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Original article

Propolis produced in Bulgaria and Mongolia: phenolic compounds and plant origin

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Summary — Phenolic composition of Bulgarian and Mongolian propolis and their possible plant sources (the resinous secretions of different tree buds) were investigated by gas chromatography–mass spectrometry (GC/MS). The results obtained demonstrate that in both countries propolis is collected mainly from poplars: from *Populus nigra* and to some extent from *P italica* in Bulgaria, and from *P suaveolens* in Mongolia.

propolis / poplar bud / phenolics / GC/MS / *Populus*

INTRODUCTION

Propolis (bee glue) is a resinous hive product collected by bees. It possesses versatile biological activities – antibacterial, antiviral, fungicidal, antiulcer, immunostimulating, hypotensive, cytostatic, etc (Ghisalberti, 1979). Recently propolis has been successfully applied in medicine and cosmetics (Lejeune *et al*, 1984; Pochinkova, 1986) and this fact has stimulated an increasing interest in its chemical composition and origin. Its chemical composition appears to be very complex – at least 156 propolis constituents have been identified so far (Walker and Crane, 1987). The phenolic compounds of propolis constitute more than 50% of its total weight (Ivanov, 1987) and are probably connected with a substantial part of its biological activity (Ghisalberti, 1979).

It is now generally accepted that bees collect propolis from resinous tree buds, but only a few authors have discussed plant sources in particular geographical regions. Qualitative comparison of the flavonoid aglycones in propolis and bud secretions of different tree species has indicated that in the European part of Russia propolis has been collected mainly from *Betula verrucosa* bud secretions (Popravko, 1976), in the Ukraine – from *Betula verrucosa*, *Populus nigra* and *P tremula* bud secretions (Popravko and Sokolov, 1980), in France (Lavie, 1976), Great Britain (Greenaway *et al,* 1987), Hungary (Papay *et al,* 1986), and even in the Sonoran Desert, Mexico (Wollenweber *et al,* 1987) – from poplar bud secretions. More chemical evidence is needed regarding reports from other parts of the world claiming that propolis can be collected from many other sources (Koenig, 1985). In many areas of the world, especially the Balkans and Asia, almost no information is available on the plant sources of propolis. In this paper we summarize our investigations on the phenolic constituents of propolis and some tree bud exudates from Bulgaria and Mongolia, in order to compare the composition of bee glue from 2 different geographic zones and to determine the main sources of propolis in these regions.

MATERIALS AND METHODS

Bud material of *Populus nigra, P italica, P tremula, Betula verrucosa, Aesculus hippocastanum, Salix alba, Fraxinus excelsior, Alnus glutinosa, Prunus padus, Prunus cerasus,* was collected from trees near Sofia. Propolis samples were obtained from the region of Sofia in Bulgaria.

Bud material from *Populus suaveolens* and propolis samples were collected near Ulan Bator, Mongolia.

Isolation of phenolics from tree bud exudates

Immediately after collection the tree buds (2 g) were briefly extracted with acetone (3 x 20 ml) to dissolve the secretions. The solution was evaporated to dryness, the residue dissolved in MeOH (10 ml), diluted with water (5 ml) and extracted successively with petrol ether (bp 40–60 °C) (3 x 15 ml) and Et_2O (3 x 15 ml). The combined ethyl ether extracts were dried (Na_2SO_4) and evaporated to yield a brown residue. An aliquot of each extract was investigated by TLC (DC Alufolien Kieselgen F_{254} Merck, mobile phases $CHCl_3$/EtOAc (7:3) and *n*-hexane/acetone (3:1)) and HPLC (the conditions of the HPLC analysis are described by Bankova *et al,* 1982) and (for *P nigra, P italica, P tremula* and *P suaveolens*) by GC/MS after silylation (5 mg ethyl ether extract, 0.1 ml *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) 70 °C, 20 min).

Isolation of phenolics from propolis

Propolis (1 g), cut into pieces, was extracted with 10 ml boiling MeOH for 2 h. Water (2 ml) was added to the filtered extract and extracted 3 times with Et_2O. The ethyl ether extract was dried over Na_2SO_4 and evaporated to dryness (0.5 g). 5 mg of the residue was dissolved in 0.1 ml BSTFA, heated for 20 min at 70 °C and analysed by GC/MS.

