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The Triple Botanical Origin of Russian Propolis from the Perm Region, Its Phenolic Content and Antimicrobial Activity

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Russian propolis from the Perm Region was studied by GC-MS, its antibacterial activity was determined, and the bioactive compounds (total phenolics and flavonoids) were quantified by spectrophotometric methods. It was found that Permian propolis belonged to a bee-glue type of triple plant origin: aspen-birch-poplar. Such propolis type has not yet been described. It contains many constituents which could be responsible for diverse biological activities: antibacterial, antifungal, antiviral, antioxidant, anti-inflammatory, and anti-tumor. The combination of the protective excretions of three plant species, resulting in the presence of secondary plant metabolites of many different structural classes gives reason to believe that the pharmacological actions are implemented in different, complementary mechanisms. Together with the detected antibacterial activity, this observation leads to the expectation that such combination could be especially effective in treatment of infections of different origin..

Keywords: Propolis, *Populus nigra*, *Populus tremula*, *Betula pendula*, Antimicrobial.

Modern science took interest in propolis (bee glue) in the middle of the 20th century. Russian researchers were among the first to study propolis. Kivalkina [1] documented its action against a number of microorganisms, mainly bacteria. The work of Popravko and his group shed some light on the chemical composition and antibiotic activity of Russian propolis [2-5], as well as on its plant sources [6,7]. Later on, many other researchers proved diverse biological activities of propolis from different origins [e.g., 8-11]. The studies of Popravko *et al.* established that the main propolis plant sources in different parts of Russia were birch, poplar and trembling aspen, and the most common and most important one was the silver birch *Betula pendula* Roth (*B. verrucosa* Ehr.) [12].

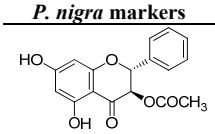
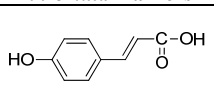
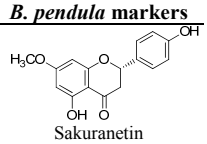
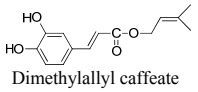
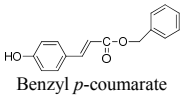
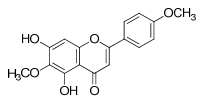
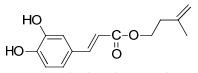
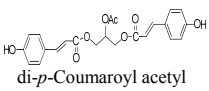
Concerning Russian propolis, recent publications consider technological problems [13-15] and mostly the application of bee glue in the treatment of different diseases, such as chronic prostatitis [16,17], stomach ulcer [18,19], and rhinological problems [20]. The present status of chemical research on Russian propolis encouraged us to apply modern analytical methods to Russian samples and to discuss the results in the light of up-to-date knowledge of bee glue. In this article, we report on our study of Russian propolis from the Perm Region, in order to characterize its potential as a raw material for food additives, cosmetics and over-the counter preparations. Bee glue from the Perm Region has not yet been studied.

Silylated ethanol extracts of five propolis samples collected at different locations in the Perm Region were analyzed by gas chromatography-mass spectrometry (GC-MS). The resolving power of capillary GC, combined with the valuable structural information provided by EIMS make this method one of the most powerful tools for chemical profiling of propolis extracts [21,22]. The GC-MS analyses allowed us to identify over 60 compounds in propolis from the Perm Region. These compounds may be grouped, as follows: flavonoid aglycones (chalcones, flavanones, flavanonols, flavones, and flavonols), aromatic acids, aromatic acid esters, glycerol esters of aromatic acids, and carbohydrates. The quantities measured correspond to the percentage of total ion current and are not a true quantitation. However, they are completely reliable in the case of comparison between samples having similar qualitative composition [23,24], as with the Perm propolis samples.

