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## Propolis chemical composition and honeybee resistance against *Varroa destructor*

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Propolis is known as honeybee chemical defence against infections and parasites. Its chemical composition is variable and depends on the specificity of the local flora. However, there are no data concerning the relationship between propolis chemical composition and honeybee colony health. We tried to answer this question, studying the chemical composition of propolis of bee colonies from an apiary near Avignon, which are tolerant to *Varroa destructor*, comparing it with colonies from the same apiary which are non-tolerant to the mites. The results indicated that non-tolerant colonies collected more resin than the tolerant ones. The percentage of four biologically active compounds – caffeic acid and pentenyl caffeates – was higher in propolis from tolerant colonies. The results of this study pave the way to understanding the effect of propolis in individual and social immunity of the honeybees. Further studies are needed to clarify the relationship between propolis chemical composition and honeybee colony health.

**Keywords:** propolis; propolis constituents; *Varroa destructor*; *Varroa*-resistant colonies

### 1. Introduction

Propolis (bee glue) is a resinous material used by honeybees (*Apis mellifera* L.) in the construction and adaptation of their nests to fill out cracks in the hives. To produce propolis, bees collect plant resins and mix it with wax (Ghisalberti 1979). It is now generally accepted that bees collect resinous plant materials, produced by a variety of botanical processes in different parts of the plants. These are substances actively secreted by intact and wounded plants through their leaves, buds and mucilages (Crane 1988). The specificity of the local vegetation is responsible for the chemical composition of propolis: in different ecosystems bees collect propolis from different source plants, choosing appropriate representatives of the local flora (Bankova 2005). Propolis contains ‘protective’ secondary plant metabolites, which play an important role in preventing microbial infestation of vulnerable plant tissues; and thus possesses antimicrobial properties against different bacteria, fungi and viruses (Burdock 1998; Sforzin & Bankova 2011).

It has been suggested that propolis plays a defensive role in the hive, too, but surprisingly, the studies dealing with the activity of propolis against bee pathogens are scarce. Recently, there is an emerging interest in the potential of propolis to combat bee pathogens (Garedew et al. 2002; Damiani et al. 2010; Simone-Finstrom & Spivak 2012; Bilikova et al. 2013). A recent study of Simone et al. (2009) has revealed the role of propolis in bees’ social immunity. In addition, some propolis constituents, such as *p*-coumaric acid, demonstrated the ability to up-regulate immunity

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genes in honeybees (Mao et al. 2013). However, none of the above-mentioned studies have considered the chemical composition of the studied propolis, while propolis chemical composition is highly variable. To date, there are no scientific data concerning the relationship between the health of the bee colonies and the chemical composition of their respective propolis.

*Varroa* mites were introduced to the European honeybee *Apis mellifera* over 30 years ago, and have since become the largest threat to apiculture around the world. It is known that a few subset populations of European honeybee races have been sustainably surviving mite infestation for periods over 10 years without mite control treatment (Fries et al. 2006; Le Conte et al. 2007). Such population exists in the area of Avignon, France, where *Varroa*-resistant honeybee colonies reduce the reproductive success of their infesting mites compared with local control colonies (Locke et al. 2012). Mechanistic explanations of the bees' ability to suppress mite reproductive success remain unknown to a large extent.

We studied the chemical composition of the propolis from resistant and susceptible colonies from Avignon from one and the same apiary in order to clarify whether there are any chemical differences in this defensive material, which might contribute to the resistance.

## 2. Results and discussion

The propolis extract obtained with 70% ethanol is known as balsam. It contains bioactive plant metabolites from plant resins, while undissolved matter is formed mainly from waxes and mechanic impurities. Balsam amount in propolis is characteristic of the amount of resin collected by the bees. The balsam content of the samples is presented in Table 1. The samples obtained from resistant colonies had significantly lower balsam content: 58% compared with 72% for propolis from susceptible colonies ( $p < 0.05$ ). This fact is an indication that bees from resistant colonies have allocated lesser resources to resin collection, than bees from susceptible colonies.

