EXPERIMENTAL RESEARCHES FOR STANDARDIZATION OF HIDROALCOHOLIC EXTRACTS OF PROPOLIS FROM THE WEST REGION OF ROMANIA

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Abstract

The large variability of the active principles concentration in the same batch of commercial propolis is well-known. Although the batch of commercial propolis acquired originates from the same apiary, it does not mean that it has a unitary chemical composition, because it is obtained from different beehives, which leads to large variations of quality and impurification degree. Consequently, the differences will be considerably greater in case the raw material comes from different apiaries. The aims of this study were: to examine the concentration variability of active components in the propolis samples collected in September 2011 in the West side of Romania, from different apiaries and to obtain a propolis extract with a reproducible composition of active principles within certain limits. From each propolis sample there were prepared three hydroalcoholic extracts with 20%, 60% and 96% ethanol, which were analysed by an HPLC method regarding the content in bioactive compounds (flavonoids and polyphenolic derivatives). The HPLC quantitative profiles of the extracts revealed significant differences in terms of the bioactive compounds content among all the three propolis sorts, but also among the samples obtained from the same propolis sort; these variations can be attributed to the non uniformity of the raw material. For all propolis extracts, the quantity of extracted bioactive compounds increased with ethanol concentration significantly. However, the most appropriate concentration of ethanol for extraction was that of 60%, the resulting extracts presenting the highest content of chrysin, the flavonoidic component quantitatively predominant in all the

analysed propolis samples. Moreover, the propolis extract obtained by blending batches of extracts can represent a starting point of further research for standardization.

Rezumat

Este binecunoscută variabilitatea largă a concentraţiei principiilor active în aceeaşi şarjă de propolis comercial. Chiar dacă produsul natural achiziţionat provine de la aceeaşi prisacă, nu înseamnă că acesta are o compoziţie chimică unitară, deoarece este obţinut de la stupi diferiţi, fapt ce conduce la variaţii mari ale calităţii şi gradului de impurificare. În consecinţă, diferenţele vor fi semnificativ mai mari în cazul în care materia primă provine de la diverse prisăci. Obiectivele acestui studiu au fost: cercetarea variabilităţii concentraţiei componentelor active în sorturile de propolis colectate în luna septembrie anul 2011 din zona de vest a României, de la diferite stupine şi obţinerea unui extract de propolis cu o compoziţie a principiilor active reproductibilă în anumite limite. Din fiecare sort de propolis au fost preparate trei extracte hidroalcoolice cu 20%, 60% şi 96% etanol, care au fost analizate printr-o metodă HPLC privind conţinutul în principii active (flavonoide şi derivaţi polifenolici). Profilele HPLC cantitative au evidenţiat diferenţe semnificative în ceea ce priveşte conţinutul în compuşi bioactivi, atât între cele trei sorturi de propolis, cât şi între probele obţinute din acelaşi sort; aceste variaţii pot fi atribuite neuniformităţii materiei prime. În cazul tuturor extractelor de propolis, cantitatea de compuşi bioactivi extraşi a crescut semnificativ cu concentrația soluției de etanol. Totuşi, concentraţia de etanol adecvată pentru extracţie a fost de 60%, extractele rezultate prezentând cel mai mare conţinut în crizină, componenta flavonoidică predominantă cantitativ în toate sorturile de propolis analizate. În plus, extractul de propolis obţinut prin amestecarea loturilor de extracte poate reprezenta punctul de plecare al unui viitor studiu în vederea standardizării.

Keywords: propolis extracts, standardization, flavonoids, chrysin.

Introduction

Propolis, one of the by-products of the beehive, has been used by man since ancient times for its medicinal properties, and it is currently one of the biggest challenges for nutritionists and the medical field, due to its many biological actions such as antibacterial, antiviral, antifungal, anti-inflammatory, anaesthetic and cytostatic [1]. Therefore, in present there is an increasing interest on the propolis products, which are consumed as health supplements and alternative medicines.

Propolis (bee glue) is a heterogeneous, resinous or wax-like mass, of solid consistency, sometimes compact, sometimes becoming malleable and adherent particles, and other times granular or friable, taking the appearance of powdery crumbs. The consistency of propolis depends on temperature, therefore it is hard at room temperature, it becomes brittle at low temperatures (below – 15ºC), soft and pliable around the temperature of 30ºC, it becomes sticky and viscous at the temperature of 60-70ºC, when it starts to melt, but the melting domain expands to temperatures of up to

100ºC or higher [1, 2]. It possesses a pleasant, aromatic smell, and varies in colour, depending on its source and age, from pale yellow to dark brown, even black [3]. As for the chemical composition of propolis, the main groups of compounds are waxes, resins, balsams, aromatic and ethereal oils, pollen and other organic matter [4-6].

