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COMPARATIVE STUDY OF POLYPHENOLS FROM PROPOLIS EXTRACTS OF DIFFERENT ORIGIN

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ABSTRACT. The propolis, a resinous substance produced by bees, was used from ancient times as one of the best general panaceea. The main active compounds known in propolis are the phenolic acids and flavonoids from the class of polyphenols. This paper presents the flavonoids and phenolic acids evaluation by chromatographic (TLC and HPLC) methods of some propolis samples originating from Arad (4 samples) and Bihor (3 samples) counties, from west of Romania. There were identified the caffeic, ferulic and gallic acids respectively the chrysine and kaempferol. The chrysine content ranges from 0.15 to 1.95 mg/ml and the kaempferol from 0.07 to 8.88 mg/ml. The caffeic acid content ranges from 0.05 to 0.70 mg/ml and the ferulic acid from 0.01 to 1.39 mg/ml.

Keywords: *propolis, polyphenols, flavonoids, phenolic acids, TLC, HPLC.*

INTRODUCTION

Propolis means, in greek language, some that defend the city or the hive. The propolis is a resinous product, made by the honey bees (*Apis mellifera* L.) from the waxes and resinous compounds collected from the trees and other plants. The propolis can have different aspects, generally it is a solid product with gumy aspect, with a color from ocker yellow to red, brown, light brown or greenish [1]. The bees use the propolis to protect the hive against infections, bacterias or fungus.

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The propolis has been used since ancient times because of its therapeutic effects. It can be used for the treatment of many diseases, because it shows antibacterial, antiseptic and detoxifying effects. It is a basic product used in apitherapy, an alternative medicine very popular in the antic Egypt, Greece and China.

The composition of propolis depends on various factors such as season and vegetation of the area. The chemical analysis of raw propolis show the presence of resins, waxes, essential oil, polyphenols, sugars, aminoacids, vitamins, enzymes, mineral salts, pollen and other solid impurities [2]. The recent studies do not have quantified more than 2-3 % of essential oil represented by aromatic compounds like benzyl-derivatives, vanilin, eugenol in European propolis, sesquiterpenes in Asian propolis and monoterpenes in South American propolis [3]. An other active compound class that is quantitatively important in propolis is the polyphenols represented by flavonoids, mostly aglyka of the glycosidic flavonoids from the plant species from that the bees collect the substances, respectively phenolic acids, mostly hydroxybenzoic derivatives like caffeic acid, ferulic acid or gallic acid [4].

European, Chinese and Argentinian propolis are characterized by the presence of phenolic acids and flavonoids, the most abundant being chrysin (2–4%) [5]. The total phenolic compound content found in ethanolic extract of red propolis (232 mg/g) was higher than that ever found for Brazilian propolis samples [6,7]. These values were similar to those find in temperate climate propolis originating from the species *Populus* sp., a resin-producing plant rich in polyphenols [7,8]. The low flavonoid concentration (43 mg/g) observed in ethanolic extract was similar to that normally found for Brazilian green propolis [8].

Due to its complex chemical composition, a lot of benefic effects of propolis and its extracts were identified. Recent studies highlighted the beneficial effect of hive products on health as they improve circulation, reduce inflammation and stimulates immunity. Propolis is known as one of the most powerful natural antibiotic. The presence of polyphenols and essential oil impart antimicrobial, antifungal, antitumoral, anti-inflammatory, hepatoprotective, antidiabetic, cardioprotective, antiangiogenic and immunomodulatory properties. Moreover, it was proved that propolis contains compounds which regenerate the damaged tissues, improve the liver and pancreas functions and have epithelisant, anti-edematous, radioprotective and antiasthmatic effects [9-11].

Due to its complex composition and special powerful therapeutic effects propolis was used to prepare several medicinal products, administered internally or applied externally and special cosmetic products – shampoo, creams, etc. This wide range of uses is based on its antioxidant effect conferred by the high content of polyphenols belonging mainly to flavonoids and caffeic acid derivatives.