Over 60 individual constituents were identified completely or tentatively in each sample by gas chromatography/mass spectrometry (GC/MS) profiling (data not shown). The chemical profiles of all the samples were very similar in qualitative composition. These profiles can be presented in a concise form by the relative amounts of the main compound classes identified (Table 1): aromatic acids [main components (MC): benzoic, caffeic, *p*-coumaric acids], phenolic acid esters – coumarates, ferulates/isoferulates and caffeates; chalcones (MC: pinocembrin chalcone), flavones and flavonols (MC: chrysin and galangin), flavanones and dihydroflavonols (MC: pinocembrin, pinobanksin-3-*O* acetate); sugars, and others [fatty acids, triterpenes, and so on – all minor components under 0.5% of total ion current (TIC)]. In order to analyse the large amount of data, we applied principal component analysis (PCA). The PCA of the peak areas corresponding to each compound from both sample groups resulted in two groups correlated with their origin from mite-resistant and mite-susceptible colonies (Figure 1).

The chemical distinctions between propolis from resistant and susceptible colonies were less obvious than the ones in balsam percentage. The most important difference was the fact that relative concentration of caffeic acid and caffeic acid pentenyl esters: 3-methyl-3-butenyl caffeate, 2-methyl-2-butenyl caffeate and 3-methyl-2-butenyl caffeate (Figure 2) were higher in propolis of resistant colonies and the differences observed were statistically significant ( $p < 0.05$ ) (Figure 3). Higher concentrations of two further caffeates, caffeic acid phenethyl ester (CAPE) and cinnamyl caffeate, were present in the samples from resistant colonies but the differences were not statistically significant. A possible explanation of these differences could be the difference in chemical profiles of the bud exudates collected by the resistant and the susceptible bee colonies.

It is important to note that caffeic acid and caffeates are among propolis components with pronounced and diverse biological properties. A mixture of CAPE, prenyl caffeates and benzyl

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Table 1. Balsam content and chemical composition (compound groups, GC/MS, percentage of TIC) of propolis from mite-susceptible and mite-resistant colonies.

Compound class	1	2	3	4	5	Mean		6	7	8	9	10	Mean
	Susceptible							Resistant					
Balsam content	82	75.6	74	68.2	62.9	72 ± 7 <sup>a</sup>	66	45.0	54	66.0	60.3	58 ± 9 <sup>b</sup>	
Aromatic acids	8.9	10.6	7.9	7.8	10.3	9 ± 1 <sup>c</sup>	12.2	10.1	10.6	6.5	4.9	9 ± 3 <sup>c</sup>	
Coumarates	2.5	2.2	2	1.4	1.5	1.9 ± 0.5 <sup>d</sup>	2.1	0.6	0.2	0.4	0.7	0.8 ± 0.7 <sup>d</sup>	
Ferulates and isoferulates	5.6	2.8	1.8	2.5	2.6	3 ± 1 <sup>e</sup>	2.1	1.3	1.8	1.5	1.3	1.6 ± 0.3 <sup>e</sup>	
Caffeates	12.9	12.2	10.3	12.6	11.4	12 ± 1 <sup>f</sup>	17.3	13	18.9	13.9	13.4	15 ± 3 <sup>g</sup>	
Chalcones	13.8	16.0	13.4	10.7	13.6	14 ± 2 <sup>h</sup>	11.8	11.2	11.4	15.1	15.9	13 ± 2 <sup>h</sup>	
Flavanones and dihydroflavonols	18.1	19.8	15.7	21.5	15.8	18 ± 2 <sup>i</sup>	15.1	15.4	15.9	15.7	14.9	15.4 ± 0.4 <sup>j</sup>	
Flavonones and flavonols	20.9	19.0	24.3	21.3	18.3	20 ± 2 <sup>k</sup>	18.3	18.1	19.0	22.3	16.7	19 ± 2 <sup>k</sup>	
Sugars	1.4	1.5	2.2	0.8	2.5	1.7 ± 0.7 <sup>l</sup>	2.1	17.4	5.2	3.8	3.1	6 ± 6 <sup>m</sup>	
Others	2.2	1.4	2.3	1.6	2.2	1.0 ± 0.4 <sup>n</sup>	1.9	1.6	2.0	2.5	2.0	2.0 ± 0.3 <sup>n</sup>	

Note: Different letters denote mean values that are statistically different.

