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Chemical composition of propolis and American foulbrood: Is there any relationship?

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Dedicated to Acad. Ivan Juchnovski on the occasion of his 80th birthday

American foulbrood (AFB) is the most destructive brood diseases of honeybees, causing significant losses to beekeepers. Propolis is an important element of the bee colony social immunity and has demonstrated activity against the causative agent of AFB *Paenibacillus larvae.* However, knowledge on the relationship between propolis chemical composition and the health of the bee colony is still scarce. We studied by GC-MS the chemical profiles of propolis samples from healthy bee colonies and colonies with clinical symptoms of AFB. Healthy colonies produced propolis with higher content of balsam. Although the qualitative composition of all samples was the same, there were quantitative differences: propolis from healthy colonies contained much higher levels (statistically significant, $p<0.01$) of ferulic acid and coniferyl benzoate, than the propolis from colonies with AFB. Our results are only preliminary, further research should be performed to clarify whether these differences are indeed related to the health of the colonies.

Key words: propolis; American foulbrood; GC-MS

INTRODUCTION

Propolis (bee glue) is a sticky material collected by honeybees from plants and used in the hive both as building material and chemical defense against infections. It is well known to possess diverse beneficial biological activities, such as antimicrobial, immunostimulating, antitumor, antiinflammatory, etc., and is widely used in health foods and over-the-counter preparations [1,2]. Recently, propolis has been attracting growing attention with respect to its potential to combat bee pathogens and the possibility to replace pesticides and antibiotics in beekeeping [3]. Several studies revealed the role of propolis as an important element of bees' "social immunity" [4]. Propolis extracts have been found to act against varroa mites [5,6], and extracts as well as some individual propolis constituents demonstrated *in vitro* significant activity against the causative agent of American Foulbrood [7,8]. American Foulbrood (AFB) is an infectious disease of honeybees caused by *Paenibacillus larvae*, a gram positive spore forming bacterium which is distributed worldwide [9]. AFB is considered the most destructive brood disease [10]. Some antibiotics are effective against *P.larvae* but antibiotics are poorly metabolized by honeybees, and their residues or those of their metabolites can be stable in honey for over a year [11]. Moreover, the use of antibiotics in beekeeping is banned in the EU countries. The current most typical solution to deal with an AFB affliction is to burn the entire hive [12]. Thus, finding alternatives is an important issue in beekeeping.

Although propolis is known to be a defensive material against microorganisms, the knowledge on the relationship between propolis chemical composition and the health of the bee colony is still scarce. We have recently found a significant difference between the composition of propolis of colonies susceptible and resistant against *Varroa destructor* in France [13]. In the present study, we continued our attempt to clarify some aspects of the relationship propolis chemistry-colony health, studying the chemical composition of propolis (by GC-MS) and correlating it with health issues of the colonies, specifically the presence or lack of clinical symptoms of AFB.

EXPERIMENTAL

Propolis samples

Propolis samples were collected in Northern parts of Sweden by Prof. Ingemar Fries, Swedish University of Agricultural Sciences, Uppsala, Sweden. Five samples, 1 - 5 were collected from healthy colonies and three samples: 6, 7 and 8, from colonies with clinical symptoms of AFB.

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Propolis extraction

Frozen propolis (freezer) was grated and 1 g was dissolved in 30 mL 70% ethanol in a 100 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.

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.

GC-MS analysis

5 mg of the propolis dry extract was dissolved in 50 µl of dry (water-free) pyridine, and 75 µl of bis(trimethylsilyl)-trifluoroacetamide (BSTFA) were added. The mixture was heated at 80 °C for 20 min and analyzed by GC-MS. 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 ionisation voltage 70 eV, as described elsewhere [13]. Identification of the compounds was performed using comparison of mass spectra and retention times of reference compounds (21 compounds), and the rest was tentatively identified using their mass spectra and retention time analysis. The semi-quantification was carried out by internal normalisation with the area of each compound. The addition of individual areas of the compounds corresponds to 100% area.

RESULTS AND DISCUSSION

We studied 5 healthy and 3 American foulbrood infected colonies, starting with the content of balsam. The extract with 70% ethanol is known as propolis balsam and contains the biologically active secondary plant metabolites of the resins collected by bees [14]. The undissolved material consists mainly of waxes and mechanical impurities. The balsam percentage characterizes the amount of resins that bees have collected from plants and used to produce propolis. The balsam content of the studied samples is presented in Table 1. The mean value of the percentage of propolis balsam in the bee glue of colonies with AFB was significantly lower, compared to healthy colonies: 55% against 72% ($p = 0.008$). This is an indication that honeybees from infected colonies have allocated lesser resources to resin collection, than bees from healthy colonies. The reason for this fact however remains unclear.