In the last 30 years, there are numerous literature reports dealing with bee glue chemistry, which prove that in its complex chemical composition the proportion of different types of substances varies and depends both on the composition of local flora at the site of collection and on the time of collection [4, 7-11]. Moreover, it was revealed that propolis samples from different geographical and climatic regions always presented biological activity, although their chemical composition was completely different. Consequently, a chemical characterization of the propolis sample is necessary before the investigation of its biological activity. Furthermore, the chemical characterization of propolis is considered the starting point for its chemical standardization, that guarantees its quality, efficacy and safety, assuring the officially acceptance of propolis in therapeutics [12, 13]. Therefore, chemical characterization of propolis samples, including determination of main biologically active substances, is essential for the process of standardization and quality control, as it allows defining the propolis chemical types, determined by its plant origin and presenting a specific biological activity [13].

From an analytical point of view, standardization involves two components: identification of the characteristic compounds in the product (which condition those physical-chemical, biological and antimicrobial features) and their dosage and the development of validated analytical methods in order to provide quantitative reproducible results regarding the compounds of interest. In case the physical-chemical methods cannot be applied, standardization is achieved through biological control methods.

In the case of propolis, both aspects of standardization have been studied by Bankova and collaborators, who published validated methods for the quantitative determination of phenolic compounds (complete phenols, flavonoids) in the poplar propolis from the temperate region [13-15].

In the general monograph *Extracts*, the European Pharmacopoeia 7th Edition [16] refers to standardized extracts as being adjusted preparations within an acceptable tolerance to a given content of constituents with known therapeutic activity. Standardization is achieved by adjustment of the extract with inert material or by blending batches of extracts.

The standardization of natural extracts is a condition imposed for the characterization of medicinal preparations. The standardized extract may be

administered in a quantity containing a controlled concentration of active principles, in order to be fully devoid of toxicity, to offer a maximum therapeutical efficiency and allow for the individualization of the treatment. Generally, for the production of propolis nutritional supplements, the Romanian pharmaceutical industry uses standardized extracts such as: the fluid propolis extract with a content of 10% bioflavonoids expressed in cresol; the dry extract containing 5% bioflavonoids (expressed in chrysin) and the lyophilized water-soluble propolis extract obtained through an original procedure patented at the Institute for Beekeeping Research and Development Bucharest, Romania [17].

Starting from these premises, the purposes of this study were: to investigate the concentration variability of active components in the propolis samples collected in September 2011 in the West region of Romania, from different apiaries and to obtain a propolis extract with a reproducible composition of active principles within certain limits, through the quantitative determination of some flavonoids in the two main groups (flavones and flavonols) and some polyphenolic derivatives such as the caffeic acid.

Materials and Methods

Materials

For the extraction and quantitative analysis of the biologically active substances in propolis, there were used three batches of this natural product, collected in September 2011 in the Western part of Romania, from an alpine apiary located in Bârzava (46°07′00″ N; 21°58′59″ E; altitude 218 m), with a main arboretum of resinous plants and from two river meadow apiaries located in Timisoara (45°44′57″ N; 21°13′37″ E) and Minis (46°08'00" N; 21°36'00" E), with a vegetation rich in plane melliferous plants such as the linden tree, the poplar tree, the acacia tree. The organoleptic properties of the three studied samples of propolis are provided in Figure 1 and Table I.

Ethanol p.a. (Chimopar, Bucharest, Romania) and distilled water were used for propolis extraction. For the HPLC analysis of extracts, acetonitrile (HPLC grade, Fluka Chemie AG, Germany) and bidistilled water were used. For the quantification of flavonoids from extracts by HPLC, rutin (>90%, Fluka Chemie AG, Germany), cinnamic acid, caffeic acid (>98%, Sigma-Aldrich, Germany), quercetin, apigenin, kaempherol, acacetin, chrysin, and pinocembrin (>99%, Sigma-Aldrich, Germany) were used as standards.