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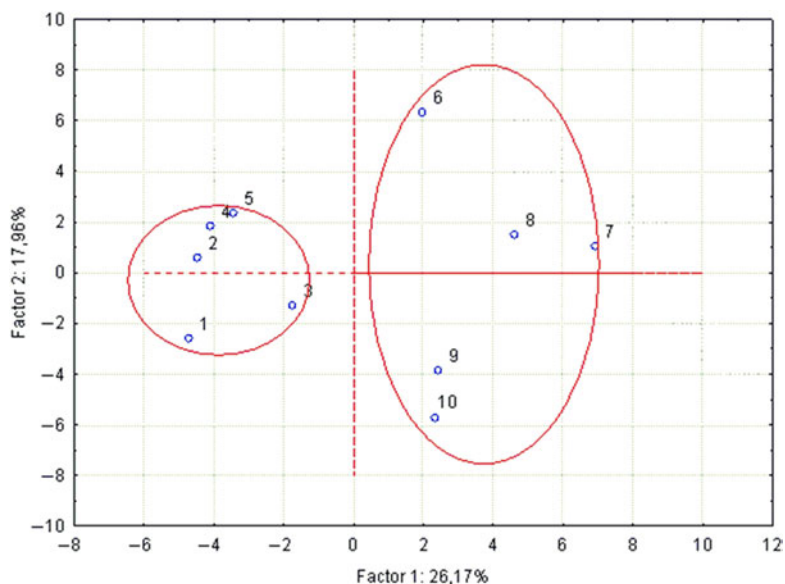


Figure 1. PCA of propolis secondary metabolite profiles from resistant and susceptible colonies. 1–5, samples from mite-susceptible colonies; 6–10, samples from mite-resistant colonies.

caffeate was recently found to inhibit the growth of the bee pathogen *Paenibacillus larvae*, the causal agent of American foulbrood (Bilikova et al. 2013). The way in which these compounds affect the health of the colony is yet to be established. They might possess acaridic activity or have the potential to strengthen the immune responses of honeybees. The compounds in question might affect the surviving potential of the bee colonies in yet another way: it could be speculated that those propolis constituents which are in higher concentration in bee glue of resistant colonies might have favourable effect on the respective colonies by reducing the damage caused by *Varroa*-vectored viruses (Le Conte et al. 2010). Further studies have to be performed in order to clarify whether any of those factors or a combination of them is of importance for the *Varroa* resistance of the colonies.

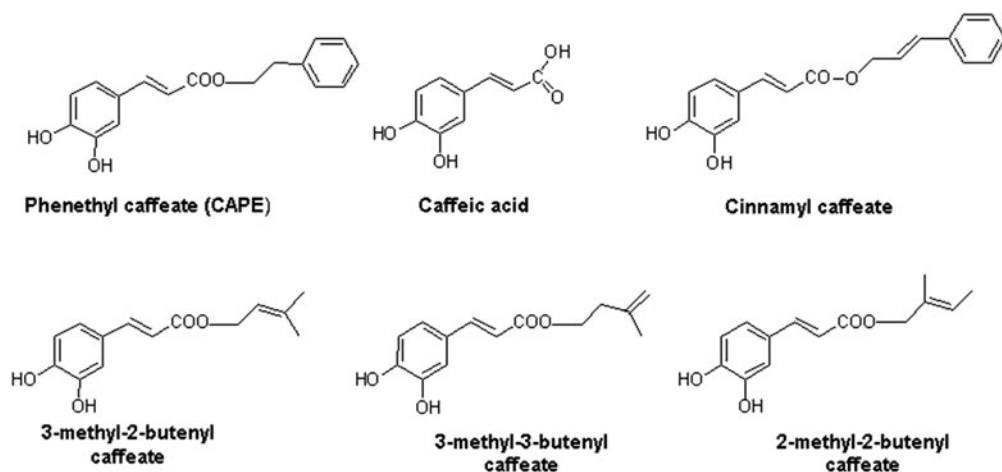


Figure 2. Plant metabolites found in higher concentration in propolis from resistant colonies.

