

Flavonoid Contents of Propolis from the West Side of Romania and Correlation with the Antioxidant Activity

G. Coneac^{*}, E. Gafițanu^{**}, D.I. Hădărușă^{***}, N.G. Hădărușă^{****},
I.A. Pînzaru^{***}, G. Bandur^{***}, L. Urșica^{*}, V. Păunescu^{*****}, A. Gruia^{*****}

^{*} "Victor Babeș" University of Medicine and Pharmacy Timișoara, Faculty of Pharmacy,
300041-Timișoara, P-ța Eftimie Murgu 2, Romania

^{**} University of Medicine and Pharmacy "Gr. T. Popa" Iași, Faculty of Pharmacy, 700115-Iași, Universității Street 16, Romania

^{***} Department of Applied Chemistry and Organic-Natural Compounds Engineering, "Politehnica" University of Timișoara,
Faculty of Industrial Chemistry and Environmental Engineering, 300006-Timișoara, P-ța Victoriei 2, Romania
Phone: +40 256 404224, Fax: +40 256 403060, E-Mail: daniel.hadaruga@chim.upt.ro

^{****} Department of Food Control, Banat's University of Agricultural Sciences and Veterinary Medicine – Timișoara,
Faculty of Food Products Technologies, 300645-Timișoara, Calea Aradului 119, Romania
Phone: +40 256 277373, Fax: +40 256 277326, E-Mail: nico_hadaruga@yahoo.com

^{*****} Immunology and Transplant Centre, 300723-Timișoara, Liviu Rebreanu 159, Romania
Phone: +40 256 489705, E-Mail: gruiia_alexandra@yahoo.com

Abstract: Propolis is a gum-like product which are gathered by bees from various plants, and which vary in color from light yellow to dark brown. It is known that the propolis have antibacterial, anticarcinogenic, and immunostimulating biological activities. Parts of these activities are probable due to the antioxidative effect of some compounds which occur in relatively higher concentrations in propolis (especially flavonoids). In this paper are presented the identification and quantification by HPLC analysis of the main flavonoids from propolis collected from the west side of Romania and the correlation of the antioxidative activity of the propolis extracts (evaluated by the DPPH method) with the flavonoid contents. The significant concentration was observed for quercetin and rutin, caffeic acid, and chrysin; other flavonoids were apigenin and kaempferol (up to 5 mg/g). The antioxidative activity was significant for the 60% and 96% ethanol propolis extracts and this activity has a good correlation with the propolis flavonoid concentrations. The rate of the DPPH consumption in the presence of these extracts were up to tens $\mu\text{M/s}$, this rate being in good correlation with the flavonoid concentration of the propolis extracts.

Keywords: propolis, flavonoids, quercetin, antioxidant activity, DPPH method, HPLC

1. Introduction

Propolis (or "bee glue") is the generic name of the resinous product which is collected by bees from various plant sources [1]. The composition of propolis varies with the source; generally, it is composed by 50% resin and vegetable balsam, 30% wax, 10% essential oils and aromatics, 5% pollen, and 5% other substances [1,2].

Propolis is well known from ancient time due to its biological and pharmacological properties, like immunomodulatory, antitumoral, antimicrobial, antiinflammatory, and antioxidant effects [1-3]. Methods of propolis extraction used in biological assays may influence its activity. The common method is solid-liquid extraction, which use ethanol in different concentrations, methanol or water [4]. The extract contains amino acids, flavonoids, terpenes, and cinnamic acid derivatives. The water extract also contains lectin [4].

The biological activity of propolis is mainly due to the presence of flavonoids. The biochemical effects of

flavonoids can be divided into four categories [1]: (1) binding affinity to biological polymers; (2) binding of heavy metal ions; (3) catalysis of electron transport; and (4) ability scavenge free radicals. Thus, the antitumoral effects of propolis was due to the flavonoids inhibiting the incorporation of thymidine, uridine, and leucine into carcinoma cells, thus leading to an inhibition of DNA synthesis. The flavonoids pinocembrin, galangin and pinobanksin, in addition to *p*-coumaric acid benzyl ester and caffeic acid phenethyl ester (CAPE), demonstrate antimicrobial activity [4-6].