Figure 1.
The appearance of the propolis samples investigated

Table I
The origin and macroscopic characteristics of the propolis samples collected in 2011 and used for obtaining the extracts

Origin and physical properties studied	**Propolis samples studied**		
	Propolis from Timişoara (P1 -Timişoara)	**Propolis from Bârzava (P2- Bârzava)**	**Propolis from Miniş (P3-Miniş)**
Origin	Timişoara, 2011, from a region with vegetation rich in linden trees, acacia, poplars, plane melliferous plants	Bârzava, 2011, from an alpine region with resinous vegetation, beech, fir tree, common spruce	Miniş, 2011, from a region with vegetation rich in linden tree, acacia, poplars, plane melliferous plants
Aspect	Solid, heterogenous, resinous mass		
Colour	Dark coffee brown with yellow-green reflections	Dark coffee brown	Dark coffee brown with yellow-green reflections
Taste	Sourish, flavoured, mucosa-adherent		
Odour	Characteristic flavour (vanillin), mixed with honey odour, wax	Flavoured, typically resinous	Characteristically flavoured (vanillin), mixed with honey odour, wax
Consistency	Viscous, sticky, when mixed it leaves traces on filter paper		
Impurities	Appeared accidentally (wood splinters, fragments of bee body)		

Methods

Obtaining the propolis extracts

Propolis extracts that reach the market are usually prepared with a ratio of the starting material to the genuine extract (DER) of 5 or 10 g of propolis for 100 mL of solution, the alcohol concentrations used as solvent varying in most of the cases between 60 and 82%. Due to the fact that in the case of a 10% g/mL DER, the residuum left after extraction is not exhausted

by active principles, the procedure is not economical. For this reason, in this study it was preferred a DER= 5:100.

From each propolis sample, there were drew three samples with the most different macroscopic aspect possible and then there were prepared three types of hydroalcoholic extracts (one in ethanol 20%, one in ethanol 60% and the third in ethanol 96%), at DER 5:100 (g/mL). Consequently, there were obtained 9 hydroalcoholic extracts from each sample of propolis (P1-Timisoara, P2-Bârzava and P3-Minis), which were analysed by an HPLC method regarding the active principle content such as: flavonols (quercetol and kaempherol), flavones (apigenol, acacetol and chrysin) and flavanone (pinocembrol). Also, it was examined the presence of rutoside in the analysed types of propolis (although this is not mentioned by literature as a component of propolis) and of the caffeic acid in the category of polyphenol carboxylic acids. Each quantitative determination was performed in triplicate.

HPLC analysis

For the quantification of flavonoids, caffeic acid and its derivatives in propolis extracts and, furthermore, for the evaluation of these bioactive propolis compounds, an Agilent 1100 HPLC apparatus was used; for the HPLC analysis a Zorbax SB-C 18 column, with 250 mm length, 4.6 mm i.d., and 5µm particle diameter was used; for flavonoid detection the wavelength of 337 nm was set; the mobile phase was acetonitrile:water in an 48:52 ratio; the temperature was 25^{o}C, with a flow of 0.3 mL/min; the injected sample volume was 20 µL. Diluted standard solutions of rutoside, quercetol, apigenol, kaempherol, acacetol, chrysin, pinocembrol and caffeic acid were analyzed in the same HPLC conditions and, furthermore, the calibration of the detector response was performed. The calibration curves were used for the quantification of the main bioactive propolis compounds.

Obtaining the calibration curve. For the semiquantitative determination of active substances contained by propolis (flavonoid and polyphenolic derivatives such as the caffeic acid), solutions with known concentrations in compounds of interest (quercetol, apigenol, kaempherol, acacetol, chrysin, pinocembrol, rutoside and caffeic acid) were prepared.

Results and Discussion

In the Figures 2-5 are presented the HPLC chromatograms of the hydroalcoholic extracts of the three Romanian propolis samples, obtained using solutions of ethanol 20%, 60% and 96% respectively. The mean values of the flavonoids and caffeic acid content, obtained by HPLC analysis of hydroalcoholic extracts of the three propolis samples are

represented in table II (P1-Timisoara propolis sample), table III (P2-Bârzava propolis sample) and table IV (P3-Minis propolis sample).