In Romania the apiculture is a wide spread agricultural activity. The bee products were used and studied from long time. Mărghitaș et al. have studied the propolis from Transylvania, Cluj, Hunedoara, Brașov counties from agricultural, chemical and therapeutic point of view. They found that the Transylvanian propolis contains 0.55 – 3.91 mg/g chrysin, 0.56 – 2.66 mg/g galangine and 0.46 – 1.49 mg/g caffeic acid [12,13]. The Moldavian propolis was studied by Croci et al., finding in three different origin samples high quantities of phenolic acid, mainly caffeic acid, ferulic acid, 3,4-dimethoxycinnamic acid and protocatehic acid and also a high content of total flavonoids, around 25 % expressed in quercetine [14,15]. Coneac et al. [16,17] have studied the propolis from Timiș county, Banat, west part of Romania. The aim of their research was to optimize the extraction conditions of polyphenols from propolis and to standardize the hydroalcoholic extract. They have found in the three studied samples important quantities of quercetine (0.386 – 13.2 mg/g), apigenine (0.213 – 13.8 mg/g), kaempferol (0.137 – 3.198 mg/g), rutoside (0.496 – 37.184 mg/g), chrysin (0.638 – 31.9 mg/g) respectively caffeic acid (0.316 – 19.365 mg/g). The qualitative and quantitative analyzes were performed by chromatographic (TLC, HPLC) and spectral methods [12-17], being employed also image analysis combined with appropriate fuzzy clustering method [18].

This study presents the evaluation of the chemical quality of propolis from west part of Romania.

RESULTS AND DISCUSSION

The first step of monitoring of polyphenols from propolis was made by TLC. This analysis show the polyphenol fingerprint of studied samples and can be evaluated the similarities and the differences. In figure 1 and 2 are presented the TLC chromatogram of the studied propolis samples.

The TLC chromatogram show the presence of more polyphenols in the samples originating from Covăsânț, Dorgos and Ștei respectively Livada Beiuș. Caffeic acid and chrysine were identified in all samples; Covăsânț, Dorgos, Ștei and Livada Beiuș samples having the highest concentrations. The less concentrated in polyphenols is the sample from Lipova. It can be observed a lot of similarities and differences between the studied samples, both from qualitative and quantitative point of view. The similarities are due probably by the collection of the pollen and waxes from the same species, the differences are due by the specific species from the bees harvesting areas. In the sample from Dorgos can be seen some special compounds colored in red that are not present in other studied propolis samples.

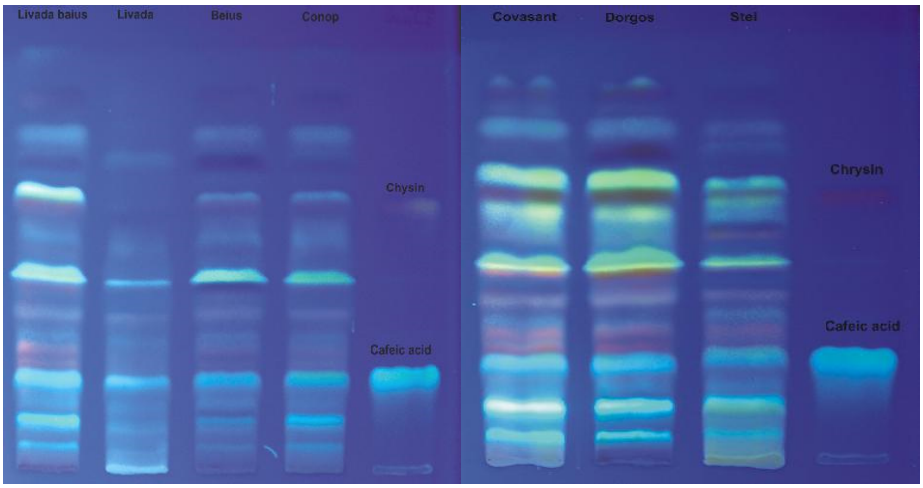

Figure 1. TLC chromatogram in fluorescence at 365 nm, after spraying with Neu-PEG reagent

