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Content of biologically active compounds in Bulgarian propolis: a basis for its standardization

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Dedicated to Acad. Bogdan Kurtev on the occasion of his 100th birth anniversary

In Bulgaria, propolis tincture is among of the most popular home-made remedies. Bulgarian propolis has been studied and it was found to originate from the bud resin of the black poplar *Populus nigra* L.; the chemical constituents responsible for its biological activity are flavonoid aglycones (flavones, flavonols, flavanones, dihydroflavonols), substituted cinnamic acids and their esters. However, the specific quantitative characteristics of Bulgarian poplar propolis have not been studied. Validated spectrophotometric procedures were used to quantify the three main groups of bioactive substances: total phenolics, total flavones/flavonols, total flavanones/dihydroflavonols, in 22 samples of Bulgarian propolis from different regions of the country. Based on the results, we characterized raw poplar propolis in terms of minimum content of its bioactive components (antimicrobial and antioxidant) as follows: 46% resin, 24% total phenolics, 7% total flavones/flavonols; 5.4% total flavanones/dihydroflavonols. These values can be used as a basis for Bulgarian propolis standard. They are somewhat higher that the ones suggested by the International Honey Commission for poplar type propolis. This is a proof that Bulgarian propolis is a valuable bee product of high quality, higher than that of the average poplar propolis samples coming from other regions.

Key words: propolis; Bulgarian propolis; chemical characterization; standardization

INTRODUCTION

Propolis, a sticky material collected by bees and used in their hives as a general purpose sealer, is well known for its diverse and useful biological activities: antimicrobial, antioxidant, anti-inflammatory, immuno-stimulating, and many others [1]. In the last decades, it has become the subject of growing scientific and commercial interest as a major ingredient of health food, cosmetics, food additives, *etc.* Recently, propolis has also been found very useful as a food preservative and as an active agent in food biopackaging materials [2]. A peculiarity of propolis, which is also an obstacle to its wide application in medicine and industry, is its variable chemical composition. Propolis composition depends on the plants available to bees for resin collection/propolis production [3]. Taking into account the fact that bees inhabit almost all ecosystems on Earth, obviously propolis chemistry is very far from constant and thus a universal propolis chemical standard is impossible. On the other hand, in any particular environment, bees have preferred source plants and this provides a good basis for standardization of specific chemical types of propolis [4].

In Bulgaria, propolis tincture is among of the most popular home-made remedies. Bulgarian traditional medicine applies it for healing of wounds and burns, sore throat, stomach ulcer, *etc.* Bulgarian propolis has been studied and it was found to originate from the bud resin of the black poplar *Populus nigra* L. [5]. Its chemical constituents, which are responsible for its biological activity, and especially for its antimicrobial and antioxidant properties, are well documented. These are flavonoid aglycones: flavones (chrysin, techtochrysin), flavonols (galangin, kaempferol), flavanones (pinocembrin, pinostrobin) and dihydroflavonols (pinobanskin, pinobanksin acetate), and other phenolics (mainly substituted cinnamic acids and their esters) [1]. It is important to note that a single propolis batch contains over 100 individual constituents, most of them having proven biological activity. Thus, the quantification of all active ingredients by chromatographic methods would be very inefficient as a routine approach. In addition, it has turned out that it is impossible to connect the bioactivity of propolis (and especially the antimicrobial activity) to one or a few individual propolis constituents [6, 7]. Till

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now, no individual propolis component was found to possess antimicrobial activity significantly higher than that of the total extract [6, 8]. Moreover, no statistically significant correlation has been found between minimum inhibitory concentration against *S. aureus* and the amounts of various active propolis components [8]. For this reason, earlier we developed and validated a combination of simple and rapid methods for quantification of the main groups of bioactive substances in poplar type propolis: total phenolics; total flavones and flavonols; and total flavanones and dihydroflavonols [9]. Applying it to over 100 poplar samples from all over the world resulted in determining the typical characteristics of poplar propolis in terms of the content of biologically active compounds [10]. However, the specific quantitative characteristics of Bulgarian poplar propolis have not been studied. The aim of the present work is to study the quality of Bulgarian propolis and provide a specific basis for its standardization and quality control.