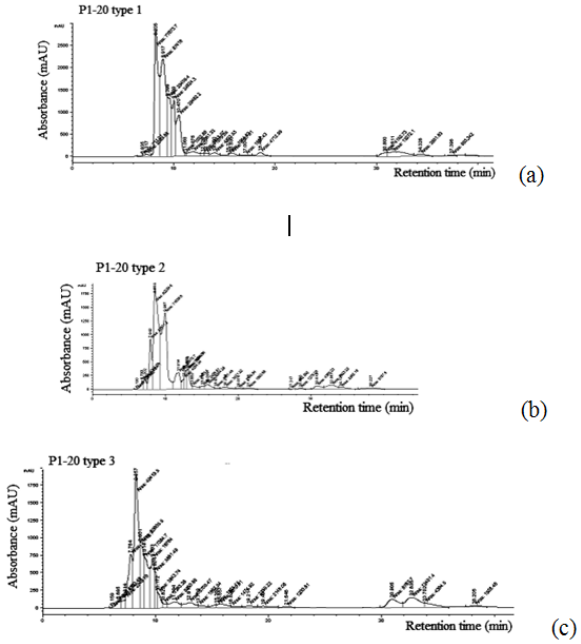

Figure 2.
HPLC chromatograms of P1- Timisoara propolis extracts in ethanol 20%: type 1 (a), type 2 (b), and type 3 (c)

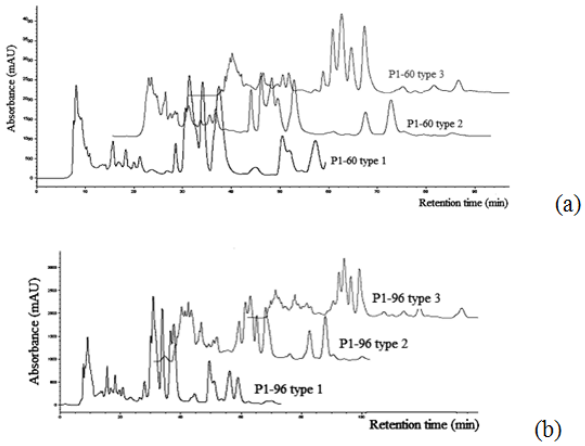

Figure 3.
HPLC chromatograms of P1- Timisoara propolis extracts in ethanol 60% (a) and in ethanol 96% (b)

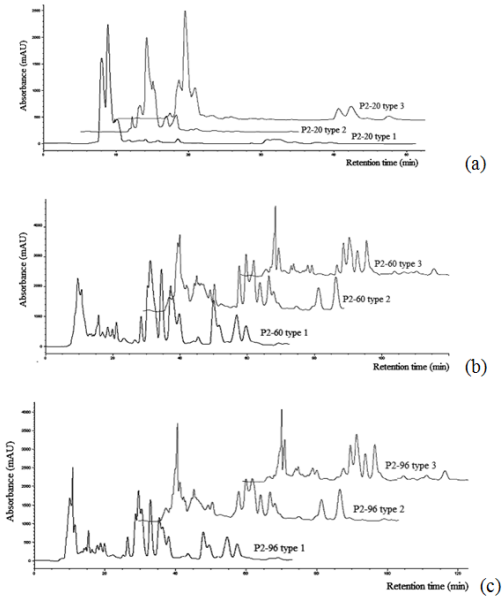

Figure 4.
HPLC chromatograms of P2-Bârzava propolis extracts in ethanol 20% (a), 60% (b) and 96% (c)

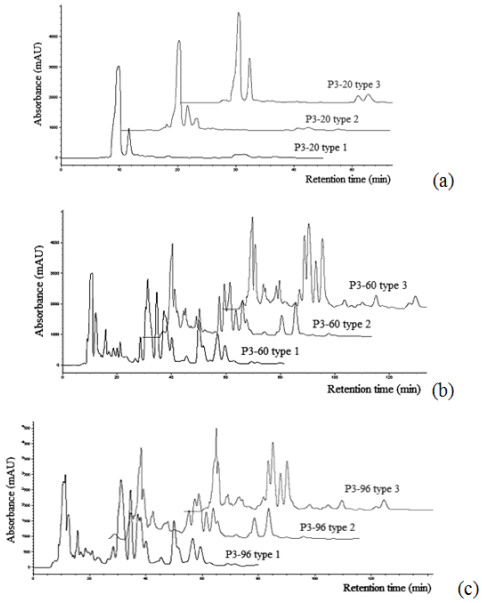

Figure 5.
HPLC chromatograms of P3-Minis propolis extracts in ethanol 20% (a), 60% (b) and 96% (c)