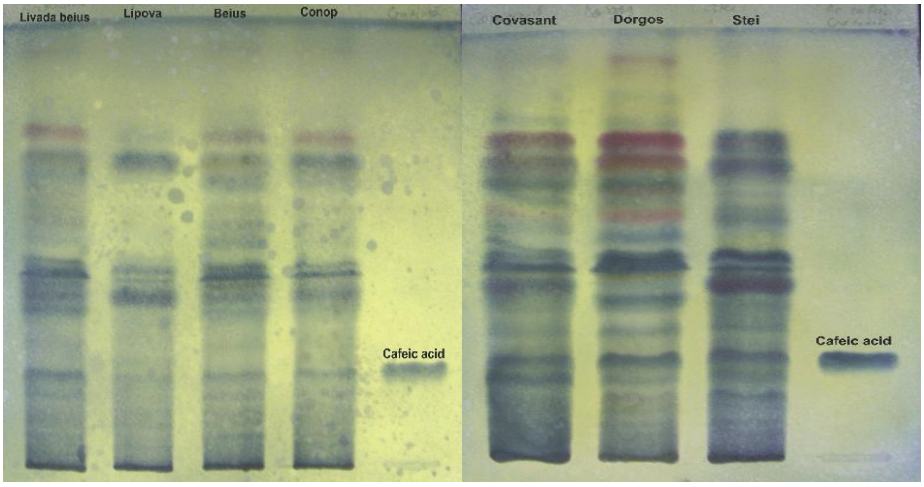

Figure 2. TLC chromatogram in visible light, after spraying with anisaldehyde and phosphomolibdenic reagents

To determine more accurately and to quantify individualy some of polyphenols HPLC analysis was performed. In figures 3-5 are presented the obtained HPLC chromatograms. In table 1 are presented the retention times and the wavelength corresponding to the maximum absorbance from UV-VIS spectra for standards and the separated compounds from the

studied propolis samples. The identification of the individual compounds is based on the comparison of retention times, maximum wavelength values and UV-Vis spectra shapes of the standards and the separated compounds.

It can be observed the presence of caffeic acid in all studied samples, the ferulic acid in the samples originating from Conop, Arad county respectively in all 3 samples from Bihor county. The gallic acid was found just in the sample from Ștei. As flavonoids, kaempferol was found in the samples from Covăsânț, Dorgos, Ștei and Beiuș, respectively chrysine in the samples from Conop, Covăsânț and Ștei.

In figure 6 are presented the calibration curves for the identified compounds (caffeic acid, ferulic acid, gallic acids, chrysine and kaempferol). In table 2 are presented the equations for the calibration curves, correlation factors and the concentrations determined in the propolis extracts.

It can be observed also some peaks that cannot be identified due to lack of standards. So, the compound separated at 1,7 minutes is present in all samples (less Beiuș); 7,6-7,8 minutes in all samples; 10,2-10,9 minutes respectively 22,2-23,4 minutes in all samples (less Ștei); 39,5-41,0 minutes is present just in the samples from Bihor county. These similarities can be explained based on the similar species from the bees harvesting area, while the differences appear probably because of some species specific only for that area.

