Chemical Characterisation Of Maltese Propolis In Relation to Botanical Sources

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Introduction

Propolis is a sticky dark-coloured resinous material produced by honeybees (*Apis mellifera* L.) from plant materials. The worker bees collect the lipophilic resins secreted from plant wounds or secreted during bud formation, mix it with wax and use it in construction and adaptation of the hive. Propolis is a multi-variant product. Its chemical constituents include waxes, resins, balsams, essential oils, amino acids, sugars and various secondary metabolites, the composition depending on the botanical sources available to the honeybees. Flavonoids and cinnamic acid derivatives, particularly the hydroxycinnamic acid caffeic acid, and its derivate caffeic acid phenyl ester, are commonly found in propolis samples (Chen *et al.*, 2001; De Castro, 2001). Over 40% of the main constituents of propolis from the temperate zones are polyphenolic acids and flavonic compounds. However tropical and sub-tropical (Mediterranean) samples do not contain such substances (Kujumgiev *et al*., 1999). Diterpenes and triterpenes have been identified in previous studies of propolis from the sub-tropical regions, specifically from Greece, Sicily and Turkey (Mellio & Chinou, 2004; Bankova *et al*., 2006). The primary objectives of this study were to chemically characterise the compounds found in Maltese propolis by GC-MS analysis and identify possible botanical sources since such studies are totally lacking locally.

Methodology

Propolis was collected from different apiaries around the Maltese Islands during the summer of 2008 and autumn of 2009. In general, each propolis sample was collected from a number of beehives located in one area. After collection, the propolis was stored in a cool dark place until further analysis. Vegetational surveys of the sites surrounding the beehives from where propolis was collected were carried out. The ethanolic extract of propolis (EEP) was prepared by mixing 70% ethanol solution with propolis at a ratio of 1:20 w/v. The propolis mixture was stored at room temperature (25^{o}C) in a dark place for seven days, with frequent shaking. On the eighth day, the propolis suspension was filtered and the residue was mixed with additional 70% ethanol keeping the 1:20 w/v ratio. This solution was stored in a dark place for 24 hours. The filtrate solution was recovered and stored. After 24 hours the residue solution was filtered and the filtrate was collected and mixed with the filtrate solution obtained from the previous day. The EEP was then evaporated *in vacuo* to dryness.Thin layer chromatography (TLC) analysis was carried out to compare Maltese propolis with propolis samples from Greece (Crete). Marker bands of Maltese propolis were also identified which were used to compare with the TLC analysis of resin samples of possible botanical sources of Maltese propolis. The compounds in Maltese propolis were analysed by gas chromatography-mass spectrometry (GC-MS). 5 mg (±0.2 mg) of the dry extracted propolis was weighed and prepared for GC-MS by silylation. The dry extract was mixed with 50 µL dry pyridine and 75 µL of bis-(trimethylsilyl) trifluoroacetamide (BSTFA). This mixture was heated in an oven set at 80°C for 20 minutes. Sample volumes were injected and analysed by GC-MS. Samples of the silyated solutions were analysed by a Hewlett Packard Gas Chromatograph 5890 Series II Plus linked to Hewlett Packard 5972 mass spectrometer system equipped with a 23 m long, 0.25 mm id, 0.5 µm film thickness HP5-MS capillary column. The temperature was programmed from 100°C to 310°C at a rate of 5°C min^{-1}. The split ratio was 1:80 with an injector temperature of 280°C and ionization voltage of 70 eV. Helium was used as a carrier gas, with a flow rate of 0.7 mL min^{-1}. Through comparison with libraries and with published GC-MS spectral data of various chemically identified propolis samples, most of the peaks of the Maltese propolis samples were identified.

Results

In this study, 25 compounds were identified in Maltese propolis, out of which seven (neoabietic acid, 13-epi-cupressic acid, totarolone, junicedric acid, 13,14-dehydrojunicedric acid, ferruginolon and sitosterol) were so far only reported in Cretan propolis (Popova *et al.*, 2009). 19 other compounds were observed in the propolis samples but could not be identified due to the lack of authentic samples and library spectra of the corresponding compounds. All 17 propolis samples analysed were qualitatively similar.

From the TLCs of Maltese propolis it was observed that Maltese propolis samples were more or less chemically identical, with a selection of strong diterpene bands. Overall, Maltese propolis samples were very similar to the Greek propolis sample. There is only one major difference which distinguished Maltese samples from Greek samples; a green/blue non-diterpene band at Rf 3.8 cm (Figure 1, Band 4), that could not be identified. This was especially abundant in M16.

The TLC of the resins from the conifer tree, *Tetraclinis articulata*, had the most similar bands to the Maltese propolis samples, followed by *Cupressus* sp. Both *Prunus persica* and *Erica multiflora* exhibited some diterpene bands on the lower range of the TLC, which had similar Rf values to bands observed on the TLCs of Maltese propolis. Clearly the gymnosperms analysed (specifically *Tetraclinis articulata*, *Cupressus* species and *Pinus halepensis*) have a number of diterpene bands similar to those of Maltese propolis.

Bands 2, 4 and 6 (Figure 1) were also identified. Band 2 is a derivative of cupressic acid (13-epicupressic, acetyl isocupressic or isocupressic acid), while Band 6 is the phenolic diterpenic acid totarol.

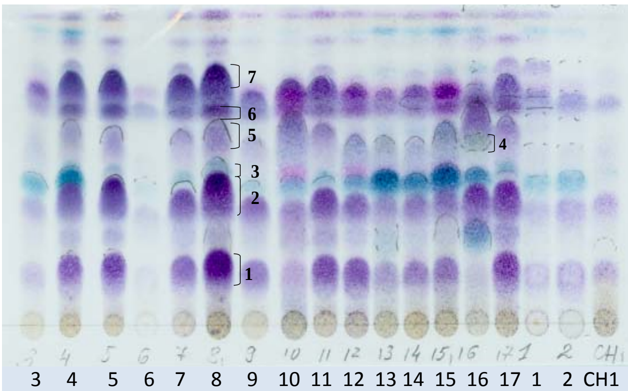

Figure 1 The TLC plate after spraying with the visualising reagent vanillin/sulphuric acid solution. A number of bands characteristic of diterpenic acids can be observed. The samples analysed are, from the left to right, Malta and Gozo (M3-M17, M1, M2), and Greece (CH1). The most prominent bands are marked on M8 and M16 (Bands 1-7).

Conclusion

The major compounds of Maltese propolis are terpene compounds, especially labdane, abietane and pimarane diterpenic acid and some triterpenic compounds. Maltese propolis does not contain any flavonoids and esters (such as caffeate esters) and only one aromatic acid compound; ferulic acid (a phenolic compound) and two types of fatty acids; tetracosanoic acid and oleic acid were identified. The results from the GC-MS analysis clearly identify species from the families of Cupressaceae and Pinaceae as being the major botanical sources of Maltese propolis. Similarly, coniferous trees have been mentioned as the major botanical sources of propolis from Greece and Sicily. The identification of totarolone, a specific chemical marker of *Tetraclinis articulata* (of the family Cupressaceae) could help narrow down some specific plant species involved in Maltese propolis production. However, further characterisation of *Tetraclinis articulata* resins by GC-MS and comparison with the GC-MS spectra of Maltese propolis is necessary to definitely ascribe *Tetraclinis articulata* as the source of totarolone and other diterpenic acids in Maltese propolis.

References

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