Table II

Identification and semiquantitative determination of flavonoids and caffeic acid in P1-Timisoara propolis extracts in ethanol 20%, 60% and 96% V/V

No.	Code	Quercetol (mg/g)	Apigenol (mg/g)	Kaemferol (mg/g)	Chrysin (mg/g)	Rutoside (mg/g)	Caffeic acid (mg/g)
Propolis extracts P1-Tm in EtOH 20 % V/V							
1	P1_20 type 1	0.298	0.289	0.222	0.955	1.082	2.560
2	P1_20 type 2	1.128	0.773	0.137	0.638	2.096	6.223
3	P1_20 type 3	0.469	0.213	0.164	1.030	1.601	0.316
Propolis extracts P1-Tm in EtOH 60 % V/V							
1	P1_60 type 1	1.357	8.132	3.544	39.789	1.709	1.745
2	P1_60 type 2	2.500	5.047	1.430	18.392	45.400	5.476
3	P1_60 type 3	1.256	2.721	1.330	19.921	4.983	1.179
Propolis extracts P1-Tm in EtOH 96 % V/V							
1	P1_96 type 1	2.280	6.323	2.923	30.223	10.590	1.947
2	P1_96 type 2	8.704	6.989	1.601	17.191	8.878	3.899
3	P1_96 type 3	1.665	1.617	1.601	12.089	5.078	1.517

Table III

Identification and semiquantitative determination of flavonoids and caffeic acid in P2-Bârzava propolis extracts in ethanol 20%, 60% and 96% V/V

No.	Code	Quercetol (mg/g)	Apigenol (mg/g)	Kaemferol (mg/g)	Chrysin (mg/g)	Rutoside (mg/g)	Caffeic acid (mg/g)
Propolis extracts P2-B in EtOH 20 % V/V							
1	P2_20 type 1	0.386	0	0	0.621	0.836	1.566
2	P2_20 type 2	1.324	0	0	0	0.496	1.619
3	P2_20 type 3	0.505	0	0	0.698	0.513	2.597
Propolis extracts P2-B in EtOH 60 % V/V							
1	P2_60 type 1	2.743	3.518	4.034	38.488	1.424	1.548
2	P2_60 type 2	3.095	4.512	3.198	22.308	4.713	5.601
3	P2_60 type 3	0.731	1.995	0.939	12.908	10.205	7.645
Propolis extracts P2-B in EtOH 96 % V/V							
1	P2_96 type 1	1.092	4.120	0.715	0	2.019	4.996
2	P2_96 type 2	3.053	5.318	2.588	11.265	15.390	4.504
3	P2_96 type 3	0.897	1.754	1.023	9.874	2.945	1.984

Table IV

Identification and semiquantitative determination of flavonoids and caffeic acid in P3-Minis propolis extracts in ethanol 20%, 60% and 96% V/V

No.	Code	Quercetol (mg/g)	Apigenol (mg/g)	Kaemferol (mg/g)	Chrysin (mg/g)	Rutoside (mg/g)	Caffeic acid (mg/g)
Propolis extracts P3-M in EtOH 20 % V/V							
1	P3_20 type 1	0.468	0.332	0	0.643	1.641	0
2	P3_20 type 2	1.784	0.349	0	0	1.830	0
3	P3_20 type 3	0.449	0.370	0	0.360	1.883	0
Propolis extracts P3-M in EtOH 60 % V/V							
1	P3_60 type 1	11.008	10.339	0	38.205	2.823	14.751
2	P3_60 type 2	3.951	7.117	0	15.125	3.657	19.365
3	P3_60 type 3	11.355	3.455	1.531	24.633	7.233	0
Propolis extracts P3-M in EtOH 96 % V/V							
1	P3_96 type 1	6.111	9.614	3.755	38.869	8.895	9.369
2	P3_96 type 2	9.362	11.140	0	14.858	37.184	0
3	P3_96 type 3	5.617	2.080	1.089	14.585	4.105	3.046

The mean values of chrysin content, determined in the three extract variants obtained with ethanol 60% from Timisoara, Bârzava and Minis propolis samples are showed in figure 6.

