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Volatile constituents of propolis from various regions of Greece – Antimicrobial activity

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Abstract

The volatiles of five samples of Greek propolis from various geographic origin (A–E) were analyzed by capillary gas chromatography, using flame ionization GC and mass spectrometric detection. Ninety-four components were identified from the oils. The major components from each sample were found to be: junipene (11.7%), α-pinene (7.9%), manoyl oxide (7.1%) (sample A), α-pinene (45.8%), *trans*-β-terpineol (6.6%) (sample B), α-pinene (17.7%), α-eudesmol (12.1%), *n*-decanal (6.2%), guaiol (5.0%) (sample C), α-pinene (18.2%), δ-cadinene (8.4%) and α-muurolene (5.0%) (sample D), α-pinene (10.9%), *n*-decanal (10.3%), cedrol (6.3%), *n*-nonanal (5.4%), and manool (5.2%) (sample E). The total profile of the volatile constituents of all samples reveals the predominance of terpenoids, especially of α-pinene. The *in vitro* antimicrobial activity of the volatiles from all five studied samples against six bacteria and three fungi is also assayed and reported.

Keywords: Greek propolis; Volatiles; Diterpenes; Antimicrobial activity

1. Introduction

Propolis, a natural substance collected by honeybees from buds and exudates of certain trees and plants, is thought to be used in the beehive as a protective barrier against their enemies. The use of propolis in the traditional medicine is known since 3000 BC in Egypt. It is claimed to improve human health and prevent diseases such as inflammation, heart disease, diabetes and cancer (Banskota, Tezuka, Adnyana, et al., 2001; Burdock, 1998). For this reason, propolis is extensively used in folk medicine (Ghisalberti, 1979), in cosmetology and in the food industry for health foods, beverages and nutritional supplements. Among the several biological activities that have been reported for propolis and its constituents, the most important are antimicrobial, anti-inflammatory, antioxidant and antiproliferative ones (Banskota, Tezuka & Kadota, 2001; Ghisalberti, 1979; Marcucci, 1995).

The chemical consistency of propolis is highly dependent on the flora of the region from which it is collected. In contrast to propolis of continental Europe, Greek propolis has a different botanical origin due to unique flora of Greece that has developed as a result of its geographical position. The Greek flora presents a generally known biodiversity with a high percentage of endemic plants. The special character of the non-volatile constituents of the Greek propolis has already been revealed by previous research (Melliou & Chinou, 2004).

Based on the above observations, we aimed to determine the character of Greek propolis concerning its volatile constituents. Our interest on propolis volatiles was also based on the fact that this type of compound has the ability to reduce the apiary aeroflora (Ghisalberti, 1979). It is remarkable, that in the field of European propolis volatile constituents, there are not sufficient data (Bankova, Christov, Popov, Pureb, & Bocari, 1994; Borčić, Radonić, &

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Grzunov, 1996; Greenaway, May, Scaysbrook, & Whatley, 1991). Additionally, the antibacterial activity of propolis oils has only occasionally been reported (Bankova et al., 1999; Keskin, Hazir, Baser, & Kürkçüoglu, 2001; Petri, Lembercovics, & Folalvari, 1988).

For these reasons in this work, we studied the volatile constituents of five samples from various geographic origin from Greece (A–E) and their antimicrobial activities.

2. Materials and methods

2.1. Plant material

The samples of Propolis were collected at Chalkidiki (North East Greece, sample A), island of Andros (Aegean, sample B), Agrinio (Central West Greece, sample C), Arta and Preveza (North West Greece, sample D, sample E) in May 2001. Voucher samples (MEL04, MEL05 MEL06 MEL07 and MEL03, respectively) have been deposited in the Herbarium of the Laboratory of Pharmacognosy and Chemistry of Natural Products, Faculty of Pharmacy, University of Athens, Greece.

2.2. Isolation procedure

The propolis samples (1 kg) were cut into small pieces and subjected to hydrodistillation for 3 h using a modified Clevenger-type apparatus to yield 0.05% sample A, 0.10% sample B, 0.04% sample C, 0.08% sample D, 0.03% sample E of essential oils in a semisolid form. The distilled oil was collected and dried over anhydrous sodium sulphate and stored at 4 °C.

2.3. GC and GC/MS analyses

The oils were first analyzed by GC-FID carried out on a Perkin–Elmer Clarus 500 gas chromatograph, fitted with a HP 5MS 30 m × 0.25 mm, 0.25 μm film thickness capillary column. The column temperature was programmed from 60 to 280 °C at a rate of 3 °C/min. The injector and detector temperatures were programmed at 230 °C and 300 °C, respectively. Helium was used as the carrier gas at a flow rate of 1 ml/min.

The GC–MS analyses were carried out using a Hewlett–Packard 6890-5973 GC–MS system operating on EI mode (equipped with a HP 5MS 30 m × 0.25 mm, 0.25 μm film thickness capillary column). He (1 ml/min) was used as carrier gas. The initial temperature of the column was 60 °C and then it was heated to 280 °C at a rate of 3 °C/min.

GC–MS analyses were also performed on a Finnigan GCQ *Plus* ion trap mass spectrometer with an external ion source in both the EI and chemical ionization (CI) modes at a flow rate of 1.0 ml/min, using CH_4 as the CI ionization reagent.

The identification of the compounds was based on comparison of their retention indices (RI), obtained using *n*-alkanes (C_9–C_{25}), and on comparison of their EI-mass spectra with the NIST/NBS, Wiley library spectra and literature (Adams, 2001). Additionally, the identity of all compounds was performed by comparison of the expected molecular weights with the results obtained from the CI spectra.

