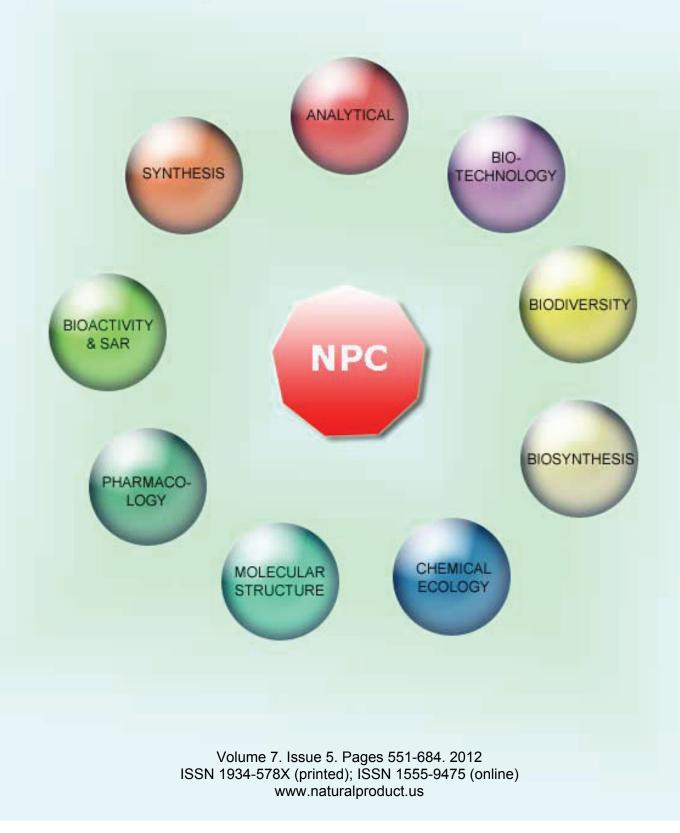
## NATURAL PRODUCT COMMUNICATIONS

### An International Journal for Communications and Reviews Covering all Aspects of Natural Products Research





## **Natural Product Communications**

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# NPC Natural Product Communications

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

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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