The aim of this study is to continue the series of studies of our team on the extraction, purification, analysis, and cyclodextrin nanoencapsulation of biocompounds from natural sources [7-10] with the identification and quantification of the main flavonoids from propolis collected from the west side of Romania and the correlation of the antioxidative activity of the propolis extracts (evaluated by the DPPH method) with the flavonoid contents.

2. Materials and method

Materials. Propolis used for flavonoid extraction and antioxidative activity evaluation was collected from the west side of Romania (Banat county) in 2006 and it's obtained by honeybees from linden, acacia, and poplar. In order to obtain propolis extracts 96% (v/v) ethanol (reagent grade, Chimopar București) was used. For quantitatively evaluation of the main compounds from propolis extracts analytical grade standard flavonoids and cinnamic acid derivatives were used (rutin, >90%, Fluka; quercetin, apigenin, kaempferol, acacetin, chrysin, pinocembrine, >99%, Sigma-Aldrich; cinnamic acid, caffeic acid, >98%, Sigma-Aldrich). DPPH (1,1-diphenyl-2-picryl-hydrazyl), reagent grade, used for the antioxidative activity evaluation, was obtained from Merck.

Propolis extracts. Fine ground propolis was extracted with water or ethanol (different concentrations) at room temperature or higher, according to our previous work [10]. Thus, one gram of propolis (finely grounded in a mixer) was extracted with 10-20 ml water or 20, 60, 96% ethanol for one hour at 65°C, or for six days at room temperature (in sealed flasks, at ~25°C). After that, the suspension was filtered, washed with one ml 96% ethanol; the extracts were sealed and stored at 4°C until HPLC and antioxidative activity analyses. The general code used for these extracts was "P(H/C)_(0/20/60/96)_(a/b/c)", where P – is for propolis sample, H/C – is used for "hot" or "cold" extraction, 0/20/60/96 – denote the concentration of ethanol used for extraction ("0" means water extract), and a/b/c – denote the duplicate/triplicate of the same sample; "hot" extracts were obtained in triplicate (a/b/c samples) and "cold" extracts were obtained in duplicates (a/b samples).

HPLC analysis. For the quantification of flavonoids, cinnamic acid and its derivatives in propolis extracts and further for the evaluation of these bioactive compounds in propolis, an Agilent 1100 HPLC apparatus was used; for the HPLC analysis a Zorbax SB-C18 column, with 250 mm length, 4.6 mm i.d., and 5 μm particle diameter was used; for flavonoid detection the wavelength of 337 nm was set; the mobile phase was acetonitrile:water at 48:52 ratio; the temperature was 25°C, with a flow of 0.3 ml/min; the injected sample volume was 20 μl. Diluted standard solutions of rutin, quercetin, apigenin, kaempferol, acacetin, chrysin, pinocembrine, cinnamic acid, and caffeic acid were analyzed in the same HPLC conditions and furthermore the calibration of the detector response was done. The calibration curves were used for the quantification of the main bioactive compounds from propolis.

Antioxidative activity. Propolis extracts were analyzed from the antioxidative point of view using DPPH method. The reaction of DPPH was spectrophotometrically measured for 180 s at 517 nm on a Perkin Elmer, Lambda EZ Series (for "hot" extracts) and on a CamSpec M 501 spectrophotometer (for "cold" extracts), and the data were analyzed using the corresponding PESSW, ver. 1.2, Revision E and UV-Vis Analyst ver. 4.67 acquisition&data handling soft, respectively. The crude propolis extracts and

tenth fold diluted samples were mixed with 1 mM DPPH ethanolic solution in 96% ethanol and the decrease of the absorbance at 517 nm was measured (the ratio of propolis extract : DPPH solution : ethanol were 1 : 1 : 13). The antioxidative activity of the propolis extracts was evaluated as relative absorbance of the sample in the presence of DPPH solution after the constance of the absorbance at 517 nm ($A\% = A(t)/A(t=0)*100$).

Rate of the DPPH consumption. The rate of the DPPH consumption in the presence of propolis extract sample was evaluated for 0-20 s for raw samples and 0-50s for the tenth-fold diluted samples, using the formula:

$$v = -\frac{dc_{DPPH}}{dt} \quad (\mu M/s) \quad (1)$$

where v is the DPPH consumption rate ($\mu M/s$), dc_{DPPH}/dt – is the first derivative of the DPPH concentration in time.