EXPERIMENTAL

Propolis samples

Propolis samples were kindly supplied by Mr. D. Dimov (Sofia, Bulgaria). The exact sites of collection are given in Table 1. The poplar origin of the samples was confirmed by screening the composition using TLC [11].

Propolis extraction

Frozen propolis (freezer) was grated and 1 g was dissolved in 30 ml 70% ethanol in a 50 mL flask and left for 24 h at room temperature. The extract was filtered and the extraction was repeated. The two extracts were combined and diluted to 100 ml with 70% ethanol in a volumetric flask. This solution was analyzed to determine the total phenolics and flavonoids.

Balsam percentage

From each crude sample, three parallel extracts with 70% ethanol were prepared as described above. Two mL of each were evaporated to dryness *in vacuo* until constant weight, and the percentages of balsam in the extracts were calculated as the ethanol soluble fraction. The mean of the three values was determined.

Flavone and flavonol content

Total flavone and flavonol content was measured by spectrophotometric assay based on aluminum chloride complex formation, as described by Popova et al. [9]. In brief, to 2mL of the test solution, 20 ml methanol and 1ml 5% $AlCl_3$ (wt/vol) were added and the volume made up to 50 ml (volumetric flask). After 30 min, the absorbance was measured at 425 nm. Blank: 2 mL methanol instead of test solution. Every assay was carried out in triplicate. Flavone and flavonol content was estimated using a calibration curve of galangin, concentration range of 4–32 mg/mL.

Flavanone and dihydroflavonol content

For flavanones and dihydroflavonols, the colorimetric method described in DAB9 was used, modified for propolis [9]. In brief, 1 ml of test solution and 2 ml of DNP solution (1g DNP in 2 ml 96% sulfuric acid, diluted to 100 ml with methanol) were heated at 50 °C for 50 min. After cooling to room temperature, the mixture was diluted to 10 ml with 10% KOH in methanol (wt/vol). One ml of the resulting solution was added to 10 ml methanol and diluted to 50 ml with methanol. Absorbance was measured at 486 nm. Blank: 1 ml methanol instead of test solution was used in analogous procedure. Every assay was carried out in triplicate. Flavanone and dihydroflavonol content was estimated using calibration curve of pinocembrin, concentration range of 0.18–1.8 mg/ml.

Total phenolics

The Folin-Ciocalteu method was applied, as described in [9]. In brief, 1 ml of the test solution was transferred to a 50 mLl volumetric flask, containing 15 ml distilled water, and 4 ml of the Folin–Ciocalteu reagent and 6 ml of a 20% sodium carbonate solution (wt/vol) were added. The volume was made up with distilled water to 50 ml. After 2 h, the absorbance was measured at 760 nm. Blank solution: 1 ml methanol instead of test solution was used in analogous procedure. Every assay was carried out in triplicate. Total phenolics content was estimated using calibration curve of standard mixture pinocembrin–galangin 2:1, concentration range 37–326 mg/ml.

RESULTS AND DISCUSSION

The study includes 22 Bulgarian propolis samples from all Bulgarian beekeeping regions (Fig. 1). All samples were proven to be of poplar (*P. nigra*) origin by thin layer chromatography

(TLC) test [11], using as references specific marker compounds for poplar propolis [12, 13]. First of all, the amount of balsam, the extract of crude propolis in 70% ethanol was determined, this being the most

