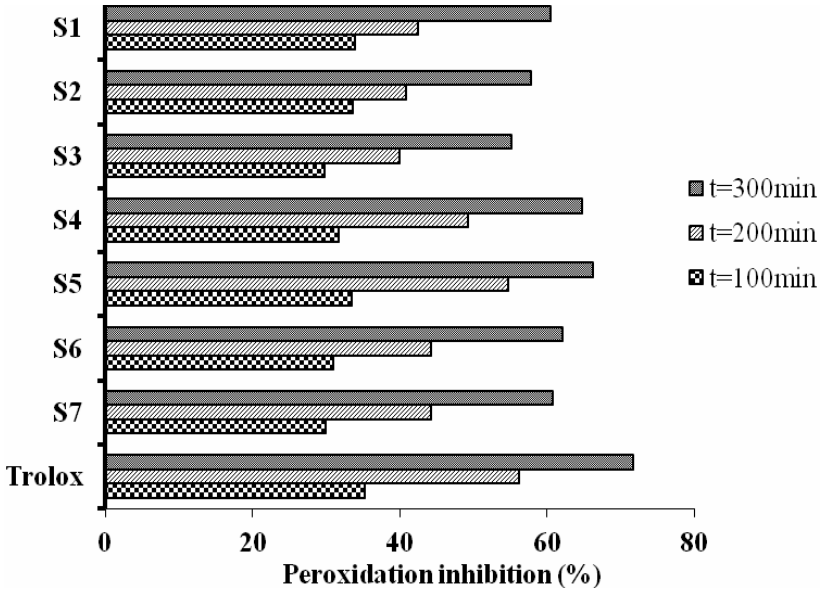

Fig. 1 – Antioxidant activity of Roumania propolis extracts evaluated by linoleic acid peroxidation assays. EEP samples were assessed at a final concentration of 40μg mL^{-1}, and Trolox at 20 μg mL^{-1} was used as the reference sample.

Table 3

Correlation coefficient (r) between the results of DPPH, ABTS, FRAP and LA inhibition assays

Assay	DPPH	ABTS	FRAP	LA
DPPH	-	0.938	0.958	0.701
ABTS	-	-	0.892	0.649
FRAP	-	-	-	0.896

Correlation coefficients were calculated for de final concentration of EEP: 20, 30 and 40 μg mL^{-1}.