3. Results and discussion

On the basis of calibration curves from **HPLC analysis** for standard flavonoids and cinnamic acid derivatives, the concentration of these bioactive compounds in extracts and furthermore in propolis (raw material) was evaluated.

Thus, for the water "hot" propolis extracts, P(H)_0_a, only rutin, caffeic acid, and quercetin were identified. For rutin, the retention time cannot be exactly established (6.7-7.0 min) probably due to the partial hydrolytic degradation of this compound under HPLC conditions (Fig. 1).

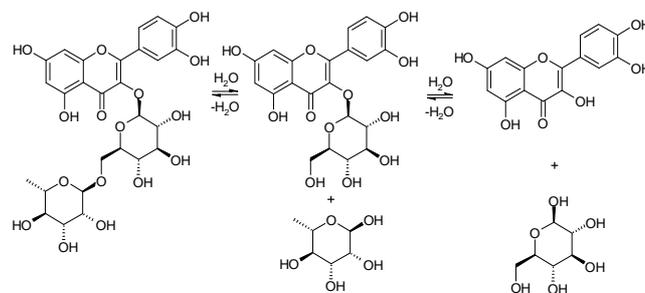


Figure 1. Hydrolytic degradation of rutin

In the case of standard quercetin, two very close peaks appear on the chromatogram (at ~13 min); this fact can be explained by the possibility of enolization of quercetin (Fig. 2).

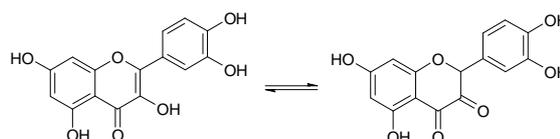


Figure 2. Enolic and ketonic forms of quercetin

The concentrations of these compounds extracted from propolis with water were 0.3 mg/g propolis for rutin, 0.1 mg/g for caffeic acid and 0.13 mg/g for quercetin. For these HPLC conditions (non-polar column), the more hydrophilic compounds (like rutin and caffeic acid) are separated in the first part of chromatogram. Not all separated compounds can be identified. For the similar "cold" extract the concentration of rutin was lower (0.06 mg/g), but the concentrations of caffeic acid and quercetin were higher (2.1 mg/g and 0.7 mg/g, respectively) (Fig. 3). Concentrations under 0.2 mg/g were obtained for all the rest of flavonoids identified in both water extracts.

For the "hot" propolis extracts conducted in 20% ethanol (in triplicate, $P(H)_{20_a/b/c}$), a great number of compounds were separated by HPLC analysis, especially those more hydrophilic, which are separated in the first part of chromatogram (Fig. 4). Among rutin, caffeic acid, and quercetin, there were also identified apigenin, kaempferol, and chrysin. The most concentrated was caffeic acid (3 mg/g), followed by rutin and chrysin (1.6 and 2.2 mg/g, respectively; Table 1). For the "cold" extract (in duplicate, $P(C)_{20_a/b}$, Fig. 4 and Table 1), rutin have been identified in similar concentration (2.2 mg/g), but caffeic acid was identified in concentration of 5.6 mg/g, quercetin 1.4 mg/g, apigenin 1.5 mg/g, and chrysin 0.05 mg/g. No significant concentration of kaempferol was identified in "cold" extract.