By GC-MS, we were able to identify completely or tentatively over 70 individual compounds in the studied samples (data not shown). The chemical profiles of all eight samples were very similar qualitatively and quantitatively. They were all of practically pure trembling aspen (*Populus tremula*) origin and thus displayed a very low flavonoids concentration [15,16]. Instead, they contained high amounts of phenolic acids and their esters, and the typical aspen chemical markers: glycerol esters of substituted cinnamic acids. The chemical profiles of the studied samples can be represented in a concise manner by the percentage of the main structural groups of chemical constituents (Table 1): aromatic acids (major components [MC] coumaric, ferulic and benzoic acid), esters of aromatic acids (MC benzyl *p*-coumarate and coniferyl benzoate), flavonoids, including chalcones, flavones/flavonols, and flavanones/dihydroflavonols (no individual flavonoid was found in amount over 2%, most were under 1%); glycerol esters of cinnamic acids (MC 2-acetyl-1,3-di-p-coumaroylglycerol), and sugars, fatty acids and others (all minor components under 0.5% of total ion current)

The large amount of data obtained from the GC-MS studies were analyzed using Principle Component Analysis (PCA). The central idea of PCA is to reduce the dimensionality of a data set in which there are a large number of correlated variables, while retaining as much as possible the total information. We selected for PCA analysis the relative amounts of the main groups of chemical constituents of propolis: aromatic acids, phenolic acid esters, flavonoids, sugars, and others. The application of PCA produced a two-dimensional plot (Fig. 1) which covered 94% of the total variation and formed two distinct groups of samples: from healthy colonies and from colonies with clinical symptoms of AFB.

Table 1. Balsam content and chemical composition (compound groups, GC/MS, percentage of TIC) of propolis from healthy and AFB infected colonies

Compound	1	2	3	4	5	6	7	8
	CLEAN					American Foulbrood		
Balsam content	68,1	79,8	69,7	66,4	76,00	51,3	51	62,5
Aromatic acids	32,0	39,1	32,6	29,9	32,2	25,8	24,9	28,8
Esters of aromatic acids	28,1	26,7	29,6	17,4	25,2	12,7	16,5	19,0
Chalcones	1,4	0,6	1,3	5 ,2	2,8	3,5	1,8	1,3
Flavanones and dihydroflavonols	0,1	0	0,2	0,8	0,7	0,4	0,1	0,1
Flavones and flavonols	1,2	0,3	0,8	6,9	2,0	5,8	1,8	0,5
Glycerols esters of cinnamic acids	7,9	8,0	10,3	9,7	10,0	13,9	7,1	11,0
Sugars	7,1	5,0	3,7	7,2	4,2	6,1	17,1	17,4
Fatty acids	0,4	0,4	0,4	0,8	0,6	2,0	1,9	0,9
Others	3,6	3,7	3,4	4,7	4,1	4,0	3,6	4,0