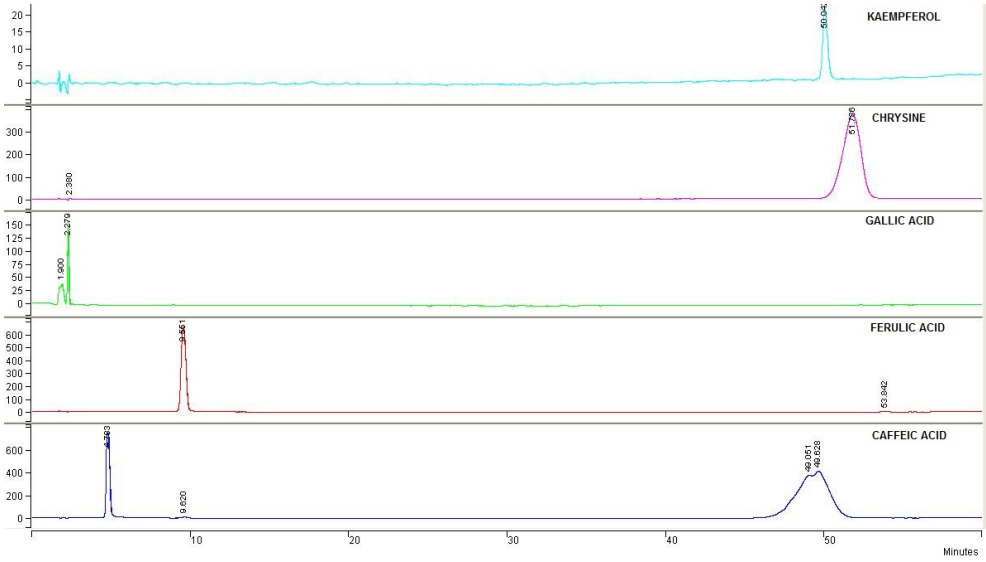

Figure 3. The HPLC chromatogram of standards

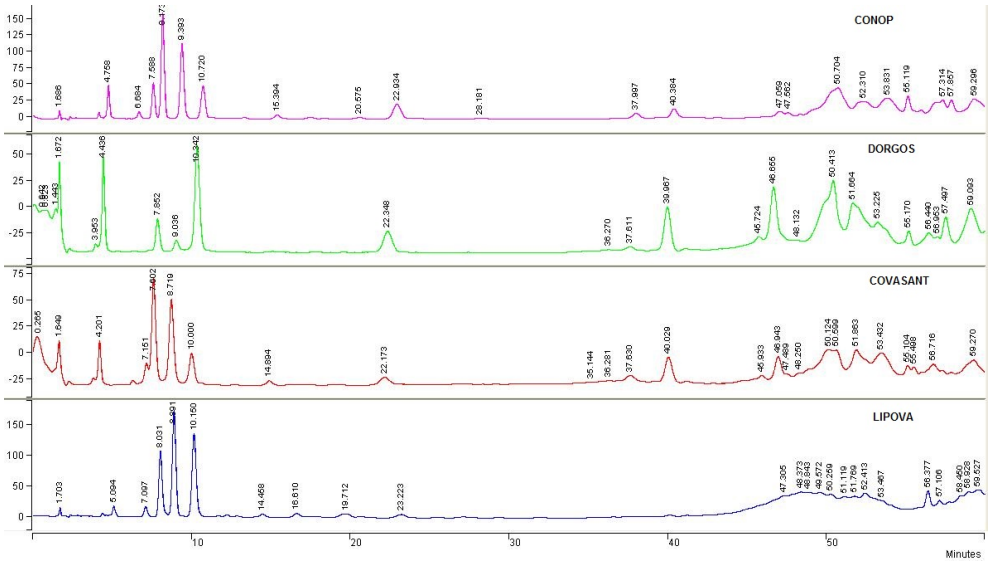

Figure 4. The HPLC chromatogram of the samples from Arad county

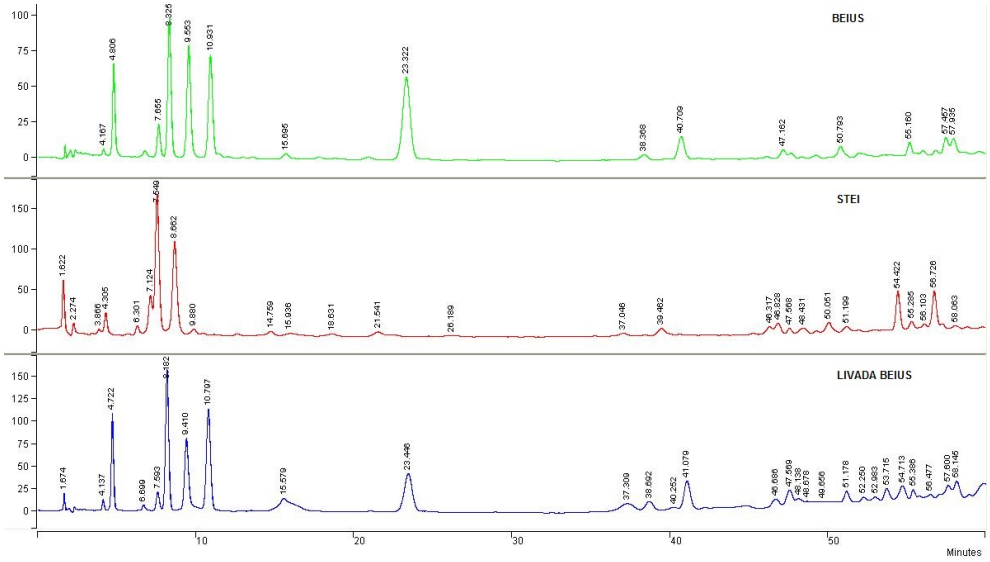

Figure 5. The HPLC chromatogram of the samples from Bihor county

The HPLC results confirm those obtained in the first TLC monitoring step.