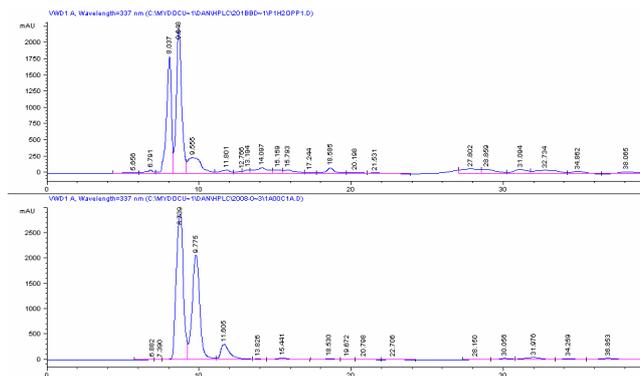


Figure 3. HPLC chromatograms of "hot" (up) and "cold" (down) water extracts

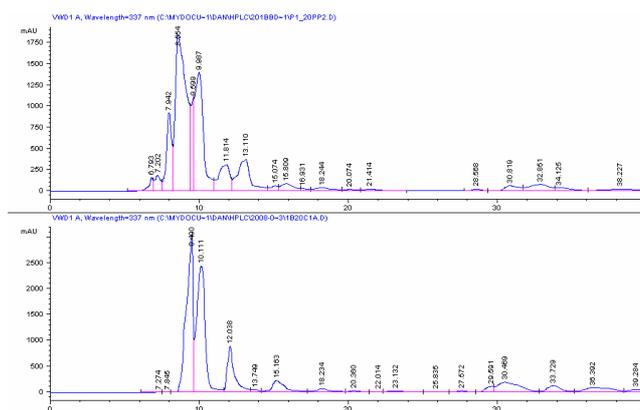


Figure 4. HPLC chromatograms of "hot" (up) and "cold" (down) 20% ethanol extracts

TABLE 1 Bioactive compounds (flavonoids and cinnamic acid derivatives) identified and quantified in "hot" and "cold" 20% ethanol propolis extracts

No	Code	Rutin (mg/g)	Caffeic acid (mg/g)	Quercetin (mg/g)	Apigenin (mg/g)	Kaempferol (mg/g)	Chrysin (mg/g)
1	$P(H)_{20_a}$	1.082	2.56	0.298	0.289	0.222	0.955
2	$P(H)_{20_b}$	2.096	6.223	1.128	0.773	0.137	0.638
3	$P(H)_{20_c}$	1.601	0.316	0.469	0.213	0.164	1.03
$P(H)_{20}$ (Mean \pm SD)		1.59 ± 0.51	3.03 ± 2.98	0.63 ± 0.44	0.43 ± 0.3	0.17 ± 0.04	0.87 ± 0.21
4	$P(C)_{20_a1}$	0.07	1.258	0.098	0.736	-	0.05
5	$P(C)_{20_a2}$	0	5.524	0.104	1.133	-	0.069
6	$P(C)_{20_b1}$	0	8.004	2.645	2.137	-	0.059
7	$P(C)_{20_b2}$	2.246	7.733	2.595	1.836	-	0
$P(C)_{20}$ (Mean \pm SD)		0.58	5.63 ± 3.12	1.36	1.46 ± 1.64	-	0.05 ± 0.03

For the propolis extracts conducted with ethanol at higher concentrations (60% and 96%, v/v) a significant increase of the ratio of more hydrophobic compounds can be observed (over 25 min, Figs. 5-8). Some of them cannot be identified.

The quantification of bioactive compounds from propolis (evaluation from extracts) in the case of using 60% and 96% ethanol as extraction solvent is shown in Tables 2 and 3.

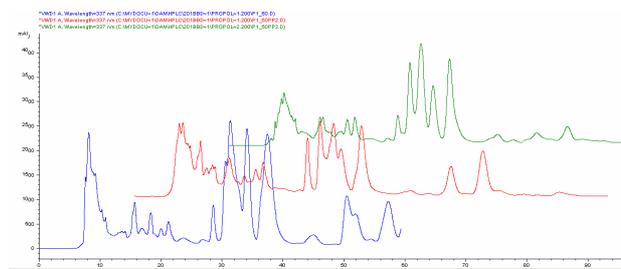
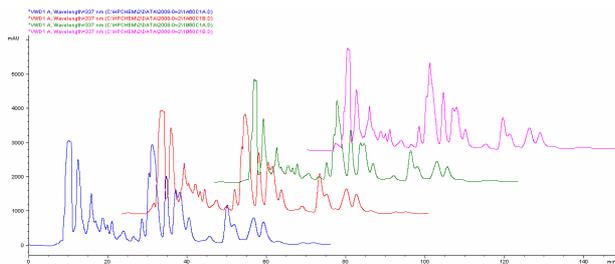
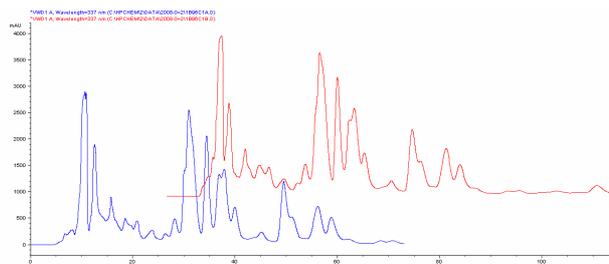
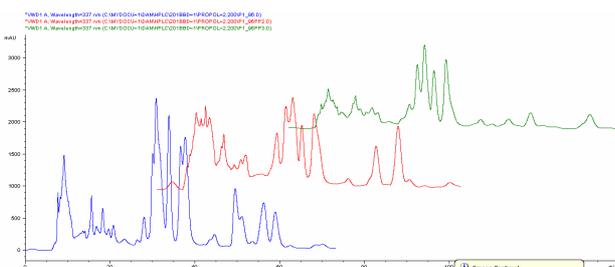


