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CHARACTERIZATION OF PROPOLIS FROM MOLDOVA'S CENTRAL REGION: CHEMICAL COMPOSITION, ANTIOXIDANT AND ANTIMICROBIAL PROPERTIES

Serghei Pogrebnoi [a], Nicolae Eremia [b], Dmitri Bilan [a], Lucian Lupascu [a], Natalia Bolocan [a], Gheorghe Duca [a], Svetlana Armasu [a,c], Dumitru Terteac [a,c], Vitalie Cebanu [c], Serghei Tincu [d], Alexandru Znagovan [d], Iulia Neicovcena [e], Olga Coseleva [e], Valerina Slanina [f], Fliur Macaev [a*]

[a]*Institute of Chemistry, Moldova State University, 3, Academiei str., Chisinau MD-2028, Republic of Moldova*
[b] *Technical University of Moldova, 168, Stefan cel Mare bd, Chisinau MD-2004, Republic of Moldova*
[c]*Scientific and Practical Institute of Horticulture, Viticulture and Food Technologies, 59, Hincesti hwy., Chisinau MD-2021, Republic of Moldova*
[d]*State University of Medicine and Pharmacy "Nicolae Testemitanu", 165, Stefan cel Mare blvd., Chisinau MD-2004, Republic of Moldova*
[e]*State University of Comrat, 17, Galatzana str., Comrat MD-3805, Republic of Moldova*
[f]*Institute of Microbiology and Biotechnology, Technical University of Moldova,1, Academiei str., Chisinau MD-2028, Republic of Moldova*
[*]*e-mail: fliur.macaev@sti.usm.md; fax: 373 22 739775*

Abstract. The chemical composition, antioxidant and antimicrobial activity of propolis ethanolic and water-ethanolic extracts from the central zone of Moldova have been investigated by GC-MS and liquid chromatography. There were found 20 amino acids, of which the most abundant are glutamic acid, alanine, leucine and isoleucine. The main constituents of the alcoholic extract are pinocembrin, *n*-heptacosan and naringenin. The aqueous-alcoholic extract was characterized by the content of sakuranin, 4-methoxy sakuranetin, caryophylline oxide, isocaryophylline oxide, *trans*-longipinocarveol. The propolis extracts exhibited strong antioxidant (53.7 mg ascorbic acid eq./g extract or 113.4 mg Trolox eq./g extract and 87.5 mg ascorbic acid eq./g extract or 162 mg Trolox eq./g extract for ethanol, and water-ethanol extract, respectively) and antimicrobial activity (from 0.0055 up to 0.07%), suggesting their potential as natural agents for therapeutic use.

Keywords: amino acid, gas chromatography–mass spectrometry, liquid chromatography, antifungal activity, antibacterial activity.

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Introduction

Propolis is a material collected by the bees from plant exudate (poplar, alder, birch and others) and mixed with bee wax. Propolis serves the purpose of sealing the cracks within the hive and also acts as an embalming agent for intruders that were killed by the bees but couldn't be removed from the hive. The exact composition of propolis can vary depending on the geographic location, climate, and plant species available to the bees. Over 300 compounds have been identified in propolis so far, making it a complex and highly diverse substance. For the so-called European propolis, the poplar serves as a source. For the most of the analysed samples of this type of propolis, the following average composition was found: 50% resin-like components (flavonoids - 5%, aromatic acids, aromatic acid esters), 30% wax (esters, fatty acids, fatty acid alcohols, marginal hydrocarbons), 10% essential and aromatic oils, 5% pollen (free amino acids and proteins), 5% other substances (minerals, ketones, lactones, quinones, steroids, vitamins and sugars) [1].

The flavonoid group includes chrysin, pinocembrin, apigenin, galangin, kaempferol, quercetin, tectochrysin, pinostrobin, pinobenchin, isorhamnetin and others. Another group of compounds is formed of aromatic acids, among which the most abundant ones are ferulic, cinnamic, caffeic, benzoic, salicylic and *p*-cumaric acids. Terpenes are represented (terpineol, camphor, geraniol, nerol, farnesol) which are responsible for its characteristic

fragrance [2-4]. The composition and ratio of substances in the solution depend on the solvent used for extraction. Various solvents, both individually and in mixtures, are used as the extractants: ethanol, methanol, water, hexane, acetone, dichloromethane, petroleum ether, ethyl ether and chloroform [2-4]. Typically, an excess of solvent, ranging from 10 to 30 times the mass of the sample, is employed, and extraction is carried out for 1-7 days at room temperature or by boiling [2,4,5,6].