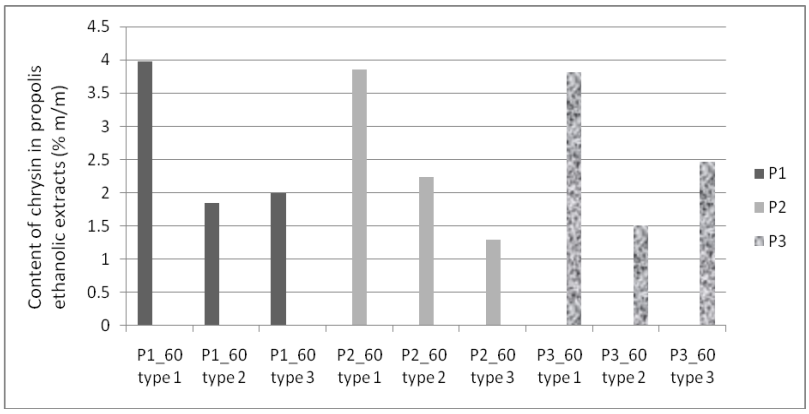

Figure 5.
The content of chrysin (g/100 g propolis) determined through HPLC in the extracts obtained from Timisoara (P1), Bârzava (P2) and Minis (P3) propolis with ethanol 60% (Mean±S.D., n=3)

Determination of the flavonoids and caffeic acid content in P1-Timisoara hydroalcoholic propolis extracts showed that there were differences for the same biologically active compound, although in all cases it was used the same ethanolic concentration and the same quantity of processed propolis. These differences can be explained through the non-uniformity of the raw material, which represents the same sample of propolis. The quantity of extracted active principles varied directly proportional to the increase in alcoholic concentration. In the case of the 60% hydroalcoholic extract there were observed significant differences from one type to another in terms of the rutoside content. Consequently, rutoside cannot be significantly quantified from a statistical point of view, probably on account of the interference with other compounds for which we lacked standards. Moreover, the use of 96% ethanol as an extraction solvent leads to obtaining quantitatively convenient propolis extracts.

In the case of P2-Bârzava hydroalcoholic propolis extracts having the same ethanolic concentration and prepared with the same quantity of propolis, the content of flavonoids and caffeic acid varied greatly; important differences between the variants of the same extract type being observed when 60% and 96% ethanol was used as an extraction medium. This considerable variability of an active component, in the same type of extract,

coming from the same propolis sample can be explained, as in the previous case, through the non uniformity of the raw material. With regard to ethanolic concentration of extracts, it was noticed that the extracts obtained with 20% ethanol contained a reduced quantity of active components in comparison with the ones prepared with high concentration of ethanol (60% and 96%). The most appropriate concentration of ethanol for extraction was shown to be that of 60% and the main component extracted was chrysin (1290.8 to 3848.8 mg/100 g propolis).

Similarly, in the third case (P3-Minis hydroalcoholic propolis extracts), the obtained results regarding the flavonoids and caffeic acid content, revealed the concentration variability in the same bioactive compound, which was attributed to the non uniformity of the raw material. Also, the alcoholic concentration convenient for extraction was 60% and the main extracted flavonoid component was chrysin (1512.5 to 3820.5 mg/100 g propolis).

The analysis of the three variants in ethanolic extracts (20%, 60% and 96%) of each propolis sample showed that, for obtaining a certain propolis extract, the entire sample must be processed at once and subjected to a quantitative determination which comprises all flavonoid derivatives. Consequently, by mixing a multitude of extract lots of the same type, it can be obtained, in the end, a final extract which will have a certain content in such compounds. Another method would be the standardization of extracts in chrysin, the main component of extracts containing 60% ethanol, this being very well represented from a quantitative point of view.

Comparing the obtained values, it was noticed extremely important differences in terms of chrysin concentration determined in propolis by the HPLC technique. Therefore, the producer may stipulate in the specification sheet a minimum content of chrysin extractable in 60% ethanol, namely 1 g/100 g propolis. Thus, the obtaining of certain extracts with reduced chrysin content could be limited by processing a poor quality vegetal material, and, at the same time, the obtaining of extracts reaching a certain titer in chrysin could be facilitated.