HPLC quantitative assessment reveals a high content of flavonoids and phenolic acids in the Dorgos (Arad) sample and less in the Lipova (Arad). These results confirm also the TLC analysis findings.

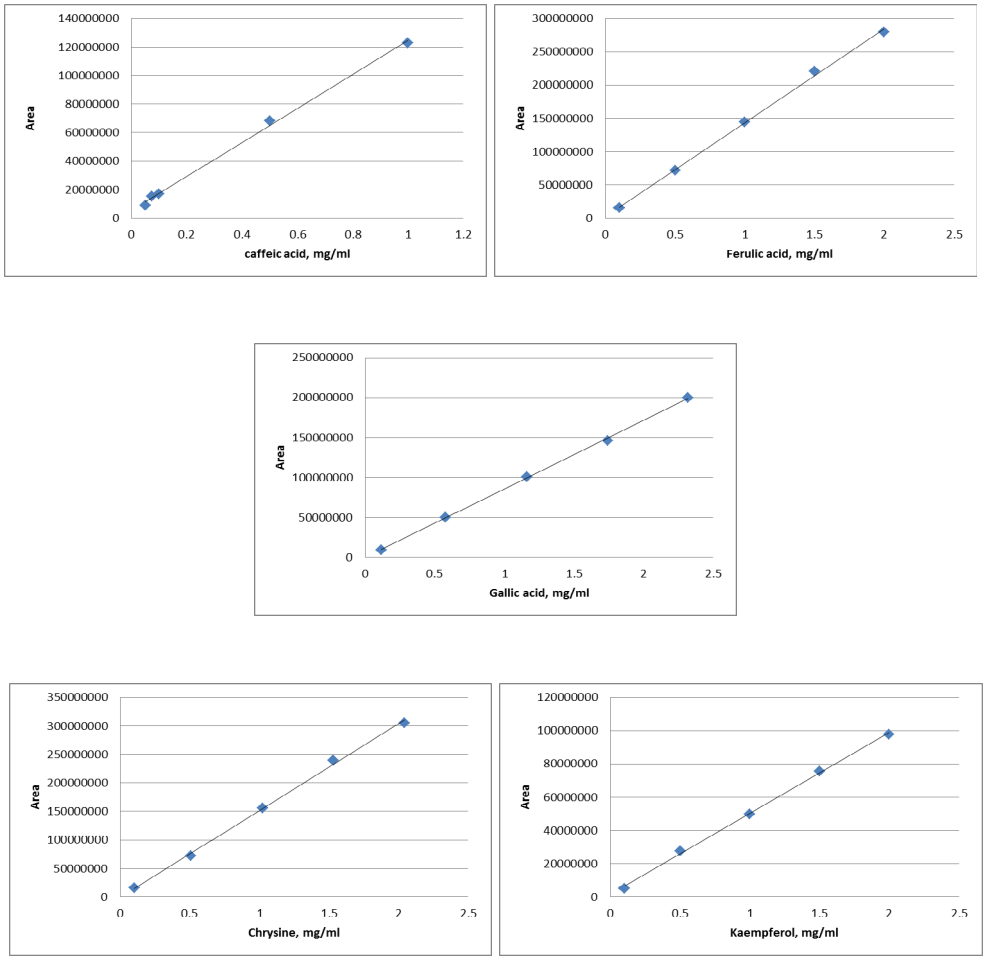

Figure 6. The HPLC standards calibration curves

Table 1. Retention times and maximum wavelength from UV-Vis spectra

Samples		Caffeic acid	Ferulic acid	Gallic acid	Kaempferol	Chrysine
Standards	RT, min	4.9	9.6	2.3	50.0	51.8
	UV-Vis max. abs., nm	238 322	236 321	270	265 365	267 311
Conop	RT, min	4.8	9.4			50.7
	UV-Vis max. abs., nm	240 322	237 320			267 311
Lipova	RT, min	5.1				
	UV-Vis max. abs., nm	238 322				
Covăsânț	RT, min	4.2			50.0	51.9
	UV-Vis max. abs., nm	237 322			267 366	267 312
Dorgos	RT, min	4.4			50.4	
	UV-Vis max. abs., nm	238 322			266 366	
Știei	RT, min	4.3	9.6	2.3	50.0	51.2
	UV-Vis max. abs., nm	239 322	237 321	271	266 366	266 310
Beiuș	RT, min	4.8	9.6		50.8	
	UV-Vis max. abs., nm	239 322	237 321		264 364	
Livada Beiuș	RT, min	4.7	9.4			
	UV-Vis max. abs., nm	238 321	237 321			

If we compare our results with the results obtained for European, Chinese and Argentinian propolis we can observe that the sample from Conop has a comparable chrysine content (1.95 mg/ml in 1:10 extract respectively 1.95 % in raw propolis).

Table 2. HPLC quantitative determinations

Samples	Caffeic acid (mg/ml)	Gallic acid (mg/ml)	Ferulic acid (mg/ml)
Calibration curves equation	$A = 10^8*c + 5*10^6$	$A = 9*10^7*c - 128491$	$A = 10^8*c + 3*10^6$
Correlation factor	0.9979	0.9995	0.9986
Conop	0.05		1.68
Lipova	0.04		
Covăsânț	0.09		
Dorgos	0.70		
Știei	0.20	0.16	0.01
Beiuș	0.40		1.09
Livada Beiuș	0.65		1.39
	Kaempferol, mg/ml	Chrysine, mg/ml	
Calibration curves equation	$A = 5*10^7*c + 2*10^6$	$A = 2*10^8*c - 851324$	
Correlation factor	0.9985	0.9995	
Conop		1.95	
Lipova			
Covăsânț	0.36	0.87	
Dorgos	8.88		
Știei	0.36	0.15	
Beiuș	0.07		
Livada Beiuș			

Comparing the obtained results with the literature data of Romanian propolis we can observe that the chrysin and caffeic acid contents are similar with those from Timiș county and higher that those from Transylvania samples. The kaempferol content of Dorgos sample from Arad county being much higher that was found in the samples from Timiș county.

CONCLUSIONS