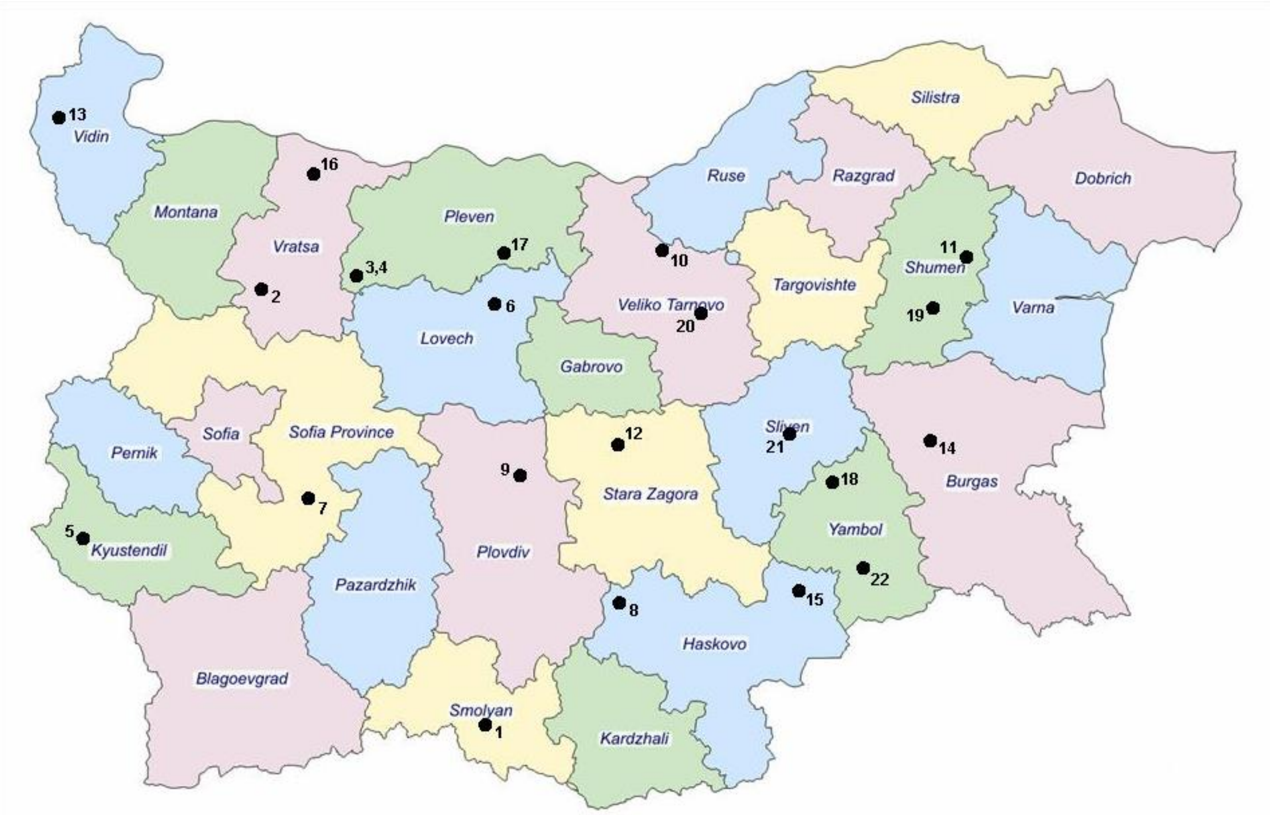

Fig. 1. Locations where propolis samples were collected in Bulgaria (●).

Table 1. Percentage of balsam and main biologically active compounds* in propolis from different regions of Bulgaria.

Sample No	**Location of origin**	**Balsam**	**Total phenolics**	**Total flavones and flavonols**	**Total flavanones and dihydroflavonols**
		% in the sample			
1	Smolyan	62	41.9	13.2	9.3
2	Vratza	77	40.0	12.3	9.4
3	Cherven bryag	65	35.8	11.8	7.3
4	Cherven bryag	82	42.0	13.5	8.6
5	Kyustendil	48	28.0	9.1	5.6
6	Lovech	39	25.7	7.2	6.5
7	Ihtiman	64	40.2	12.2	9.4
8	Gorski Izvor village, Haskovo region	57	30.5	10.4	5.9
9	Prolom village, Plovdiv region	64	34.2	11.0	6.7
10	Polski Trambesh	45	24.3	7.7	5.4
11	Novi Pazar	56	30.5	10.3	6.8
12	Kazanlak	66	24.8	8.8	6.3
13	Kula, Vidin region	38	11.2	3.6	3.5
14	Karnobat	67	40.3	13.2	8.6
15	Topolovgrad	67	39.7	13.0	9.3
16	Krushovitsa village, Vratsa region	48	27.0	9.0	6.4
17	Pelishat village, Pleven region	58	21.2	7.2	5.4
18	Yambol	54	29.7	10.0	8.1
19	Ivanski village, Shumen region	71	36.0	11.8	7.5
20	Kozarevets village, Gorna Oryahovitsa region	33	17.0	4.5	4.1
21	Sliven	46	18.7	2.9	4.5
22	Dobrich village, Yambol region	75	38.2	9.7	6.8