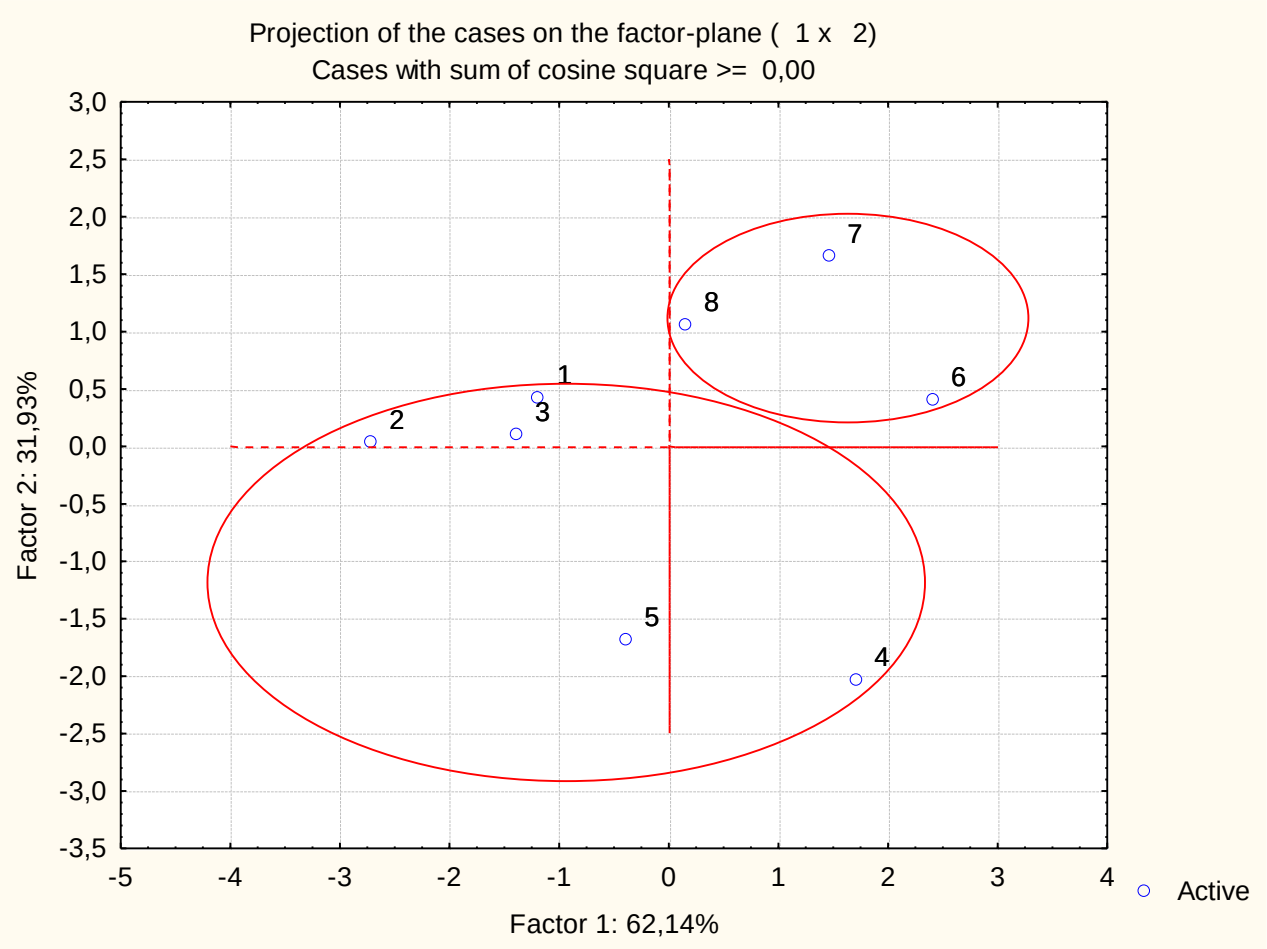

Fig. 1. PCA of propolis secondary metabolite profiles from healthy and AFB infected colonies. 1–5, samples from healthy colonies; 6–8, samples from infected colonies.

Looking into detail, it became evident that the chemical difference between propolis of healthy and AFB infected colonies are less obvious than the ones in balsam content. The most substantial distinction between the two groups was the content of two individual propolis constituents: propolis from healthy colonies contained much higher levels (statistically significant, $p<0.01$) of ferulic acid and the benzoic acid ester coniferyl benzoate, than the propolis from colonies with AFB (Fig. 2). Especially the concentration of coniferyl benzoate was 3 – 4 times higher. Recently we found considerable activity of some propolis flavonoids and phenolic acid esters against *P. larvae*. Those active compounds were isolated from propolis originating from *P. nigra* and are practically absent

in aspen (*P. tremula*) propolis. It is interesting to note that coniferyl alcohol and some of its esters have antibacterial activities [17,18].

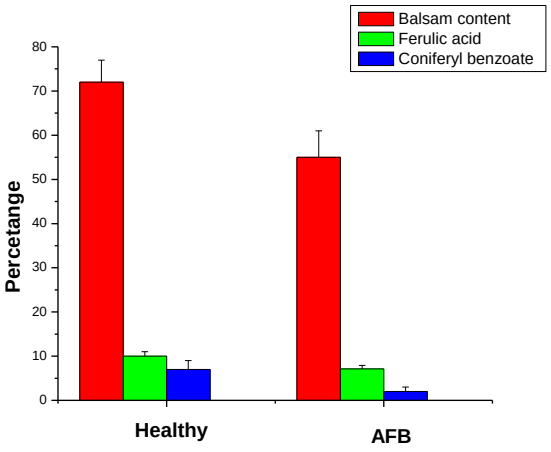

Fig. 2. Content of balsam, ferulic acid and coniferyl benzoate in propolis from healthy and AFB infected colonies.

CONCLUSIONS

Our results are only preliminary and they cannot give an unambiguous answer to the question about the possible relationship between propolis chemical composition and bee colony health. Nevertheless, they give some indications that such a relationship might be present. We established that chemical differences exist between propolis from colonies with AFB and healthy colonies. Further research should be performed to clarify whether these differences are indeed related to the health of the colonies. Special attention should be paid to the specific compounds that are more abundant in healthy colonies

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СЪСТАВ НА ПРОПОЛИСА И АМЕРИКАНСКИ ГНИЛЕЦ: ИМА ЛИ ВРЪЗКА?

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(Резюме)

Американският гнилец (АГ) е най-разрушителното заболяване, засягащо пчелното пило и причинява значителни загуби на пчеларските стопанства. Прополисът е важен елемент от социалния имунитет на пчелните семейства и е показал активност срещу причинителя на АГ *Paenibacillus larvae*. При все това познанията за връзката между химичния състав на прополиса и здравето на пчелното семейство са много ограничени. С помощта на газова хроматография – масспектрометрия ние изучихме химичните профили на проби прополис от здрави кошери и от такива с клинични симптоми на АГ Оказа се, че здравите пчелни семейства произвеждат прополис със значително по-високо съдържание на балсам. Макар че качественият състав на всички проби беше практически идентичен, бяха намерени количествени различия – прополисът на здравите пчелни семейства съдържаше по-висок процент (статистически значими разлики, $p<0.01$) ферулова киселина и кониферилбензоат в сравнение с болните от АГ. Получените резултати са само предварителни и са необходими по-нататъшни изследвания, за да се изясни дали тези разлики наистина са свързани със здравето на пчелните семейства.