2012 Vol. 7 No. 5 569 - 570

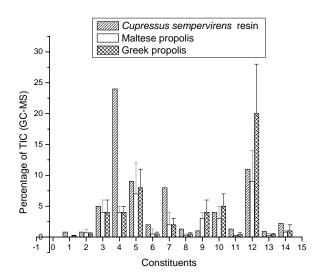


Figure 1: Diterpenic profile of Maltese and Greek propolis (data from [7]), and of *C. sempervirens* resin (mean values  $\pm$  standard deviation). 1 – ferruginol, 2 – sempervirol, 3 – communic acid, 4 – totarol + 13-*epi*-torulosal, 5 – pimaric acid + imbricataloic acid, 6 - 13-*epi*-torulosol, 7 - 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 Had-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  $\mu$ m 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 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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<b>Composition of the Essential Oil of Wild Growing</b> <i>Artemisia vulgaris</i> from Erie, Pennsylvania Jack D. Williams, Ayman M. Saleh and Dom N. Acharya	637
Chemical Composition of the Essential Oils from the Flower, Leaf and Stem of <i>Lonicera japonica</i> Nenad Vukovic, Miroslava Kacaniova, Lukas Hleba and Slobodan Sukdolak	641
<i>Jasminum sambac</i> Flower Absolutes from India and China – Geographic Variations Norbert A. Braun and Sherina Sim	645
<i>In vitro</i> Bioactivity of Essential Oils and Methanol Extracts of <i>Salvia reuterana</i> from Iran Javad Safaei Ghomi, Reihaneh Masoomi, Fereshteh Jookar Kashi and Hossein Batooli	651
Investigation of the Volatile Constituents of Different <i>Gynura</i> Species from Two Chinese Origins by SPME/GC-MS Jian Chen, An Adams, Sven Mangelinckx, Bing-ru Ren, Wei-lin Li, Zheng-tao Wang and Norbert De Kimpe	655
Volatiles from <i>Michelia champaca</i> Flower: Comparative analysis by Simultaneous Distillation-Extraction and Solid Phase Microextraction	
Disnelys Báez, Diego Morales and Jorge A. Pino	659
Chemical Composition and Antibacterial Activity of the Essential Oil of <i>Espeletia nana</i> Alexis Peña, Luis Rojas, Rosa Aparicio, Libia Alarcón, José Gregorio Baptista, Judith Velasco, Juan Carmona and Alfredo Usubillaga	661
The Composition and Antimicrobial Activities of Cyperus conglomeratus, Desmos chinensis var. lawii and Cyathocalyx zeylanicus Essential Oils	
Abdulkhader Hisham, Koranappallil B. Rameshkumar, Neelam Sherwani, Salim Al-Saidi and Salma Al-Kindy	663
Composition, Antimicrobial and Free-radical Scavenging Activities of the Essential Oil of <i>Plectranthus marrubatus</i> Kaleab Asres, Solomon Tadesse, Avijit Mazumder and Franz Bucar	667
Constituents and Antimicrobial Activity of the Essential Oils from Flower, Leaf and Stem of <i>Helichrysum armenium</i> Khodam-Ali Oji and Ali Shafaghat	671
<u>Review/Account</u>	
Plant Essential Ails and Mastitis Disease: Their Potential Inhibitory Effects on Pro inflammatory Cytaking Production in	

 Plant Essential Oils and Mastitis Disease: Their Potential InhibitoryEffects on Pro-inflammatory Cytokine Production in
 Response toBacteria Related Inflammation