GC/MS analysis

For GC/MS analysis a 25 m OV-101 fused silica capillary column was used in a JEOL JGC-20K gas chromatograph directly coupled to a JEOL JMS-D 300 mass spectrometer. The samples were introduced *via* a metal injector working in a split mode, with helium as the carrier gas, and a temperature of 150–280 ° at 3°/min. The mass spectrometer was run in the electron impact mode, the ionization potential was 70 eV, ionization current 300 μA and the ion source temperature 170 °C.

Identification of the compounds

The identification of the compounds is shown in table I. Most of them were identified by comparison with authentic samples (RT and mass spectra) while the remaining part was identified by their mass spectral fragmentation.

RESULTS

In Bulgaria buds from *Populus nigra, P italica, P tremula, Betula verrucosa, Aesculus hippocastanum, Alnus glutinosa, Fraxinus*

Table I. Composition of the phenolic compounds of poplar buds and propolis from Bulgaria and Mongolia. The percentage figures refer to the ion current generated by the compound in the mass spectrometer.

Compound	*Bulgarian propolis*	P nigra	P italica	P trem	*Mongolian propolis*	P suav
Flavonoids						
Pinostrobin [b]	t	t	–	–	–	–
Pinostrobin chalcone [a]	–	–	–	–	4	4
Pinocembrin [b]	23	25	2	t	11	12
3,7-Dihydroxy-5-methoxyflavanone [b]	t	t	–	–	–	–
2,5-Dihydroxy-7-methoxyflavanone [b]	t	t	–	–	–	–
Pinobanksin [b]	7	6	3	–	–	–
Pinobanksin-3-*O*-acetate [b]	6	6	4	–	6	7
Galangin [b]	6	4	< 1	t	2	2
Derivatives of cinnamic acid						
p-Coumaric acid [b]	< 1	1	< 1	2	–	–
Dimethoxycinnamic acid [a]	< 1	< 1	9	–	–	–
Isoferulic acid [b]	< 1	< 1	4	–	–	–
Ferulic acid [b]	< 1	< 1	1	37	–	–
Caffeic acid [b]	2	2	3	–	4	2
3-Methyl-3-butenyl ferulate [b]	< 1	< 1	–	–	–	–
Pentenyl ferulate [a]	t	–	–	–	–	–
3-Methyl-2-butenyl ferulate [b]	1	1	–	–	–	–
Benzyl ferulate [b]	–	–	–	5	–	–
Ethyl caffeate [a]	–	–	–	–	–	< 1
Butenyl caffeate [a]	–	–	–	–	< 1	< 1
Butyl caffeate [a]	–	–	–	–	< 1	–
Pentyl caffeate [a]	–	–	–	–	2	< 1
Pentenyl caffeate [a]	–	–	4	–	–	t
Pentenyl caffeate (isomer) [a]	–	–	6	–	–	–
3-Methyl-3-butenyl caffeate [b]	5	4	17	–	4	3
Pentenyl caffeate (isomer) [a]	2	2	3	–	–	–
3-Methyl-2-butenyl caffeate [b]	6	5	22	–	27	24
Benzyl caffeate [a]	3	1	< 1	–	–	–
β-Phenylethyl caffeate [a]	7	6	12	–	1	2
Other acids						
Benzoic acid [b]	–	–	t	1	–	–
Miristic acid [b]	t	–	–	–	–	–
Palmitic acid [b]	< 1	–	–	–	–	–
Stearic acid [b]	t	–	–	–	–	–
Acetophenone derivatives						
Dihydroxyacetophenone [a]	t	–	–	–	–	–
Dihydroxymethoxyacetophenone [a]	t	t	t	–	–	–
Unknown components						
X-1	–	–	–	–	4	3
X-2	–	–	–	–	2	1
X-3	3	2	< 1	–	–	–
X-4	2	2	2	–	–	–
X-5	2	2	3	–	–	–
X-6	2	2	–	–	–	–
X-7	4	5	–	–	–	–
X-8	2	3	1	–	–	–

t : traces; < 1 : less than 1%. X-1 to X-8 : unidentified components. a: identification on the basis of mass-spectral characteristics. We have not confirmed our identification by comparison with an authentic reference standard, as we do not have one. b: identification on the basis of comparison with an authentic reference standard.

excelsior, Salix alba, Prunus padus and *Prunus cerasus,* as well as propolis were collected in the Sofia region. In Mongolia propolis and buds from *Populus suaveolens* were collected in the region of Ulan Bator, poplars *P suaveolens* being the only trees in this area. The bud secretions (after a brief extraction with acetone) were analysed by TLC and HPLC. Phenolics similar to those of Bulgarian propolis were found only in bud secretions from *Populus nigra, P italica* and *P tremula,* so they were further investigated using GC/MS. The other tree bud secretions were not analyzed by GC/MS, since they did not contain the main propolis phenolic constituents. The preliminary investigation of Mongolian propolis and *P suaveolens* bud secretions showed a similarity in their phenolics, so both extracts were also analyzed by GC/MS. The results obtained are summarized in table I.