Figure 5. Superimposed HPLC chromatograms of the $P(H)_{60_a/b/c}$ extracts

Figure 6. Superimposed HPLC chromatograms of the $P(C)_{60_a1/a2/b1/b2}$ extractsFigure 8. Superimposed HPLC chromatograms of the $P(C)_{96_a/b}$ extractsFigure 7. Superimposed HPLC chromatograms of the $P(H)_{96_a/b/c}$ extracts

A higher concentration of more hydrophobic bioactive compounds (apigenin, kaempferol, chrysin) can be observed in these cases, compared with the more hydrophilic ones (caffeic acid and quercetin). Rutin cannot be quantified, probably due to the interference with other similar compounds (like saccharide derivatives). In both cases apigenin, kaempferol, and chrysin were in comparable concentrations, probably close to the real ones in raw propolis (5 mg/g propolis for apigenin, kaempferol 2 mg/g, chrysin 20-26 mg/g in the case of "hot" extracts, and apigenin 8-14 mg/g, kaempferol 3 mg/g, chrysin 10-32 mg/g for "cold" extracts; Tables 2 and 3).

TABLE 2. Bioactive compounds (flavonoids and cinnamic acid derivatives) identified and quantified in "hot" and "cold" 60% ethanol propolis extracts

No	Code	Rutin (mg/g)	Caffeic acid (mg/g)	Quercetin (mg/g)	Apigenin (mg/g)	Kaempferol (mg/g)	Chrysin (mg/g)
1	$P(H)_{60_a}$	1.709	1.745	1.357	8.132	3.544	39.789
2	$P(H)_{60_b}$	45.4	5.476	2.5	5.047	1.43	18.392
3	$P(H)_{60_c}$	4.983	1.179	1.256	2.721	1.33	19.921
$P(H)_{60}$ (Mean \pm SD)			2.8 ± 2.3	1.7 ± 0.7	5.3 ± 2.7	2.1 ± 1.3	26 ± 11.9
4	$P(C)_{60_a1}$	-	2.223	8.644	7.827	2.287	14.532
5	$P(C)_{60_a2}$	-	0.52	8.742	1.686	3.234	24.69
6	$P(C)_{60_b1}$	-	8.667	10.664	8.12	2.712	-
7	$P(C)_{60_b2}$	-	13.928	11.82	15.331	3.5	31.929
$P(C)_{60}$ (Mean \pm SD)			6.34 ± 6.16	9.97 ± 1.55	8.24 ± 5.58	2.93 ± 0.54	9.81

TABLE 3. Bioactive compounds (flavonoids and cinnamic acid derivatives) identified and quantified in "hot" and "cold" 96% ethanol propolis extracts

Nr	Cod	Rutin (mg/g)	Acid cafeic (mg/g)	Quercetină (mg/g)	Apigenină (mg/g)	Kaempferol (mg/g)	Crisină (mg/g)
1	$P(H)_{96_a}$	10.59	1.947	2.28	6.323	2.923	30.223
2	$P(H)_{96_b}$	8.878	3.899	8.704	6.989	1.601	17.191
3	$P(H)_{96_c}$	5.078	1.517	1.665	1.617	1.601	12.089
$P(H)_{96}$ (Mean \pm SD)			2.45 ± 1.27	4.2 ± 3.9	5.0 ± 2.9	2.04 ± 0.8	19.8 ± 9.4
4	$P(C)_{96_a1}$	0	8.753	0	0	-	31.929
5	$P(C)_{96_a2}$	0	0.306	0	0	-	0
6	$P(C)_{96_b1}$	6.521	0	14.188	13.457	-	0
7	$P(C)_{96_b2}$	0	0	12.251	14.077	-	0
$P(C)_{96}$ (Mean \pm SD)			4.5	13.2	13.8	-	31.9