The results obtained characterize all five samples as originating from three plant sources: the bud resins of black poplar (*Populus nigra* L.), aspen (*Populus tremula* L.) and silver birch (*Betula pendula* Roth). This conclusion is based on the detection of specific taxonomic markers of every one of these three species (Table 1). The flavonoid aglycones pinocembrin and galangin, as well as hydroxycinnamic acids, are present in both poplar and aspen buds [24]. However, the presence of compounds characteristic for only *P. nigra*: pinobanksin 3-*O*-acetate, together with caffeic acid esters, and especially 2-methyl-2-butenyl caffeate and 3-methyl-2-butenyl caffeate, is unambiguous proof that the black poplar is one of the sources of the studied samples [23,25]. The importance of *P tremula* for propolis collection in the Perm Region is confirmed by the identification of glycerol esters of hydroxycinnamic acids. These compounds were found in the bud exudate of *P. tremula*, but never in that of *P. nigra*. It is noteworthy that the GC-MS conditions of analysis of black poplar bud exudate would have allowed their identification [23-25]. Further, in aspen bud resins, much higher concentrations of *p*-coumaric acid, ferulic acid, and benzyl coumarate are found than in black poplar [23,24].

The participation of silver birch as a propolis source can be established by means of its flavonoid markers. The bud exudates of *B. pendula* possess a complex flavonoids profile, rich in flavonoid aglycones of different structural classes [26-28].

Table 1: Important taxonomic markers in propolis from the Perm Region.

P. nigra markers	*P. tremula* markers	*B. pendula* markers
 Pinobanksin 3-O-acetate	 *p*-Coumaric acid	 Sakuranetin
 Dimethylallyl caffeate	 Benzyl *p*-coumarate	 Pectolinarigenin
 3-Methyl-3-butenyl caffeate	 di-*p*-Coumaroyl acetyl glycerol	

Characteristic constituents of birch bud exudates are flavonoid aglycones substituted in ring B (rare in poplar buds), as well as flavones having a substituent at position 6 in ring A. The flavone pectolinarigenin (5,7-dihydroxy-6,4'-dimethoxyflavone) is an important marker of the Betulaceae family, and of the genus *Betula* [28]. Its presence in the samples from the Perm Region, together with high amounts of flavonoids with substituents in ring B, such as sakuranetin (one of the main components of the samples), corroborates the participation of birch buds as a botanical source of these propolis samples [28, 29]. In poplar buds these compounds are present only in very low amounts. Thus, the phenolic compounds (flavonoids and cinnamic acid derivatives) in Table 1 represent the most important markers for the three source plants of propolis from the Perm Region.

All the above mentioned data lead to the reasonable conclusion that Permian propolis belongs to a new mixed aspen-birch-poplar type. The proportion of every one of the botanical sources could be tentatively evaluated using the concentrations of the most important markers of *P. nigra*, *P. tremula* and *B. pendula*, as shown in Figure 1.

Popravko has reported on propolis of mixed birch-poplar origin [7]. However, propolis of the triple aspen-birch-poplar type has not been described till now. It is interesting to note that all five samples from the Perm Region belong to this new propolis type, which seems to be typical for this geographic area.

Most of the chemical constituents of Permian propolis belong to the large group of plant phenolics, characterized by diverse biological and pharmacological activities: phenolic acids and their esters [30 – 32], such as CAPE [32-35], flavonoid aglycons [36-39], and glycerol esters of substituted cinnamic acids [40, 41].

In addition to the GC-MS analysis, quantification of the main groups of biologically active propolis constituents was performed. We used the combination of spectrophotometric methods proposed for poplar type propolis [42]. Although *Betula* flavonoids can be found in these samples, together with poplar metabolites, they belong to the same structural classes and could be analyzed using the same standards. The results obtained are represented in Table 2.

The results demonstrate that, although not of all-poplar origin, the characteristics of Permian propolis samples are within the range recommended by the International Honey Commission for poplar type propolis [43]: minimum 45% balsam, minimum 21% total phenolics, and minimum 8% total flavonoids (4% flavones and flavonols, 4% flavanones and dihydroflavonols).