Bulgarian propolis and poplar buds

It is evident from table I that composition of phenolic constituents of *P nigra* bud secretions is extremely similar to that of propolis from the same region. Only some minor phenolic constituents, as well as fatty acids are absent. In Bulgarian propolis and *P nigra* bud secretions we found phenolic acids, flavonoids and a series of phenolic acid esters: caffeates and ferulates. Phenolic fraction from *P italica* bud secretions contained more phenolic acids and their esters, and fewer flavonoids than *P nigra* bud secretions. The concentration of isoferulic and caffeic acids in *P italica* bud secretions are similar, but only caffeic acid esters are present. In *P tremula* bud secretions the number of flavonoid aglycones was low, these results being in agreement with earlier observations (Popravko and Sokolov, 1980). The phenolic composition of *P tremula* bud secretions is very different from that of *P nigra* and *P italica;* the main component appears to be ferulic acid. Esters were found in traces and we succeeded in the identification of benzyl ferulate only.

Mongolian propolis and poplar buds

To our knowledge this is the first investigation of propolis from Mongolia. Phenolic fraction from Mongolian propolis (table I) contained compounds similar to those found in Bulgarian and other kinds of European propolis: phenolic acids and their esters, as well as flavonoid aglycones. Its composition is very close to that of *P suaveolens* bud secretions. The only phenolic acid in both extracts was caffeic acid, and only caffeates but not esters of other substituted cinnamic acids were found.

DISCUSSION

It is evident from the results obtained in this study that the phenolic composition of Bulgarian propolis is very similar to that of *P nigra* bud secretions. We found 31 compounds in propolis and 26 of these were found in *P nigra* bud secretions in similar concentrations. The absence of some minor propolis constituents in *P nigra* bud secretions could be an indication of the existence of some other minor sources of propolis. The identification of some rare natural compounds (isopentenyl caffeates and ferulates, pinobanksin-3-*O*-acetate) in Bulgarian propolis and *P nigra* bud secretions is an additional indication that these bud secretions are the main source of Bulgarian propolis.

In Bulgarian propolis we found 21 phenolic compounds of the 24 found in *P italica* bud secretions. According to the results shown in table I, *P italica* bud secretions could also be a source of propolis, but to a lesser extent than *P nigra* bud secretions.

The differences in the phenolic composition of propolis and *P tremula* bud secretions is more significant; obviously *P tremula* bud secretions are of very limited importance for propolis.

We can explain the small differences observed in the propolis composition from different regions in Bulgaria (Bankova, Kuleva, 1989) with the participation of different poplar species in it. We do not know why the bees prefer *P nigra* bud secretions: because this is the most widespread poplar in Bulgaria (Velchev, 1966) or for some other reason; but it is evident that they prefer collecting propolis from *P nigra* bud secretions.

Instead of seasonal variation of propolis we investigated seasonal variations in phenolic constituents of *P nigra* bud secretions.

The samples were collected every 3 months and no significant differences in their phenolic composition was found. The phenolic composition of Mongolian propolis is very similar to this of *P suaveolens* bud secretions. In these bud secretions we found all the compounds of the corresponding propolis in similar concentrations. *P suaveolens* bud secretions contain more esters than *P nigra* bud secretions and they are caffeates only. In *P suaveolens* and Mongolian propolis we did not even find traces of phenolic acids other than caffeic acid. Caffecic acid and caffeates are very important antibacterial constituents of propolis (Ghisalberti, 1979). This is in agreement with our earlier observations that antibacterial activity of Mongolian propolis is higher than that of Bulgarian propolis (Bankova *et al*, 1989). It is evident that under different ecological conditions: in desert regions (Sonoran Desert, Mexico (Wollenweber *et al*, 1987)), in the Mongolian midlands, and in the Balkan Peninsula, *Populus* species are one of the most important sources of propolis and they are of great ecological importance to bees. These conclusions are based on the compounds soluble in organic solvents, these compounds are responsible for the main part of the biological activity of propolis (Ghisalberti, 1979).