Antioxidant activity of propolis extracts was evaluated by DPPH method, the alure of the absorbance (at 517 nm) of the propolis extract-DPPH solution in time being inverse logarithmic (Figs. 9 and 10). For unitarity, the relative absorbance, $A\%$, as ratio between absorbance at time t and initial absorbance (percentual values), was calculated, and the $A\%$ values used for comparison were those values from the t time when $A\%$ became constant. Lower $A\%$

values denote higher antioxidant activity. Thus, the best results were obtained in the case of "hot" and "cold" 60% ethanol propolis extracts ($A\% = 7\%$ and 5% , respectively), followed by the the 96% ethanolic extracts. The worst results were obtained in the case of water and 20% ethanolic extracts. All these results were in good correlation with the overall concentration of flavonoids in propolis.

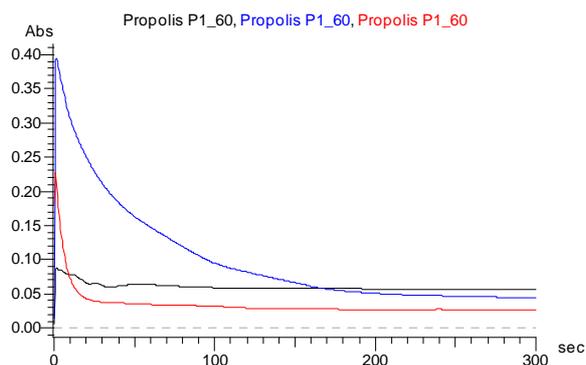


Figure 9. Superimposed *Absorbance vs. time* curves for "hot" 60% ethanol propolis extracts (undiluted and diluted ones)

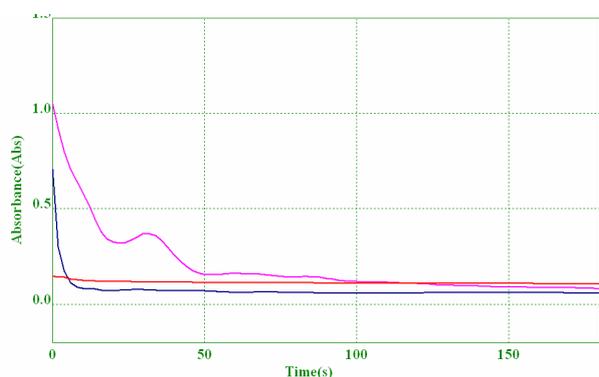


Figure 10. Superimposed *Absorbance vs. time* curves for "cold" 60% ethanol propolis extracts (undiluted and diluted ones)

Similar results were obtained in the case of **DPPH consumption rate**, calculated as mean rate for the pseudo-linear part of the *DPPH concentration (μM) vs. time (s)* curve (Figs. 11 and 12). Thus, for the "hot" water and 20% ethanol propolis extracts the rate values (for first tenth dilution of extracts) were similar ($0.6 \mu\text{M/s}$), but for the same diluted 60% and 96% ethanolic extracts these values were 4-fold higher ($\sim 2.6 \mu\text{M/s}$). In the case of "cold" extracts, these rates were higher ($\sim 2.5 \mu\text{M/s}$ for extracts with diluted ethanol, and $\sim 9.3 \mu\text{M/s}$ for the concentrated ones; values for tenth fold extract dilution), that are in good concordance with the concentration of flavonoids (in all cases the overall concentrations of flavonoids and caffeic acid in "cold" extracts were higher than in "hot" extracts).

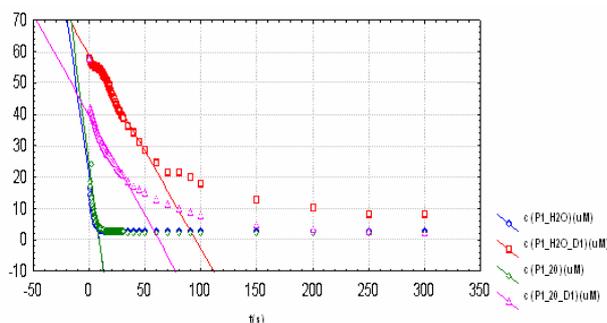


Figure 11. *DPPH concentration (μM) vs. time (s)* for the water and 20% ethanol propolis extracts antioxidant activity evaluation