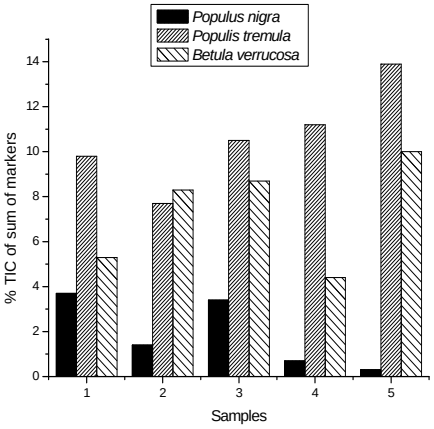


Figure 1: Proportion of the plant sources of propolis from the Perm Region, evaluated by the concentration of taxonomic markers shown in Table 1. (based on GC-MS analysis).

Table 2: Concentration of main groups of bioactive compounds in Russian propolis samples from the Perm Region.

Sample	Balsam[a], %	Total phenolics	Total FF[b],	Total FDHF[c]
		% in raw propolis		
1	57.4±0.1	23.6±0.4	3.3±0.1	6.2±0.2
2	52.3±0.6	23.6±0.4	4.1±0.03	6.5±0.2
3	56±1	22.8±0.1	3.3±0.03	5.9±0.2
4	66±2	25.5±0.5	3.1±0.1	6.7±0.1
5	62±2	26.5±0.7	4.3±0.1	6.5±0.1
		% in balsam		
1		41.1	6.0	11.3
2		45.1	7.8	12.4
3		40.7	5.9	10.5
4		34.5	4.7	10.1
5		42.7	6.9	10.5

[a] substances extracted with 70% ethanol; [b] flavones and flavonols; [c]flavanones and dihydroflavonols

The total flavone and flavonol content is somewhat lower, but the content of flavanones and dihydroflavonols is higher, thus the total flavonoids concentration remains within the range. This fact characterizes Permian propolis as a valuable raw material for different preparations.

The extracts of Permian propolis samples were tested for their action against several microorganisms. The results are presented in Table 3. All samples were active against Gram-positive bacteria and *C. albicans*, but their activity against *E. coli* was low, MIC > 2000 $\mu g.mL^{-1}$. This is in agreement with other publications reporting low activity of propolis against Gram-negative microorganisms [34]. In general, the samples with higher concentration of total flavonoids in balsam demonstrated higher antibacterial activity (lower MIC). This confirms observations made earlier [43]. Interestingly, the most active sample, No 1, is the one in which the three plant sources are "most evenly" represented.

Table 3: Antimicrobial activity of Russian propolis samples from the Perm Region.

Sample	MIC, $\mu g.mL^{-1}$			
	M. luteus	*C. albicans*	*B. cereus*	*S. aureus*
1	111.3	311.5	66.8	133.5
2	204.0	663.0	204.0	255.0
3	328.5	821.3	273.8	328.5
4	188.0	658.0	188.0	343.5
5	286.3	858.8	229.0	235.0

It could be speculated that the combination of numerous compounds with different structures and different mechanisms of action results in synergistic effects and leads to this low value for the MIC.

The results of the present study lead to the conclusion that propolis from the Perm region belongs to a specific bee glue type of triple plant origin: aspen-birch-poplar. Such propolis type has, until now, not been described. The combination of the protective excretions from three different tree species, resulting in the presence of secondary plant metabolites of many different structural classes, gives reason to believe that the pharmacological actions are implemented in different, complementary mechanisms. It is reasonable to expect that such combinations could be especially effective in treatment of infections of different origin. Together with the detected antibacterial activity, this makes Permian propolis a valuable source of biologically active compounds and a promising subject for further studies.

Experimental

Propolis samples, extraction and sample preparation: The samples were collected in different apiaries in the Perm Region in 2010. Propolis, grated after cooling, was extracted for 24 h with 70% ethanol (1:10, w/v) at room temperature. The extract was evaporated to dryness *in vacuo* and weighed to give the balsam amount. About 5 mg of the residue was mixed with 50 μL of dry pyridine and 75 μL of BSTFA and heated at 80° C for 20 min. The standard compounds (1 mg) were subjected to the same procedure for silylation (10 μL of dry pyridine and 15 μL of BSTFA). The silylated extracts and reference compounds were analyzed by GC–MS.