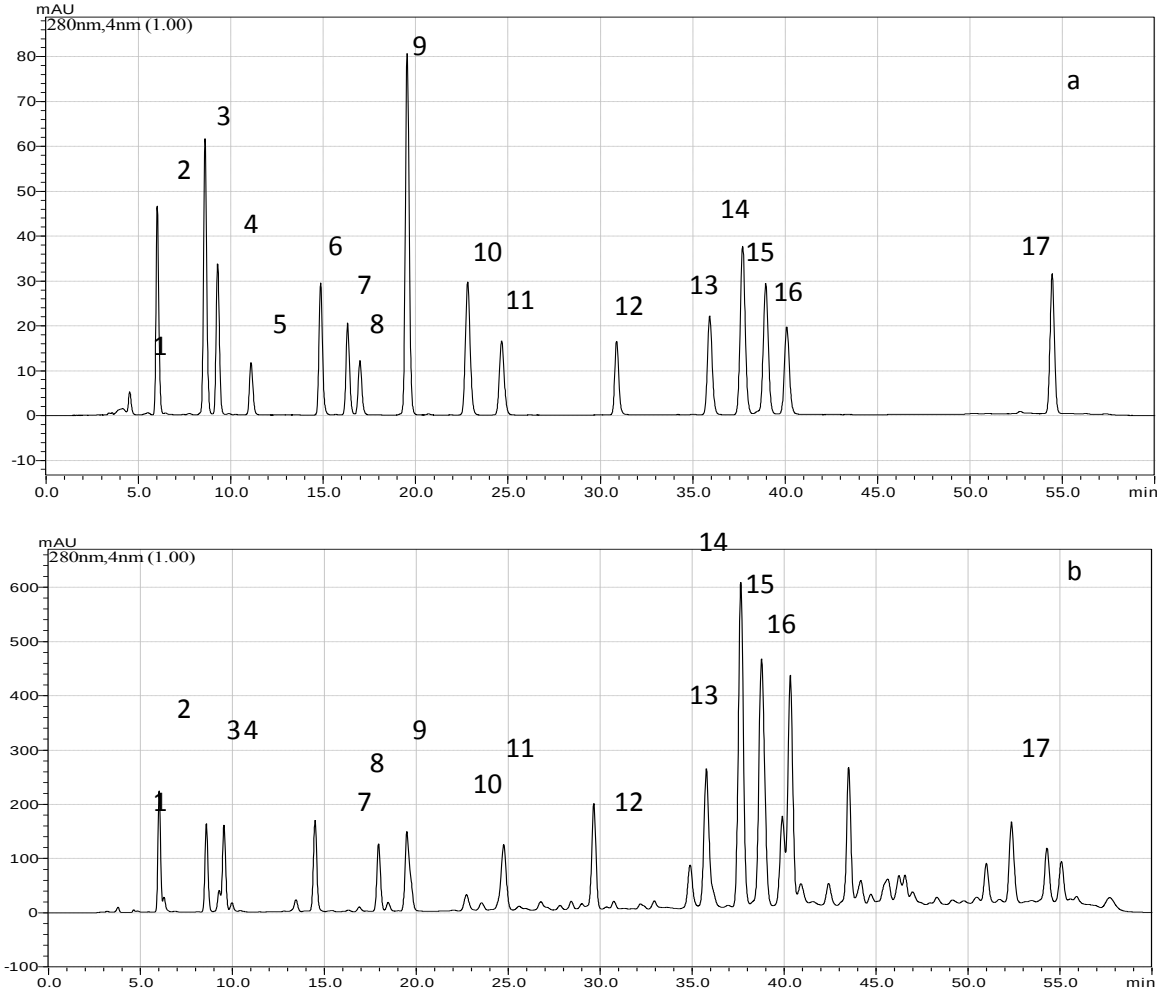

Fig. 2 – Representative schematic diagram of LC chromatograms at 280 nm: (a) Chromatogram of standard solution (a) *1* Rutin; *2* Caffeic acid; *3* Coumaric acid; *4* Ferulic acid; *5* Myricetin; *6* Daidzein; *7* Luteolin; *8* Quercetin; *9* Cinnamic acid; *10* Naringenin; *11* Kaempferol; *12* Rhamnetin; *13* CAPE (Caffeic acid 2-phenylethyl ester); *14* Chrysin; *15* Pinocembrin; *16* Galangin; *17* Pinostrobin; (b) Chromatogram of propolis extract S5.

Table 4

Some chromatographic parameters of phenolic acids and flavonoids

Compound	T_R (min)	Precision		Recovery %	R^2	LOD	LOQ
		Intra-day RSD% n=7	Inter-day RSD% n=6				
Rutin	4.61	2.52	3.28	89.47	0.999	0.16	0.48
Caffeic acid	6.03	2.62	3.97	104.60	0.999	0.05	0.16
Coumaric acid	8.62	2.5	4.69	100.84	0.999	0.04	0.10
Ferulic acid	9.28	1.41	4.53	98.63	0.999	0.05	0.15
Myricetin	11.08	3.53	1.38	89.44	0.999	0.08	0.23
Daidzein	14.86	1.34	1.74	88.12	0.998	0.05	0.15
Luteolin	16.32	3.96	1.24	92.96	0.999	0.06	0.19

Table 4 (continued)

Quercetin	16.95	1.92	1.43	97.63	0.999	0.08	0.25
Cinnamic acid	19.48	1.01	1.97	93.35	0.999	0.04	0.11
Naringenin	22.74	1.40	2.11	98.86	0.998	0.08	0.26
Kaempferol	24.60	3.43	3.06	96.16	0.999	0.05	0.16
Rhamnetin	30.77	2.59	3.96	103.70	0.998	0.07	0.20
CAPE	35.79	1.42	3.17	103.48	0.997	0.06	0.17
Chrysin	37.65	1.73	2.05	99.23	0.998	0.03	0.10
Pinocembrin	38.79	2.48	2.28	107.87	0.997	0.06	0.19
Galangin	39.97	4.40	1.18	95.67	0.998	0.07	0.23
Pinostrobin	54.29	1.80	2.13	102.43	0.998	0.08	0.24

T_R – average migration time of three consecutive analysis of standard solution; RSD – repeatability of migration (%); R^2 – correlation coefficient; LOD – limit of detection = 3xS/N (μg mL^{-1}); LOQ – limit of quantitation=10xS/N (μg mL^{-1}).