 Ibrahim Taga, Christopher Q. Lan and Illimar Altosaar
 675

# Natural Product Communications 2012

## Volume 7, Number 5

## Contents

<u>Gerald Blunden Award (2011)</u>	<u>Page</u>
<b>On-line (HPLC-NMR) and Off-line Phytochemical Profiling of the Australian Plant,</b> <i>Lasiopetalum macrophyllum</i> Michael Timmers and Sylvia Urban	551
Original Paper	
Iridoid and Phenolic Glycosides from the Roots of <i>Prismatomeris connata</i> Shixiu Feng, Jijiang Bai, Shengxiang Qiu (Samuel), Yong Li and Tao Chen	561
Effect of some <i>ent</i> -Kaurenes on the Viability of Human Peripheral Blood Mononuclear Cells Yndra Cordero, Grecia M. Corao, José A. Cova and Alfredo Usubillaga	563
Steroidal Glycosides from <i>Veronica chamaedrys</i> L. Part I. The Structures of Chamaedrosides C, C <sub>1</sub> , C <sub>2</sub> , E, E <sub>1</sub> and E <sub>2</sub> Alexandra Marchenko, Pavel Kintya, Bozena Wyrzykiewicz and Elena Gorincioi	565
Identification of the Plant Origin of the Botanical Biomarkers of Mediterranean type Propolis Milena Popova, Boryana Trusheva, Simone Cutajar, Daniela Antonova, David Mifsud, Claude Farrugia and Vassya Bankova	569
Alkaloids from Some Amaryllidaceae Species and Their Cholinesterase Activity Lucie Cahlíková, Nina Benešová, Kateřina Macáková, Radim Kučera, Václav Hrstka, Jiří Klimeš, Luděk Jahodář and Lubomír Opletal	571
Phytochemical and Biological Activity Studies of the Bhutanese Medicinal Plant Corydalis crispa Phurpa Wangchuk, Paul A. Keller, Stephen G. Pyne, Thanapat Sastraruji, Malai Taweechotipatr, Roonglawan Rattanajak, Aunchalee Tonsomboon and Sumalee Kamchonwongpaisan	575
On the Biosynthetic Pathway of Papaverine <i>via (S)</i> -Reticuline – Theoretical vs. Experimental Study Bojidarka Ivanova and Michael Spiteller	581
Diversification of Exudate Flavonoid Profiles in Further Primula spp. Tshering Doma Bhutia and Karin M. Valant-Vetschera	587
Three New Biflavonoids from Chinese Dragon's Blood, Dracaena cochinchinensis Jing Guan and Shun-Xing Guo	591
Secondary Metabolites from Polar Fractions of <i>Piper umbellatum</i> Turibio Kuiate Tabopda, Anne-Claire Mitaine-Offer, Tomofumi Miyamoto, Chiaki Tanaka, Bonaventure Tchaleu Ngadjui and Marie-Aleth Lacaille-Dubois	595
A New Antimycobacterial Furanolignan from Leucophyllum frutescens Blanca Alanís-Garza, Ricardo Salazar-Aranda, Rosalba Ramírez-Durón, Elvira Garza-González and Noemí Waksman de Torres	597
Water-soluble Constituents of the Heartwood of Streblus asper Jun Li, Mao-Tong Tang, Qiang Wu, Hong Chen, Xiao-Tao Niu, Xin-Lan Guan, Jian Li, Sheng-Ping Deng, Xiao-Jian Su and Rui-Yun Yang	- 599
Lichen Depsides and Depsidones Reduce Symptoms of Diseases Caused by Tobacco Mosaic Virus (TMV) in Tobacco Leaves Ingrid Ramírez, Soledad Araya, Marisa Piovano, Marcela Carvajal, Alvaro Cuadros-Inostroza, Luis Espinoza, Juan Antonio Garbarino and Hugo Peña-Cortés	603
Antioxidant, Hemolytic and Cytotoxic Activities of <i>Senecio</i> Species used in Traditional Medicine of Northwestern Argentina Emilio Lizarraga, Felipe Castro, Francisco Fernández, Marina P, de Lampasona and César A, N, Catalán	607
Isolation of Antitrypanosomal Compounds from <i>Vitis repens</i> , a Medicinal Plant of Myanmar Khine Swe Nyunt, Ahmed Elkhateeb, Yusuke Tosa, Kensuke Nabata, Ken Katakura and Hideyuki Matsuura	609
Evaluation of Analgesic Activities of Tremetone Derivatives Isolated from the Chilean Altiplano Medicine <i>Parastrephia lepidophylla</i> Julio Benites, Eunices Gutierrez, José López, Mauricio Rojas, Leonel Rojo, Maria do Céu Costa, Maria Pilar Vinardell and	
Pedro Buc Calderon Ajuganane: A New Phenolic Compound from <i>Ajuga bracteosa</i>	611
Javid Hussain, Naeema Begum, Hidayat Hussain, Farman Ullah Khan, Najeeb Ur Rehman, Ahmed Al-Harrasi and Liaqat Ali Base-mediated Transformations of 3,5-Dibromoverongiaquinol from the Sponge <i>Aplysina</i> sp. to Cavernicolins-1, -2 and	615
a Subereatensin Analogue Elena A. Santalova	617
The Origin of Virgin Argan Oil's High Oxidative Stability Unraveled Saïd Gharby, Hicham Harhar, Dominique Guillaume, Aziza Haddad and Zoubida Charrouf	621
Chemical Composition of Essential Oils from a Multiple Shoot Culture of <i>Telekia speciosa</i> and Different Plant Organs Anna Wajs-Bonikowska, Anna Stojakowska and Danuta Kalemba	625
Evaluation of Volatile Constituents of Cochlospermum angolense Michele Leonardi, Silvia Giovanelli, Pier Luigi Cioni, Guido Flamini and Luisa Pistelli	629
GC-MS Analysis of Aroma of <i>Medemia argun</i> (Mama-n-Khanen or Mama-n-Xanin), an Ancient Egyptian Fruit Palm Arafa I. Hamed, Michele Leonardi, Anna Stochmal, Wieslaw Oleszek and Luisa Pistelli	633