* standard deviations < 6%

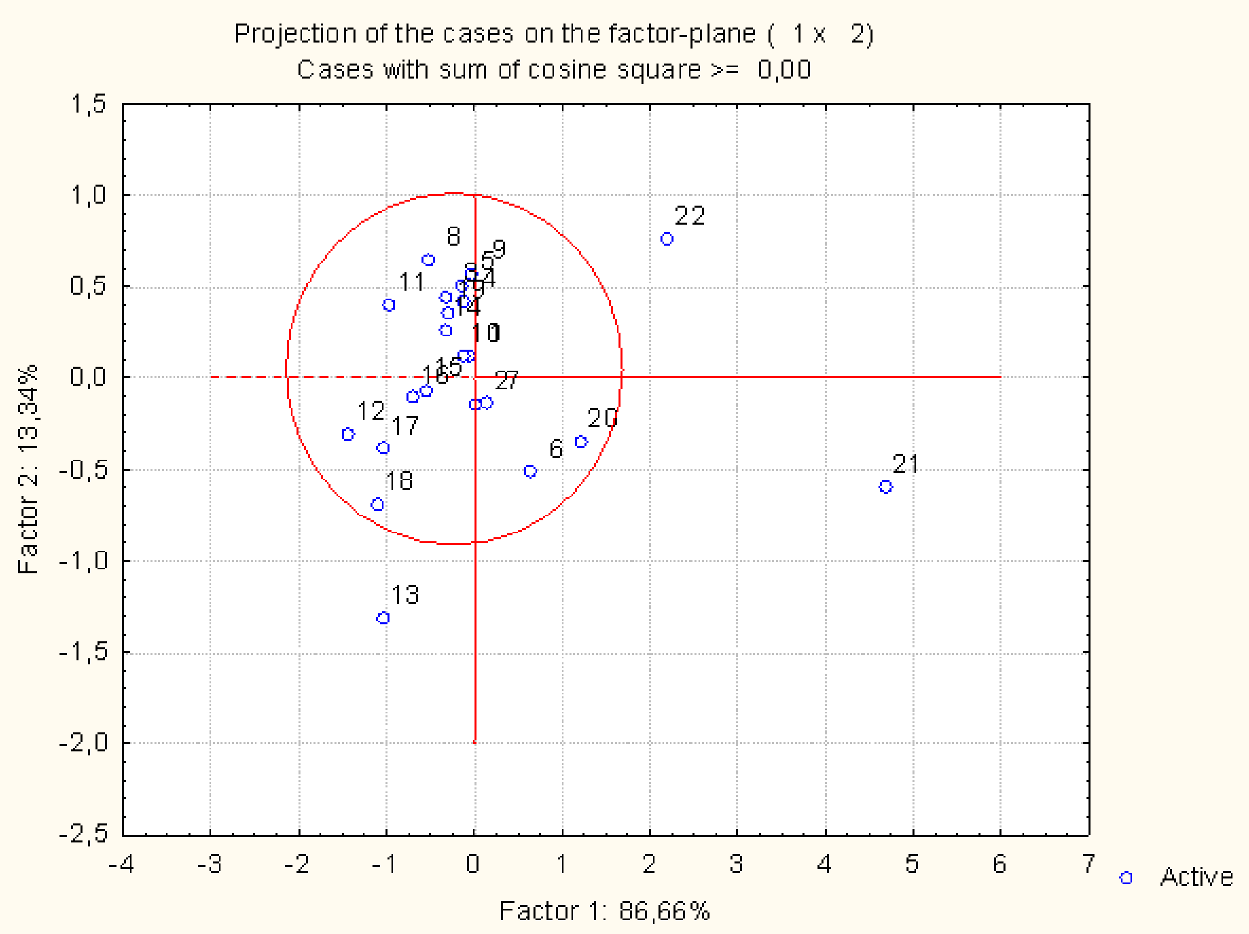

Fig. 2. PCA of propolis chemical composition of propolis samples.

Table 2. Characteristics of Bulgarian propolis samples (based on 22 samples).

Parameter (content, %)	Mean value	Median	Minimum	Maximum	P80*	P20
Balsam	58	60	33	88	67	46
Total phenolics	30.7	30.5	11.2	42	40	24.3
Total flavones and flavonols	9.6	10.2	2.9	13.5	12.3	7.2
Total flavanones and dihydroflavonols	6.8	6.8	3.5	9.4	8.6	5.4

usual way to extract propolis for use in medicine and cosmetics [14]. The balsam percentage is an important characteristic of propolis quality: in general, high percentage of balsam means that propolis contains a low percentage of wax and mechanical impurities, and higher concentration of biologically active components [6].