Table 5

The distribution of some individual constituents in Roumanian EEP samples

Compound	Content[a] (mg g^{-1} EEP)						
	Arad	Caras-Severin	Ialomita	Dambovita	Teleorman	Harghita	Olt
	S1	S2	S3	S4	S5	S6	S7
Rutin	1.27	1.67	1.37	1.61	1.49	1.39	2.09
Caffeic acid	0.54	0.88	9.34	13.15	14.12	0.62	1.40
Coumaric acid	12.68	7.73	4.22	8.33	9.26	17.30	6.80
Ferulic acid	7.13	2.75	2.23	10.45	2.63	29.13	2.92
Myricetin	0.37	0.45	-	0.48	-	0.45	0.67
Daidzein	5.97	6.23	0.44	0.43	-	2.68	5.22
Luteolin	0.55	0.76	1.43	1.82	1.61	0.80	1.15
Quercetin	1.71	1.91	5.20	6.08	6.21	1.45	3.41
Cinnamic acid	8.52	7.64	3.35	1.50	1.94	4.65	4.92
Naringenin	3.74	3.96	5.67	8.77	6.19	3.33	4.45

Table 5 (continued)

Kaempferol	2.97	2.88	5.77	6.24	5.52	2.49	4.73
Rhamnetin	1.99	2.14	4.66	4.55	4.92	1.66	3.45
CAPE	39.45	36.45	45.73	48.52	41.73	12.83	39.56
Chrysin	28.04	36.72	50.31	61.12	52.67	22.08	37.37
Pinocembrin	53.24	48.65	80.85	87.53	72.60	37.56	49.49
Galangin	29.64	27.59	48.26	49.47	49.29	18.40	32.62
Pinostrobin	12.52	15.56	14.30	13.21	11.92	9.82	13.74

[a] Values are expressed as mean of triplicate analyses for each sample.

The results of the HPLC-DAD quantitative analysis are shown in Table 5. Rutin, myricetin and luteolin are found in the lowest concentrations in the EEP samples. Caffeic and coumaric acids are found in all samples, and their quantities varied significantly, from 0.54 to 14.12 mg g^{-1} EEP and from 4.22 to 17.30 mg g^{-1} EEP respectively. The highest quantity of ferulic acid (29.13 mg g^{-1} EEP) are found in sample S6, sample S2 contains the highest quantity of daidzein (6.23 mg g^{-1} EEP) and sample S5 the highest quantity of quercetin (6.21 mg g^{-1} EEP). All the samples contain cinnamic acid, naringenin, kaempferol and rhamnetin between 1.5 mg g^{-1} EEP and 8.77 mg g^{-1} EEP. The compounds with the highest concentrations in all Roumanian propolis samples were CAPE (caffeic acid 2-phenylethyl ester), chrysin, pinocembrin, galangin and pinostrobin (in good correlation with other results on propolis from other countries.[38,39] The CAPE content in samples varied from 12.83 to 48.52 mg g^{-1} EEP. The highest contents in chrysin (61.12 mg g^{-1} EEP), pinocembrin (87.53 mg g^{-1} EEP) and galangin (49.47 mg g^{-1} EEP) were found in sample S4 while sample S2 had shown the highest content of pinostrobin (15.56 mg g^{-1} EEP).

The variability of individual polyphenols between samples from different sites of Roumania is noticeable but is in accordance with results obtained for total polyphenols, flavonoids, and radical scavenger activities. We could conclude that the sample S4 (Dâmbovita, South Roumania) presented the best characteristics, and the sample S6 (Harghita, Central Roumania) presented less qualities, but further studies are required for more samples from all the regions of Roumania to drow certain conclusions.

CONCLUSIONS

The present work established that ethanolic extracts of Roumanian propolis are an important sources of polyphenols and posses a strong antioxidant activity. The positive, but different correlation coefficients between the total phenolics and the antioxidant activities of samples revealed a complex action of propolis samples against free radicals and lipid peroxidation. The HPLC-DAD analyses of 17 polyphenolic compounds in Roumanian propolis extracts from 7 sites of collection showed the presence of large quantities of flavonoids (crhysin, pinocembrin, galangin and pinostrobin), considered responsible for antimicrobial, antiinflammatory and antioxidant effect, CAPE (caffeic acid phenethyl ester) with antitumor effect, and also caffeic, coumaric and cinnamic acids (with antioxidant effect).[6,13] These results confirmed that Roumanian propolis is a good source of antioxidants and it could be used in food and pharmaceutical industries in order to protect and improve the human health.

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