Even that Romania was one of the first countries that promoted the propolis study, this paper is one of the first that report the chemical characteristics of the propolis originating from the west part of country, namely from Arad and Bihor counties. This paper present a comparative study of more samples from most important apicultural centers from these counties with the purpose to can have also statistically clear image of the propolis quality of the region.

This study highlight that the propolis from west of Romania have a high polyphenols, flavonoids (chrysine and kampferol) and phenolic acids (caffeic acid, ferulic acid), content that can lead us to presume that it will have also an

important antioxidant capacity. The results shown higher values as those reported for propolis originating from China, Europe or Brasilia and similar or higher than that reported from Timiș county, west of Romania or other regions from Romania. These results propose the propolis collected from this part of Romania to be used in food, cosmetic and pharmaceutical fields, to be raw material for safe and efficient medicinal products, cosmetics or food supplements.

Because the propolis is a natural product obtained by bees from the resins collected from different species, the vegetation from the bees harvesting areas influenced the chemical composition of propolis samples originating from different places. The used chromatographic methods (TLC and HPLC) showed these differences and highlight also the similar compounds.

The used chromatographic methods can be used for the quality evaluation of propolis.

EXPERIMENTAL SECTION

The propolis samples origin and preparation for study

The studied propolis were collected from various beekeepers from west part of Romania, Arad respectively Bihor counties. There were collected 4 samples from Arad county: Conop, Lipova, Covăsânț and Dorgos respectively 3 samples from Bihor county: Beiuș, Livada Beiuș and Ștei (figure 7).

To prepare the propolis for analysis the samples were extracted by grinding the samples and than mixed with 70 % vol. ethanol. The extraction was performed by maceration (cold extraction) using 10 g of propolis and 100 ml solvent. The mixtures were well shaken, and then 48 hours kept in dark, during which were occasionally shaked. At the end, each mixture was filtered. For each sample were prepared three extracts [19].

