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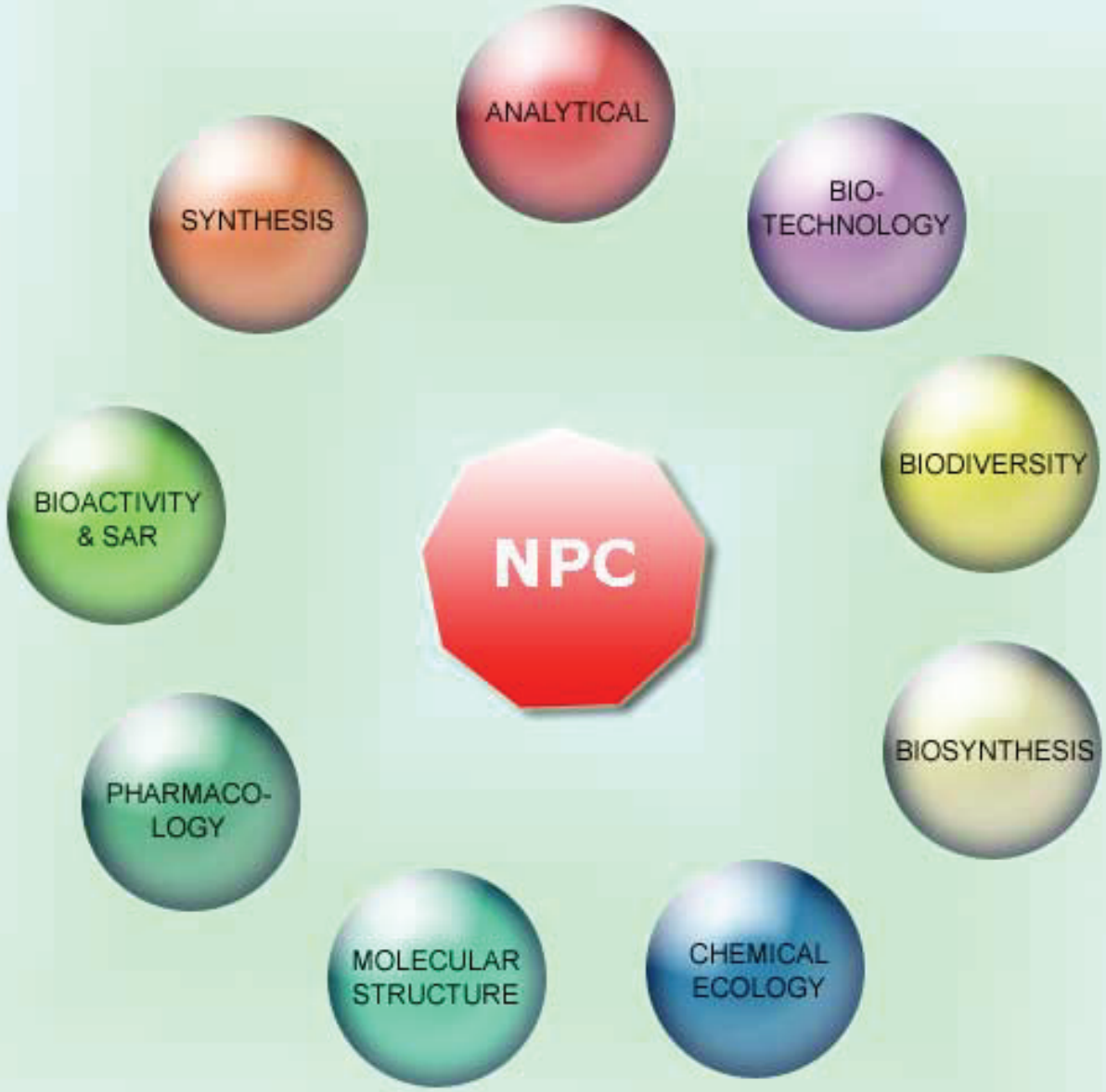

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Identification of the Plant Origin of the Botanical Biomarkers of Mediterranean type Propolis

Milena Popova[a], Boryana Trusheva[a], Simone Cutajar[b], Daniela Antonova[a], David Mifsud[c], Claude Farrugia[b] and Vassya Bankova[a*]