The antioxidant activity of propolis essentially depends on the method of preparing the extract. Banskota, A.H. *et al*. reported that water extracts of Chinese and Brazilian propolis possessed strong 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical-scavenging activity compared with the corresponding methanol extracts, whereas the methanol extracts of Netherlands and Peruvian propolis exhibited stronger DPPH free radical-scavenging activity than water extracts [7]. Different methods for determining antioxidant activity are applied. Ahn, M.-R. *et al*. used three assay systems for evaluating of the ethanol extracts of propolis (EEP): the inhibition of linoleic acid oxidation by *b*-carotene bleaching, the frМесто для формулы.ee radical-scavenging activity on 1,1-diphenyl-2-picryl-hydrazil (DPPH), the scavenging activity on 2,2-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical cation and ferric reducing ability of plasma (FRAP) [6,8]. It was found that the content of flavonoids and polyphenolic compounds correlates with antioxidant activity [9,10]. Flavonoids make the most significant contribution to antioxidant activity; their content is relatively high and at the same time they are good traps for radicals.

Since ancient times propolis has been used in folk medicine as a drug, it exhibits a wide range of biological activities as antibacterial, antiviral, fungicidal, local anaesthetic, antiulcer, immunostimulant, hypotensive and cytostatic properties, some reports were published about successful clinical use of propolis to aid the healing of wounds, ulcers, and tuberculosis, for the treatment of mycotic infections and eczema [9,11-16]. At the same time propolis is an alternative therapeutic agent that rarely causes side effects [17,18].

The objective of this study was to select the conditions for the preparation and comparative assessment of ethanolic and water-ethanolic extracts of propolis obtained at room temperature, with regards to the study of the amino acid composition, and evaluation of the antioxidant and antimicrobial activity.

Experimental

Materials

Reagents used: ethanol 96%, ascorbic acid, 1,1-diphenyl-2-picrylhydrazyl (DPPH) and Trolox were purchased from Sigma-Aldrich. The solid samples of propolis were collected in the central zone of the Republic of Moldova in 2020 (provided by State Agrarian University of Moldova).

Methods

Extract preparation of propolis

The solid samples of propolis were cooled and crushed in an agate mortar; further, the ethanol extracts were prepared using 96% and 48% alcohol solutions by stirring at room temperature in a ratio of 1:100 by weight. After stirring the mixture for 5 days at room temperature, the resulting extracts were filtered, the solvent was distilled off in vacuum at a temperature not exceeding 30°C, and the residue was used for analysis. The mass fraction of the extraction was 600±7.1 mg and 400±6.7 mg from 100 mL of 96% and 48% alcohol solutions, respectively.

GC-MS spectroscopy

The analysis of ethanolic and water-ethanolic extracts of propolis was carried out on an Agilent Technologies 7890A system with 5975C Mass-Selective Detector equipped with split-splitless injector (split, 250ºC, split ratio 1:50, 1 μL) and HP-5 ms capillary calibrated column (30 m x 0.25 mm x 0.25 μm); the carrier gas: helium 1.1 mL/min; oven: 70^{o}C-2 min, 5ºC/min-200ºC-20/min-300ºC/5 min; MSD in scan 30-300 amu, 15 min, 30-450 amu, solvent delay 3 min 40 s; EI= 60eV.

Liquid chromatography

The analysis of the amino acid content was carried out by ion exchange liquid chromatography on an AAA T 339M amino acid analyser using Li-citrate buffer solution with pH 2.90±0.01, 2.95±0.01, 3.20±0.02, 3.80±0.02, 5.00±0.02. The samples were hydrolyzed by 6 M HCl according to the method [19].

Antioxidant activity evaluation

Ethanol and water were used to prepare the solutions, the DPPH was dissolved in ethanol, ascorbic acid in water. The measurements were carried out on a Perkin Elmer Lambda25 spectrophotometer in a 1 cm path length quartz cuvette, at 517 nm [16]. Equal volumes of solutions of ascorbic acid of various concentrations were added to DPPH solutions

with a concentration of 78 μg/mL. The prepared solutions were kept in a dark place for 30 min, and then absorption *A* was measured. The relative inhibitory concentration *IC*(%) was calculated by the Eq.(1) [16].

$$IC = \frac{A_0 - A}{A_0} \cdot 100\% \quad (1)$$

where, A_0 – absorption of the DPPH solution of 39.4 μg/mL concentration;
A – absorption of a sample.

A plot of relative inhibitory concentration (*IC*,%) - concentration of ascorbic acid solutions (*C*, μg/mL), was drawn, which was used to determine the IC_{50} concentration value, and the IC_{50} values for Trolox and propolis extracts were determined in a similar way.