GC-MS analysis: The GC–MS analysis was performed with a Hewlett–Packard gas chromatograph 5890 series II Plus linked to a Hewlett–Packard 5972 mass spectrometer system equipped with a 30 m long, 0.25 mm i.d., and 0.5 μm film thickness HP5-MS capillary column. The temperature was programmed from 60 to 300°C at a rate of 5°C/min, and a 10 min hold at 300° C. Helium was used as a carrier gas at a flow rate of 0.8 mL/min. The split ratio was 1:10, the injector temperature 280°C, the interface temperature 300°C, and the ionization voltage 70 eV. The identification of the compounds was performed using commercial libraries and comparison of mass spectra and retention times of reference compounds. The semi-quantification of the main compounds was carried out by internal normalization with the area of each compound. The addition of individual areas of the compounds corresponds to 100% area.

Balsam determination: Propolis (cooled) was grated and two successive extractions were performed with 70% EtOH: 1 g was dissolved in 30 mL, left for 24 h at room temperature, filtered, and then the procedure was repeated. (A third extraction gave a negative reaction to 5% ferric chloride, which means that the extraction was complete.) The extracts were filtered, combined and diluted to 100 mL in a volumetric flask. This extract was analyzed for total flavanone and dihydroflavonol content. From this extract, 3mL was transferred into a 50 mL volumetric flask, filled up with methanol and this solution was further analyzed for total phenolics and for flavones and flavonols.

Flavone and flavonol content: Total flavone and flavonol content was measured by spectrophotometric assay based on aluminum chloride complex formation [42,44]. In brief, 2 mL of the test solution, 20 mL methanol and 1mL 5% $AlCl_3$ (w/v) were mixed and the volume made up to 50 mL. 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 were estimated using a calibration curve of galangin, with a concentration range of 4–32 mg/mL.

Flavanone and dihydroflavonol content: For flavanones and dihydroflavonols, the colorimetric method from DAB9 was used, modified for propolis [42, 45]. One 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 (w/v). 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 an analogous procedure. Every assay was carried out in triplicate. Flavanone and dihydroflavonol content was estimated using a calibration curve for pinocembrin, in the concentration range of 0.18–1.8 mg/mL.

Total phenolic substances: For quantification of total phenolics, the Folin–Ciocalteu method [42,46] was used. One mL of the test solution was transferred to a 50 mL volumetric flask, containing 15 mL distilled water, to which 4 mL of Folin–Ciocalteu reagent and 6 mL of 20% sodium carbonate solution (w/v) 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: 1mL methanol instead of test solution was used in an analogous procedure. Every assay was carried out in triplicate. Total phenolics content was estimated using a calibration curve of standard mixture of pinocembrin–galangin (2:1), in the concentration range 37–326 mg/mL.

Antibacterial test: Antibacterial activity of the 5 studied samples was determined according to the National Standard of the Russian Federation (GOST R 53124 – 2008). In brief, the test was performed using the agar dilution method with a *Staphylococcus aureus* inoculum corresponding to McFarland turbidity standard 1. The activity of 0.02, 0.04, 0.06 …. 0.20 mL of a propolis extract in ethanol was determined in 10 mL Mueller-Hinton agar. Propolis extract was obtained from 5 g raw propolis in 100 mL 70% ethanol; the propolis concentration was determined after evaporation to dryness of a known volume, in triplicate. The plates were incubated at 36°C for 24 h. If bacterial growth appeared on the plate, the microorganism was considered to be resistant to the corresponding concentration. As a control, 0.20 μL 70% ethanol was used. Microorganisms used: *S. aureus* strain 209p (FDA 209p, ATCC 3569P), *Bacillus cereus* ATCC 11778, *Escherichia coli* ATCC 25922, *Micrococcus luteus* ATCC 9341, and *Candida albicans* VKPGU 401/885-653.

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