[a]*Institute of Organic Chemistry with Centre of Phytochemistry*, *Bulgarian Academy of Sciences*, *Acad. G. Bonchev Str. Bl. 9*, *1113 Sofia*, *Bulgaria*

[b]*University of Malta*, *Department of Chemistry*, *Msida MSD 2080*, *Malta*

[c]*Junior College*, *University of Malta*, *Department of Biology*, *Msida MSD 1252*, *Malta*

Bankova@orgchm.bas.bg

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Propolis is a honeybee product which bees produce by collecting resins from various botanical sources. The chemical composition of propolis is directly dependant on the availability of resinous plant materials in different geographic regions. This study was undertaken to evaluate the resinous plant sources used by bees to produce Mediterranean type propolis. Although this propolis type has already been the subject of numerous studies, its major botanical source had not yet been identified. In this study, using GC-MS analysis, we identify the resin of the common cypress, *Cupressus_sempervirens*, as the major plant source of the characteristic diterpene fingerprint profile of Mediterranean propolis.

Keywords: Propolis, Plant origin, *Cupressus sempervirens*.

Propolis is a bee product that has gained significant popularity in the last decades in alternative medicine, apitherapy, and the production of 'health foods' and beverages due to its numerous biological activities [1]. It has been proved to play a significant role in bees' social immunity; contributing to the overall good health of honeybee colonies [2]. It is known that bees collect propolis from resinous plant materials and in different geographic regions, propolis might be of very specific chemical composition due to the specificity of the local flora and the choice it offers to the bees. Until now, a number of propolis types have been identified [3] according to their chemistry and plant origin, the most popular being poplar (European) type propolis, Brazilian green propolis, and red propolis (Brazilian, Cuban, Colombian). During the last decade, numerous studies have demonstrated the existence of a new European propolis type: Mediterranean, which is characterized by high diterpene concentration and remarkable antibacterial activity [4]. Its plant source, however, is as yet undetermined. The answer to this question is important with respect to future standardization because it gives the possibility to pinpoint the typical bioactive constituents of this propolis type which have to be quantified for purposes of quality control [5]. In this study we report on the identification of the source of the most abundant and important diterpenes in Mediterranean propolis: the resin of the common cypress, *Cupressus sempervirens.*

Mediterranean propolis possesses a distinct chemical profile, particularly rich in diterpenes. This propolis type was found for the first time less than 10 years ago in Sicily [6]. Soon, it was also detected in Crete [4], South-Eastern Greece [7], Malta [8], and the Adriatic coast of Croatia [6]. Its plant derivation has not been recognized until now. Based on the identified diterpenes, the source plant was suggested to be some conifer species, most probably of the Cupressaceae family, for which the flora of the Mediterranean Region is very rich. This conclusion [7] was based on the fact that the propolis samples contained ferruginol, totarol, oxygenated ferruginol and totarol derivatives, and sempervirol, which are characteristic for Cupressaceae [9]. However, *Pinus* species could not be disregarded as resin sources.

Conclusive evidence for the botanical sources of propolis could be found in its chemical composition by comparing it with appropriate plant materials, collected in the vicinity of the hives. For this reason we compared the GC-MS diterpenic profile of propolis after silylation (a number of Maltese samples, and our published data of six Greek samples [7]), and the profiles of the resin of the most common coniferous trees in the regions where Maltese propolis samples were collected: common cypress, *Cupressus sempervirens* L., and Aleppo pine, *Pinus halepensis* Mill.

The diterpenic profile of Maltese propolis, as already established [8], was close to the one of propolis from South-Eastern Greece [7]. They both displayed significant similarity to the profile of the resin of *C. sempervirens* (Fig. 1). The most important diterpenic constituents, 19 individual compounds, were the same in all three materials. On the other hand, the resin of *P. halepensis* did not contain any of these compounds. The major constituents of the latter were abietic, dehydroabietic, neoabietic, isopimaric and palustric acids. Of them, isopimaric and palustric acids were not identified in propolis, and only traces of the acids with an abietane skeleton were detected. This fact clearly allowed us to exclude *P. halepensis* as a plant source for Mediterranean type propolis.