Antimicrobial activity testing

The method of serial double dilutions was used with the determination of the minimum concentration of inhibition of bacteria and fungi, followed by inoculation of cultures on solid agar nutrient media (peptone agar for bacteria and Sabouraud agar for fungi) and the determination of the minimum bactericidal and fungicidal concentrations [20]. Two strains of phytopathogenic bacteria were used: *Erwinia caratovora* and *Xanthomonas campestris*; two non-pathogenic for plants bacterial strains: *Pseudomonas fluorescens* and *Bacillus subtilis*, two yeast-fungi: *Candida albicans* and *Saccharomyces cerevisiae* and the necrotrophic fungus *Botrytis cinerea*.

Results and discussion

Chemical composition of propolis extracts

Ethanol and water-ethanol mixture were used as non-toxic solvents for the extraction of substances from propolis. Extraction was carried out at room temperature to avoid unwanted destruction of substances. It should be mentioned that, the chemical composition of propolis of samples of central zone of the Republic of Moldova was investigated for the first time. The obtained results show that approximately the same content of 5.0, 5.6, 5.1% was registered for 4-methylthiochalcone, *N*deacetylcolcyquin *N*-(*E*)-4-(4-hydroxy-3,5-dimethoxyphenyl) butene-2-oic acid, and techtochrysine, while the content of oleic acid amide was 0.9%. Sakuranin (31.8%), 4-methoxy sakuranetin (20.0%), caryophylline oxide (1.1%), isocaryophylline oxide (6.7%), *trans*-longipinocarveol (5.3%) were identified as components in the water-ethanolic extract (Table 1).

Table 1

Main compounds determined in propolis extracts by GC-MS.

Compound	*Empirical ormula*	*m/z*	*RT, min*	*Content, %*
96% EtOH extract				
Oleamide	$C_{18}H_{35}NO$	281	19.100	0.9
Pinostrobinhalcon	$C_{16}H_{14}O_4$	270	19.486	12.8
Pinocembrin	$C_{15}H_{12}O_4$	256	19.919	17.6
Unidentified		487	20.050	5.0
Gibberellic acid methyl ester	$C_{20}H_{24}O_5$	344	20.347	11.9
Tekhtochrizin	$C_{16}H_{12}O_4$	268	20.525	5.1
n-Heptakosan	$C_{27}H_{56}$	380	20.721	16.7
Naringenin	$C_{15}H_{12}O_5$	272	20.810	19.4
N-Deacetylcolcyquine	$C_{31}H_{33}NO_9$	563	20.934	5.6
N-E-4-(4-Hydroxy-3,5-dimethoxyphenyl) butene-2-oic acid				
4-Methylthiohalcone	$C_{16}H_{14}OS$	254	21.035	5.0
Total				100.0
48% EtOH extract				
Unidentified		281	14.499	7.1
trans-Longipinocarveol	$C_{15}H_{24}O$	220	14.505	5.3
Caryophylline oxide	$C_{15}H_{24}O$	220	14.763	1.1
Unidentified		446	15.267	4.8
Isocaryophylline oxide	$C_{15}H_{24}O$	220	15.267	6.7
Unidentified		281	15.688	8.6
Unidentified		281	16.554	0.9
4-methoxy sakuranetin	$C_{17}H_{16}O_5$	300	20.943	20.0
Sakuranin	$C_{22}H_{24}O_{10}$	448	21.332	31.8
Unidentified		529	25.618	13.7
Total				100.0

The results of the study showed that the total amount of amino acids in propolis varied from 7.0589 mg/g to 10.2161 mg/g, while the largest contributions are made by glutamic acid, alanine, leucine and isoleucine (Table 2). It was revealed that the total amount of essential amino acids in propolis was 4.223 mg/g, nonessential - 4.359 mg/g, immunoactive - 3.975 mg/g, glycogenic - 3.233 mg/g, ketogenic - 2.570 mg/g, proteinogenic - 8.582 mg/g, and sulphur-containing - 0.090 mg/g (Table 3).

Antioxidant and antimicrobial activity evaluation

Propolis extracts were tested for antioxidant activity in the DPPH radical scavenging assay (Table 4), and the IC_{50} values were determined, *i.e.* the concentrations at which the samples quenched 50% of DPPH radicals. The water-ethanol extract showed 1.5-fold higher antioxidant activity than ethanol extract. Ascorbic acid (IC_{50}= 3.30±0.42 μg/mL) and Trolox (IC_{50}= 4.70±0.31 μg/mL) were used as standards, for which the values 3.21 [16], 4.4 [21], 3.7 [22], and 6.1 [23], 2.78 [24], 9.71 [25] were previously given, respectively.