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Résumé — Propolis produite en Bulgarie et en Mongolie : composés phénoliques et origine botanique. Des bourgeons de *Populus nigra, P italica, P tremula, Betula verrucosa, Aesculus hippocastanum, Alnus glutinosa, Fraxinus excelsior, Salix alba, Prunus padus* et *Prunus cerasus* ainsi que des échantillons de propolis ont été prélevés dans la région de Sofia, Bulgarie. En Mongolie, de la propolis et des bourgeons de *Populus suaveolens* ont été récoltés dans la région d'Ulan Bator, les peupliers de cette espèce étant les seuls arbres à pousser dans cette région. Les sécrétions des bourgeons, après une brève extraction à l'acétone, ont été analysés en chromatographie sur couche mince et chromatographie liquide haute pression. Seules les sécrétions de bourgeons de *Populus nigra, P italica* et *P tremula* ont fourni des phénols semblables à ceux trouvés dans la propolis bulgare. Aussi ont-elles été étudiées en chromatographie gazeuse/spectrométrie de masse. Les résultats obtenus sont présentés dans le tableau I. Il est clair que les composés phénoliques des sécrétions de bourgeons de *P nigra* ressemblent énormément à ceux de la propolis de la même région. La fraction phénolique de la propolis mongole (tableau I) contient des composés similaires à ceux trouvés dans la propolis bulgare et dans celles provenant d'autres régions européennes : acides phénoliques et leurs esters aussi bien que les aglycones-flavonoïdes. Sa composition est très proche de celle des sécrétions de bourgeons de *P suaveolens*. Ceci prouve que dans des conditions écologiques variées, régions désertiques du Mexique (Désert Sonoran, Wollenberger *et al*, 1987), régions centrales de Mongolie et péninsule balkanique, les espèces de *Populus* constituent l'une des sources les plus importantes de propolis et qu'elles sont d'une grande importance écologique pour les abeilles. Ces conclusions reposent sur l'analyse de composés solubles dans des solvants organiques et responsables en grande partie de l'activité biologique de la propolis (Ghisalberti, 1979).

propolis / bourgeon / *Populus* / phénol / GC/MS

Zusammenfassung — Propolis aus Bulgarien und aus der Mongolei: Phenolverbindungen und pflanzliche Herkunft. In Bulgarien wurden Knospen von *Populus nigra, P italica, P tremula, Betula verrucosa, Aesculus hippocastanum, Alnus glutinosa, Fraxinus excelsior, Salix alba, Prunus padus* und *Prunus cerasus* gesammelt, sowie auch Propolis aus der Region von Sofia. In der Mongolei wurden in der Region von Ulan Bator Propolis sowie auch Knospen von *Populus suaveolens* (dem einzigen Baum dieser Region) gesammelt. Die Knospensekrete wurden nach kurzer Extraktion mit Aceton mittels TLC und HPLC analysiert. Ähnliche Phenole wie in der bulgarischen Propolis wurden nur in den Knospensekreten von *Populus nigra, P italica* und *P tremula* gefunden. Deshalb wurden diese mittels GC/MS weiter analysiert. Die erzielten Resultate sind in Tabelle I zusammengefaßt. Aus dieser Tabelle wird deutlich, daß die Zusammensetzung der Phenolbestandteile von den Knospensekreten von *P nigra* derjenigen der Propolis aus derselben Region extrem ähnlich ist. Die Phenolfraktionen der mongolischen Propolis (Tabelle I) enthielten Verbindungen, die den in bulgarischer Propolis so wie an, europäischer Herkunft sehr ähnlich sind: Phenolsäuren und ihre Ester, so wie Flavon-Aglykone. Ihre Zusammensetzung ist sehr ähnlich wie die der Knospensekretion von *P suaveolens*. Es ist klar, daß unter den verschiedenen ökologischen Verhältnissen von Wüstenregionen (Sonora Wüste, Mexiko (Wollenweber *et al*, 1987)), im mongolischen Mittelland und am Balkan Pappelarten zu den wichtigsten Propolisquellen gehören und für die Bienen von großer ökologischer Bedeutung sind. Diese Schlußfolgerungen beruhen auf den in organischen Mitteln lösbaren Verbindungen, die auch für den Hauptteil der biologischen Aktivität von Propolis verantwortlich sind (Ghisalberti, 1979).

Propolis / Knospe / *Populus* / Phenol / GC/MS

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