Major compounds in Greek and Maltese propolis, as well as in the cypress resin, were isocupressic acid, pimaric and imbricataloic acids. However, some quantitative differences between propolis and cypress resin are visible. Most obvious is the higher concentrations in the resin of totarol + *epi*-torulosal, and *epi*-cupressic acid, which is structurally related to *epi*-torulosal. This could be explained by possible variations in the resin composition and the fact that bees might have collected propolis at a different time from that when the resin samples were obtained. It is known that resin diterpenes may demonstrate significant seasonal variations [10]. The observed

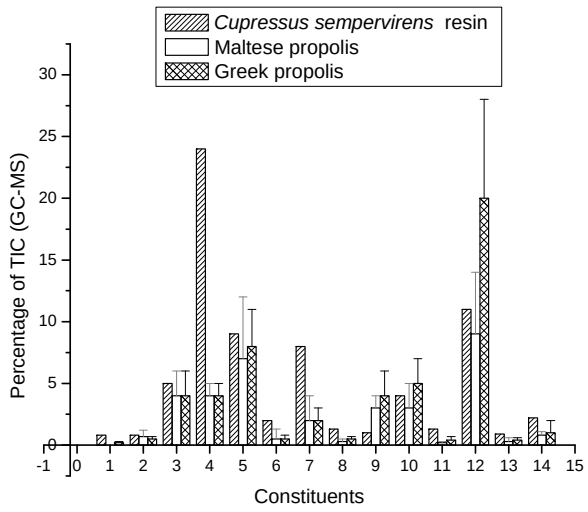

Figure 1: Diterpenic profile of Maltese and Greek propolis (data from [7]), and of *C. sempervirens* resin (mean values ± standard deviation). 1 – ferruginol, 2 – sempervirol, 3 – communic acid, 4 – totarol + 13-*epi*-torulosal, 5 – pimaric acid + imbricataloic acid, 6 - 13-*epi*-torulosol, 7 - 13-*epi*-cupressic acid, 8 – ferruginolon, 9 – isoagatholal, 10 – agathadiol + imbricatoloic acid, 11 – totarolon, 12 – isocupressic acid, 13 - 6/7-hydroxyferruginol, 14 - acetylisocupressic acid. (TIC: Total Ion Current).

profile of cypress resin was very similar to the one found by Isidorov (private communication), where totarol, *epi*-cupressic acid and isocupressic acid were the most abundant constituents. In all propolis samples and *Cupressus* resins, isocupressic acid was among the constituents with the highest concentration.

Our data support the hypothesis that the diterpene-rich Mediterranean propolis originates mainly from the resin of the common cypress *C. sempervirens*. This propolis type has demonstrated remarkable antibacterial activity [4, 11], due to the presence of antimicrobial diterpenic acids. Conifer resins have been used since immemorial times as a medicine for infected wounds, boils, and pyodermas, as well as rubefacients and vesicants in poultices and creams in veterinary practice [12]. Apparently, in the Mediterranean Region, honeybees have chosen an excellent source of anti-infective compounds to incorporate in their propolis in order to protect the hive. It is obvious that further studies are needed to reveal in more detail the chemistry and biological activity of Mediterranean propolis and to develop appropriate procedures for its standardization based on its major diterpenic constituents originating from the resin of *C. sempervirens.*

Experimental

Propolis and resin samples: Propolis samples were collected from apiaries at 14 different locations in Malta and at 3 locations on the island of Gozo. The vegetative material and external resin of *Cupressus sempervirens* (2 samples) and *Pinus halepensis* (2 samples) were collected in Malta, in 2010, from the Buskett area, very close to the hives from where some of the propolis samples were collected (outskirts of Rabat area and Ħad-Dingli area).

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.

Identification and semiquantification process: The identification of the compounds was performed using commercial libraries and comparison of mass spectra and retention times of reference compounds. The isolation of the diterpenes used as reference compounds was described earlier [4]. In the cases of lack of the corresponding reference compounds, the structures were proposed on the basis of their general fragmentation and using reference literature spectra [9], where possible. The semiquantification 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. Some pairs of compounds do not produce well resolved peaks, although the mass spectra of each of the two components were clearly observed; in this case the two compounds were quantified as a sum [7].

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