It was found that the ethanol extract of propolis inhibits the growth of *Bacillus subtilis*, *Erwinia caratovora, Pseudomonas fluorescens*, *Xanthomonas campestris* with the same minimum bactericidal concentration (MBC, %), equal to 0.0035%, which is higher than the activity of ampicillin, equal to 0.0057%. The minimum fungicidal concentration (MFC, %) level for *Saccharomyces cerevisiae* and *Candida albicans* was of 0.0085 for the ethanol extract, which is higher than for the fungus *Botrytis cinerea Pers*. A similar trend of antibacterial and antifungal action was noted for the water-ethanol extract, with a general increase in concentration, with the exception of activity against *Erwinia caratovora* and *Xanthomonas campestris*. The antimicrobial activity of propolis is most likely due to the action of chemical compounds that make up the extract of the product [18,26-29]. All the levels of minimum concentration of inhibition (MIC) were the same as corresponding MBC and MFC.

Table 2

Amino acid composition of propolis samples.

Amino acid	*Content in the average sample*	
	mg/g	*%*
Cysteic acid	0.023± 0.000	0.266
Aspartic acid	0.494±0.108	5.77
Threonine	0.499±0.113	5.82
Serine	0.518±0.036	6.05
Glutamic acid	1.239±0.161	14.46
Proline	0.525±0.116	6.13
Glycine	0.539±0.174	6.29
Alanine	0.713±0.123	8.32
Valine	0.387±0.031	4.52
Cysteine	0.024±0.004	0.276
Methionine	0.043±0.006	0.50
Isoleucine	0.624±0.134	7.29
Leucine	0.665±0.121	7.75
Tyrosine	0.307±0.134	3.59
Phenylalanine	0.511±0.221	5.96
γ-Aminobutyric acid	0.019±0.006	0.225
Diaminovaleric acid	0.028±0.007	0.327
Lysine	0.460±0.123	5.37
Histidine	0.241±0.095	2.81
Arginine	0.707±0.177	8.25
Σ aminoacids	**8.766±1.565**	**100.00**

Table 3

Total amino acid composition of the analysed propolis in the average samples.

Amino acid	*Content, mg/g*
Σ indicators of nitrogen metabolism	8.85±1.61
Σ nonessential amino acids	4.36±0.61
Σ essential amino acids	4.22±0.94
Σ immunoactive amino acids	3.98±0.25
Σ glycogenic amino acids	3.23±0.26
Σ ketogenic amino acids	2.57±0.74
Σ proteinogenic amino acids	8.58±1.55
Σ sulphur-containing amino acids	0.09±0.01

Table 4

Antioxidant activity of propolis extracts.

Extract	IC_{50}, *μg/mL*	*mg ascorbic acid eq./g extract*	*mg Trolox eq./g extract*
Ethanol	61.4	53.7±7.8	113.4±15.9
Water-ethanol	29.0	87.5±12.3	162±22.7

Table 5

Minimum concentrations of antimicrobial activity of propolis extracts.

Test-microorganisms	*Antimicrobial activity, %*	*Ethanol extract*	*Water-ethanol extract*
Erwinia caratovora	MBC	0.035	0.025
Xanthomonas campestris	MBC	0.035	0.012
Pseudomonas fluorescens	MBC	0.035	0.050
Bacillus subtilis	MBC	0.070	0.050
Candida albicans	MFC	0.0085	0.012
Saccharomyces cerevisiae	MFC	0.0085	0.012
Botrytis cinerea Pers	MFC	0.0055	0.050

Conclusions

The comparative analysis of the composition of propolis ethanolic and water-ethanolic extracts from the central zone of Moldova have been investigated by GC-MS chromatography. The research results showed that the nature of the solvent significantly affects the composition of extracts, which differ in biological activity. There are five main components in ethanol extract: chalcone pinostrobinhalcon, flavonoids pinocembrin and naringenin, terpenoid gibberellic acid methyl ester and hydrocarbon *n*-heptakosan. While in the water-alcohol extract, half of quantity are glycosylated flavonoids 4-methoxy sakuranetin and sakuranin. The amino acid composition of the propolis samples was determined by liquid chromatography. The amino acid content was less than 1%, which is much lower than the average European level.

The antioxidant activity of the water-alcohol extract is 1.5 times higher than for the ethanol extract (53.7 and 87.5 mg ascorbic acid eq./g extract, respectively or 113.4 and 162 mg Trolox eq./g extract, respectively). The antimicrobial activity of both extracts on the studied strains is approximately the same (from 0.0055 up to 0.07%). The tested compounds are approximately at the same level of activity of some antibiotics like ampicillin, levomycetin or augmentin, but not exceeding them.

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