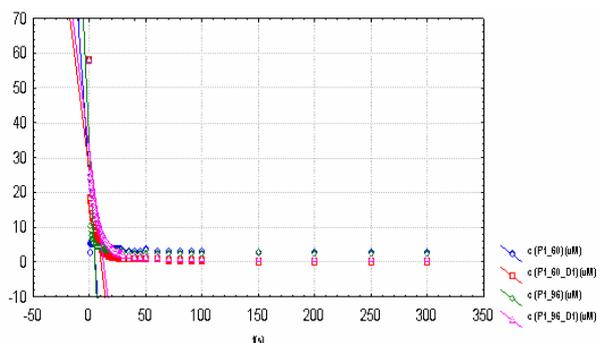


Figure 12. *DPPH concentration (μM) vs. time (s)* for the 60% and 96% ethanol propolis extracts antioxidant activity evaluation

4. Conclusion

The following conclusions can be drawn among to these analyses and antioxidative activity evaluations of propolis extracts: (1) significant concentrations of flavonoids and caffeic acid were determined in the case of romanian propolis extracts, especially by using concentrated ethanol as solvent; (2) propolis extracts obtained at lower temperature for long time have higher concentrations of flavonoids compared with those obtained at higher temperature in a short time; (3) the best results on the antioxidative activity evaluation were obtained in the case of 60% ethanolic propolis extracts; and (4) the antioxidative activity and DPPH consumption rate were in good correlation with the overall flavonoids and caffeic acid concentrations.

REFERENCES

- Burdock, G.A., Review of the Biological Properties and Toxicity of Bee Propolis (Propolis), *Food and Chemical Toxicology* Vol. 36, **1998**, pp. 347-363.
- Sforzin J.M., Propolis and the Immune System: a review, *Journal of Ethnopharmacology* Vol. 113, **2007**, pp. 1-14.
- Weston R.J., The Contribution of Catalase and Other Natural Products to the Antibacterial Activity of Honey: a review, *Food Chemistry* Vol. 71, **2000**, pp. 235-239.
- *** The Review of Natural Products, DerMarderosian A., Beutler J.A. (eds.), Wolters Kluwer Health, Inc., St. Louis, **2004**.
- Havsteen B.H., The Biochemistry and Medical Significance of the Flavonoids, *Pharmacology & Therapeutics* **2002**, 96, 67-202
- *** The Science of Flavonoids, Grotewold E. (ed.), Springer Science & Business Media, Inc., New York, **2006**.
- *** Antioxidants and cardiovascular disease, 2nd ed., M.G. Bourassa and J.C.Tardif (eds.), Springer Science & Business Media, Inc., New York, **2006**.
- Hădăruță N.G., Hădăruță D.I., Păunescu V., Tatu C., Ordodi L., Bandur G., Lupea A.X., Bioactive Nanoparticles (6). Thermal Stability of Linoleic Acid / α - and β -Cyclodextrin Complexes, *Food Chemistry* Vol. 99(3), **2006**, pp. 500-508.
- Hădăruță D.I., Hădăruță N.G., Resiga D., Pode V., Dumbravă D., Lupea A.X., Obtaining and Characterization of Sage (*Salvia sclarea* L.) Essential Oil/ β -Cyclodextrin Supramolecular Systems, *Revista de Chimie* Vol. 58(6), **2007**, pp. 566-573.
- Hădăruță N.G., Hădăruță D.I., Riviș A., Păunescu V., Costescu C., Lupea A.X., Bioactive Nanoparticles. Essential Oil from *Lamiaceae* Family Plants / β -Cyclodextrin Supramolecular Systems, *Revista de Chimie* Vol. 58(10), **2007**, pp. 909-914.
- Coneac G., Gafițanu E., Hădăruță D.I., Hădăruță N.G., Riviș A., Bandur G.N., Pinzaru I.A., Rusu G., Urșica L., Păunescu V., Gruia A., Sebeșan M., Grozescu I., Lazău C., Sfirloagă P., Propolis Extract / β -Cyclodextrin Nanoparticles: Synthesis, Physico-Chemical, and Multivariate Analyses, *Journal of Agroalimentary Processes and Technologies* Vol. 14(1), **2008**, pp. 58-70.