The TLC analysis

The flavonoides and phenolic acids were determined by thin layer chromatography using a silica chromatographic plate with fluorescence indicator at 254 nm. The mobile phase was toluene (Merck) – diethyl ether – acetic acid 10% (Merck), in proportion of 50:50:10 v/v. The used standards were caffeic acid and chrysin, each having a concentration of 1 mg/mL in methanol. It was applied 15 µL from the samples and 10 µL from each standard. After drying the plate at room temperature, the first chromatogram was observed in the fluorescence at 365 nm, after which the plate was sprayed with Neu-PEG reagent and observed in fluorescence at 365 nm. The second chromatogram was sprayed with a 10% phosphomolybdenic acid solution in methanol, followed by anisaldehyde reagent; the plate was heated at 105-110°C for 5-10 minutes and the chromatograms were observed in visible light [20]. The chromatograms were observed under a Camag reprostar lamp and documentation system equipped with a HP digital camera.

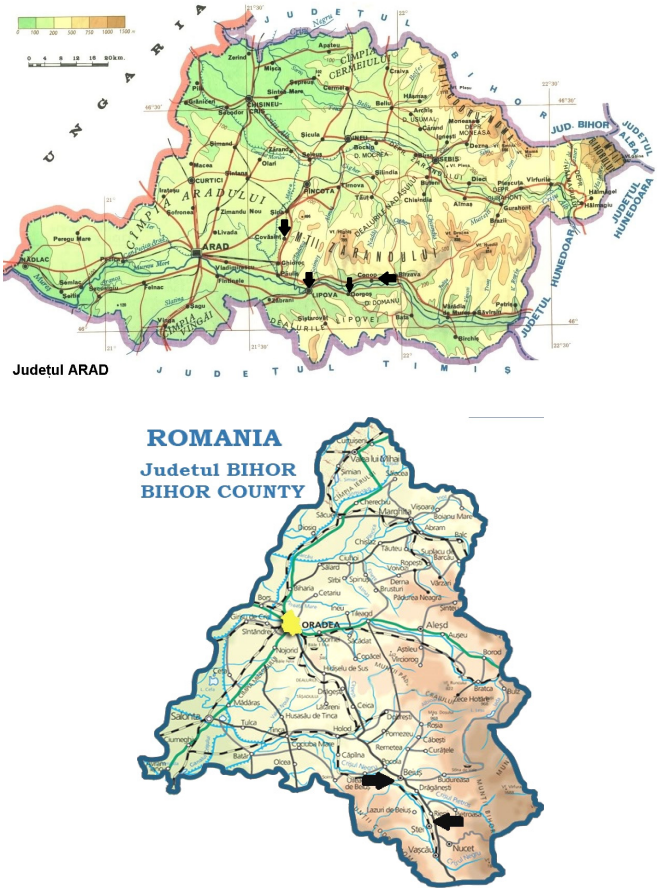

Figure 7. The collection places of studied propolis samples

The HPLC analysis

The determination was carried out on a Varian Star HPLC system. It was used a silica C18 column (Phenomenex, Luna C18, 150 x 4.6 mm, 5 µm). Like mobile phase was used a tertiary gradient prepared from 0.1% (v/v) phosphoric acid (Merck) in water, methanol and acetonitrile (Merck). The elution started with a linear gradient, beginning with isocratic elution followed for the next 30 minutes with 75 % phosphoric acid 0,1%, then for 5 minutes with 69 % phosphoric acid 0,1%, then for 5 minutes with 67 % phosphoric acid 0,1% and at the end for 20 minutes with 54 % phosphoric acid. The flow rate was 1 mL/min [21]. The DAD detector was operated at 280 and 340 nm and the

injection volume was 10 µL for each sample and standard. As standards were used chrysin (1.02 mg/mL), caffeic acid (1 mg/mL), ferulic acid (1 mg/mL), gallic acid (0,116 mg/mL), kaempferol (1 mg/mL), in methanol. For quantitative determination were used different concentrations of standards: caffeic acid (0.05 – 1 mg/ml), ferulic acid (0.1 – 2 mg/ml), gallic acid (0.116 - 2.32 mg/ml), chrysine (0.102 – 2.04 mg/ml) respectively kaempferol (0.1 – 2 mg/ml).

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