The values obtained from these measurements and from the spectrophotometric procedures, are presented in Table 1. In general, it is known that the chemical composition of poplar bud exudates is relatively constant; nevertheless, there could be significant variations in the percentage of individual constituents in specific locations or even in different individual plants [12]. However, looking at the data in Table 1, it is hard to make out any specific groups based on geographic origin. In order to analyze the relatively large amount of analytical data, we applied chemometric approach: the Principal Component Analysis (PCA), using normalized values for the content of the three groups of bioactive compounds. The obtained two-dimensional plot (Fig. 2) covers 100% of the variation. Most samples, except of three, form one well-defined group, despite the fact that they originate from all over the country. Obviously, the Bulgarian poplar trees produce resin of similar quantitative composition, concerning the total content of the three main groups of bioactive constituents. The only exceptions are the samples 21 and 22, characterized by significantly higher amount of total phenolics, and sample 13 with the lowest amount of total phenolics (as percentage of dry extract).

The statistical analysis of the data in Table 1 makes it possible to determine the range of probable values of the studied parameters and to formulate appropriate limits as a basis for standardization. The results for all of the parameters were analyzed by the Shapiro-Wilk normality test and it was found that the values were not normally distributed. The same was found earlier for poplar type propolis in general [10]. For this reason, we believe it is improper to use mean values as the basis for standardization. Instead, we suggest the 20-th percentile to be used in order to set the minimum values for content of resin and biologically active compounds, as shown in Table 2. According to these results, we suggest the typical characteristics of a Bulgarian propolis sample which can be applied as the basis for standardization and quality control, as follows:

Balsam content: minimum 46 %

Total phenolics: minimum 24 %

Total flavones and flavonols: minimum 7 %

Total flavanones and dihydroflavonols: minimum 5.4 %

These values are somewhat higher that the ones suggested by the International Honey Commission [15] for poplar type propolis. Especially the value for total phenolics content is 14% higher than the average for polar type propolis. This fact is important because a statistically significant correlation has been found between total phenolics and antibacterial effect (Minimal Inhibitory Concentration, MIC) of this propolis type: the higher the concentration of total phenolics, the lower the MIC (higher antibacterial activity) [10].

CONCLUSION

The results obtained in this study present a proof that Bulgarian propolis is a valuable bee product of high quality, higher than that of the average poplar propolis samples coming from other regions.

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СЪДЪРЖАНИЕ НА БИОЛОГИЧНО АКТИВНИ СЪЕДИНЕНИЯ В БЪЛГАРСКИ ПРОПОЛИС – ОСНОВА ЗА СТАНДАРТИЗАЦИЯТА МУ

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(Резюме)

Прополисовата тинктура е сред най-популярните домашно приготвяни лекарства в България. Българският прополис е изследван и е установено, че той произхожда от смолата на пъпките на черната топола *Populus nigra* L., като химичните съединения, отговорни за биологичната му активност са флавоноидни агликони (флавони, флавоноли, флаванони и дихидрофлавоноли), заместени канелени киселини и техни естери. Все още обаче не са изследвани специфичните количествени характеристики на българския тополов прополис. Използвахме валидирани спектрофотометрични процедури за количествено определяне на трите основни групи биологично активни вещества: тотални феноли, тотални флавони и флавоноли, тотални флаванони и дихидрофлавоноли в 22 проби прополис от различни райони на България. Въз основа на получените резултати можахме да охарактеризираме суровия български прополис по отношение на минимално съдържание на биологичноактвни вещества (антимикробиални и антиоксидантни), както следва: 46% балсам, 24% тотални феноли, 7% тотални флавони и флавоноли, 5,4% тотални флаванони и дихидрофлавоноли. Тези стойности могат да се използват като основа за стандартизация на българския прополис. Те надвишават минималните стойности, предложени от Международната комисия по меда за тополов тип прополис. Този факт доказва, че българският прополис е ценен пчелен продукт с високо качество, по-високо от средното за тополов прополис от други географски райони.