Conclusions

In summary, this investigation highlighted that the composition of the plant source (resinous or melifferous plants) determines the chemical composition of the propolis samples from different geographic locations from the western region of Romania. Moreover, it was obvious the significant variability of the concentration in each biologically active component in all three variants of 20%, 60% and 96% hydroalcoholic

extracts, prepared from the propolis samples (P1-Timisoara, P2-Bârzava and P3-Minis), processed in the same quantity and, respectively, in the same type of ethanol. The non- uniformity of the raw material proved to be responsible on the concentration variability in a bioactive compound in the same extract type corresponding to a certain batch. In case of propolis chemical types collected from different apiaries located in various regions from the western Romania, the alcoholic concentration favorable for the extraction of a satisfying quantity of biologically active substances is 60%. The flavonoidic component quantitatively predominant in the analysed propolis samples is represented by chrysin. In order to obtain extracts reaching a certain titer in the main biologically active component (chrysin), the processing the entire propolis batch, which comprises all flavonoid derivatives, it is recommended. Further studies need to be performed in order to achieve a reliable standardization of above mentioned propolis types and to investigate their biological activities in connection with the chemical composition.

References

1. Ceschel, G.C., Maffei, P., Sforzini, A., Lombardi Borgia, S., Yasin, A., Ronchi, C. In vitro permeation through porcine buccal mucosa of caffeic acid phenetyl ester (CAPE) from a topical mucoadhesive gel containing propolis. *Fitoterapia*. 2002; 73 (Suppl. 1): S44–S52.
2. Mateescu, C., Dumitru, I.F. Propolisul si extractele de propolis. In: News in biotechnologies; Ilex Publishing House, Bucharest, 2001: 64-117.
3. Marcucci, M.C. Propolis: chemical composition, biological properties and therapeutic activity. *Apidologie*. 1995; 26(2): 83-99.
4. Ghisalberti, E.L., Jefferies, P.R., Lanteri, R., Matisons, J. Constituents of propolis. *Experientia*. 1978; 34: 157-158.
5. Ramanauskiené K., Inkéniené A.M., Leskauskaité D., Modeling of the composition of a semisolid propolis preparation and evaluation of its quality, *Farmacia*. 2012; 60(4): 535-543.
6. Grădinariu V., Cioancă O., Gille E., Aprotosoaie A.C., Hrițcu L., Hăncianu M., The chemical profile of basil biovarieties and its implication on the biological, *Farmacia*. 2013; 61(4): 632-639.
7. Bankova, V., Dyulgerov, A., Popov, S., Evstatieva, L., Kuleva, L., Pureb, O., Zamjansan, Z. Propolis produced in Bulgaria and Mongolia: phenolic compounds and plant origin. *Apidologie*. 1992; 23(1): 79-85.
8. Bankova, V.S., De Castro, S.L., Marcucci, M.C. Propolis: recent advances in chemistry and plant origin. *Apidologie*. 2000; 31(1): 3-15.
9. Popova, M., Bankova, V.S., Bogdanov, S., Tsvetkova, I., Naydenski, C., Marcazzan, G.L., Sabatini, A-G. Chemical characteristics of poplar type propolis of different geographic origin. *Apidologie*. 2007; 38(3): 306-311.
10. Sawaya, A.C.H.F., Cunha, I.B.S., Marcucci, M.C., De Oliveira Rodrigues, R.F., Eberlin, M.N. Brazilian Propolis of Tetragonisca angustula and Apis mellifera. *Apidologie*. 2006; 37(3): 398-407.
11. Mărghitaş, L.Al., Dezmirean D.S., Bobiş O. Important developments in Romanian propolis research. *Evid. Based. Complement. Alternant. Med.* 2013, http://dx.doi.org/10.1155/2013/159392.
12. Kosalec, I., Bakmaz, M., Pepeljnak, S., Vladimir–Knezevik, S. Quantitative analysis of the flavonoids in raw propolis from northern Croatia. *Acta Pharm*. 2004; 54: 65-72.

13. Bankova, V. Chemical diversity of propolis and the problem of standardization. *J. Ethnopharm*. 2005a; 100: 114-117.
14. Bankova, V. Recent trends and important developments in propolis research. *Evid. Based. Complement. Alternant. Med*. 2005b, 2: 29-32.
15. Popova, M., Bankova, V., Butovska, D., Petkov, V., Damyanova, B., Sabatini, A.G., Marcazzan, G.L., Bogdanov, S. Validated methods for the quantification of biologically active constituents of poplar type propolis. *Phytochemical analysis*. 2004, 15: 235-240.
16. *** European Directorate for the Quality of Medicines & HealthCare. 2010. European Pharmacopoeia, 7th Edition: 674. Council of Europe, Strasbourg.
17. Oiţă, N., Baidan, N., Paloş, E. Produsele apicole în oftalmologie. *România Apicolă*. 1981; 8: 10-20.

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