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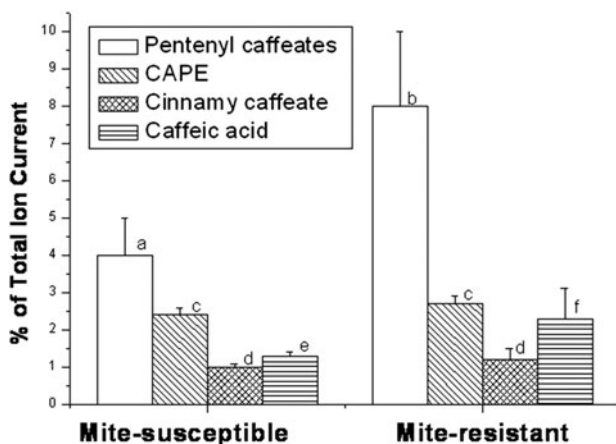


Figure 3. Content of caffeic acid derivatives in propolis from Avignon – mite-resistant and mite-susceptible colonies. Bars with the same letters are not statistically different from each other.

### 3. Experimental

#### 3.1. Propolis

Propolis was collected in May–June 2012 in an apiary near Avignon, from five mite-susceptible and five mite-resistant colonies.

#### 3.2. Chemicals

The following standards were used for the identification of compounds by GC/MS: benzoic, cinnamic, caffeic, *p*-coumaric, ferulic acids and vanillin were purchased from Merck; pinocembrin, chrysin and galangin were purchased from Extrasynthese, France; pinobanksin and pinobanksin acetate were previously isolated in our lab. CAPE, pentenyl caffeates, benzyl caffeate, benzyl and phenethyl ferulate, pentenyl ferulates, were synthesised in our lab previously.

#### 3.3. Balsam content

Propolis samples were extracted with 70% ethanol. Propolis was powdered; an exact measured sample of 0.5 g was dissolved in 15 mL 70% ethanol in a 25 mL flask and left for 24 h at room temperature. It was then filtered, and the procedure was repeated. The extracts were filtered (paper filter), combined and diluted to 50 mL with 70% ethanol in a volumetric flask. For each sample, three parallel extractions were performed. From each of the parallel extracts, 2 mL was evaporated *in vacuo* to dryness to constant weight *g*. The percentage of balsam *P* in propolis sample (*M* – weight of the propolis sample) was calculated by the formula

$$P = \frac{50g}{2M} 100\%.$$

The mean of the three values was determined.

#### 3.4. Sample preparation for GC/MS analysis

From each sample, after evaporation of the abovementioned extract to dryness, about 5 mg of the dry residue were mixed with 50  $\mu$ L of dry pyridine and 75  $\mu$ L of N,O-bis-(trimethylsilyl)-

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trifluoroacetamide (BSTFA) and heated at 80°C for 20 min. The standard compounds were subjected to the same procedure for silylation as about 1 mg of the pure compound was mixed with 10 µL of dry pyridine and 15 µL of BSTFA. The silylated ethanolic extracts and reference compounds were analysed by GC/MS.

### 3.5. GC/MS analysis

The GC/MS analysis was performed with a Hewlett–Packard gas chromatograph 5890 series II plus (Hewlett-Packard, Palo Alto, CA, USA) equipped with a 30 m long, 0.25 mm i.d. and 0.5 µm film thickness HP5-MS capillary column, linked to a Hewlett–Packard 5972 mass spectrometer system (Hewlett-Packard, Palo Alto, CA, USA). 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, injector temperature 280°C, interface temperature 300°C, ionisation voltage 70 eV. 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.

### 3.6. Statistical analysis

Multivariate analysis of propolis chemical profiles was performed by PCA, using the GC/MS data for the identified compounds expressed as a percentage of the TIC, respectively. Statistica Version 8.0 was used for the analyses.

## 4. Conclusions

The results of this study pave the way in the understanding of the effect of propolis in the honeybee immunity and give another example of the ability of honeybees to modulate their behaviour to improve their social immunity. Further studies need to be carried out to understand the relationship between the chemical composition of propolis and honeybee colony health.

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