2.4. Antimicrobial assay

In vitro antimicrobial studies were carried out by the dilution method, measuring the MIC values in 96-hole plates against two Gram-positive bacteria: *Staphylococcus aureus* (ATCC 25923), *S. epidermidis* (ATCC 12228), four Gram-negative bacteria: *Escherichia coli* (ATCC 25922), *Enterobacter cloacae* (ATCC 13047), *Klebsiella pneumoniae* (ATCC 13883) and *Pseudomonas aeruginosa* (ATCC 227853) as well as against three human pathogen fungi *Candida albicans* (ATCC 10231), *C. tropicalis* (ATCC 13801) and *C. glabrata* (ATCC 28838). Stock solutions of the tested extracts and pure compounds were prepared at 10 and 1 mg/ml, respectively. Serial dilutions of the stock solutions in broth medium (100 μl of Müller–Hinton broth or on Sabouraud broth for the fungi) were prepared in a microtiter plate (96 wells). Then 1 μl of the microbial suspension (the inoculum, in sterile distilled water) was added to each well. For each strain, the growth conditions and the sterility of the medium were checked and the plates were incubated as referred above. MICs were determined as the lowest concentrations preventing visible growth. Standard antibiotic netilmicin (at concentrations 4–88 μg/ml) were used in order to control the sensitivity of the tested bacteria, while 5-flucytocine and itraconazole (at concentrations 0.5–25 μg/ml) were used as controls against the tested fungi (Sanofi, Diagnostics Pasteur) at concentrations of 30, 15 and 10 μg/ml. The tested compounds were dissolved in dichloromethane. For each experiment pure solvent was used as blind control. The experiments were repeated three times and the results were expressed as average values.

3. Results and discussion

Propolis samples were collected from five different locations in Greece. GC/MS analysis of the oils led to the identification of the majority of the components, which are listed in Table 1 along with their quantitative data and their retention indices. The identification of components was based on comparison of their mass spectra with those of Wiley and NBS Libraries (Massada, 1976) and those described by Adams (2001), as well as on comparison of their retention indices (Van den Dool & Kratz, 1963) with the literature values (Adams, 2001).

Ninety-four constituents were identified from the five essential oils. The major volatile constituents of Chalkidiki region (sample A) were junipene (11.7%), α-pinene (7.9%), manoyl oxide (7.1%). The major constituents of the essential oil of Andros region (sample B) were α-pinene (45.8%), *trans*-β-terpineol (6.6%). From the oil of Agrinio region (sample C) α-pinene (17.7%), α-eudesmol (12.1%), *n*-decanal (6.2%) and guaiol (5.0%) were characterized as main

Table 1
Percentage composition (%) of propolis collected from five regions of Greece

Compounds	Chalkidiki	Andros	Agrinio	Arta	Preveza	RI
1. *n*-Nonane	0.2	0.2	0.1	0.3	0.5	900
2. Tricyclene	–	0.2	0.2	–	–	927
3. α-Thujene	–	–	0.3	1.0	–	930
4. α-Pinene	7.9	45.8	17.7	18.2	10.9	939
5. α-Fenchene	–	1.0	–	–	–	953
6. Camphene	0.3	0.6	0.6	–	2.9	954
7. Benzaldehyde	0.3	0.1	0.1	–	0.2	960
8. Verbenene	0.8	0.1	1.3	1.8	1.3	968
9. Sabinene	–	0.9	0.4	0.8	0.4	975
10. β-Pinene	0.6	2.2	0.8	1.4	0.4	979
11. *cis-m*-Mentha-2,8-diene	0.2	–	–	0.3	–	987
12. Myrcene	–	–	0.5	–	–	991
13. *n*-Octanal	1.5	1.8	1.7	–	2.2	999
14. Δ-2-Carene	–	0.7	0.3	0.3		1002
15. α-Phellandrene	–	–	–	–	0.3	1003
16. α-Terpinene	0.3	0.5	0.4	2.0	0.6	1017
17. *p*-Cymene	1.4	1.0	1.3	1.0	0.8	1025
18. Limonene	1.8	0.7	0.5			1029
19. β-Phellandrene	–	–	–	1.0	0.5	1030
20. 1,8-Cineole	–	–	–	–	0.3	1031
21. γ-Terpinene	0.5	1.0	0.6	2.9	1.6	1060
22. *n*-octanole	–	0.1	–	–	–	1068
23. *cis*-Sabinene hydrate	–	0.2	–	–	–	1070
24. 2-Methoxyethyl benzene	1.6	0.4	–	2.1	–	1084
25. Terpinolene	–	0.5	–	0.9	–	1089
26. *p*-Cymenene	–		0.3	–	–	1091
27. *n*-Nonanal	2.9	2.5	3.4	–	5.4	1101
28. α-Fenchol	–	0.2	–	–		1117
29. α-Campholene aldehyde	–	1.7	0.6	–	0.7	1126
30. *trans*-Pinocarveol	–	0.6	1.3	0.9	–	1139
31. *cis* -Verbenol	–	1.4	–	0.2	–	1141
32. *cis*-Sabinol	0.8	–	–	–	–	1143
33. *trans*-Verbenol	–	–	0.3	–	–	1145
34. Camphor	0.3	1.7	–	0.2	–	1146
35. Camphene hydrate	–	0.1	–	–	–	1150
36. Benzyl acetate	0.3	–	–	–	–	1162
37. *trans*-β-Terpineol	2.9	6.6	2.9	3.0	2.2	1163
38. Pinocarvone	–	0.4	–	–	–	1165
39. Borneol	–	0.9	–	0.5	–	1169
40. *p*-Mentha-1,5-dien-8-ol	–	0.6	1.0	0.2	–	1170
41. *p*-Cymen-8-ol	0.3	0.4	–	0.2	–	1183
42. α-Terpineol	0.4	1.5	–	0.6	0.6	1189
43. Myrtenal	0.4	0.6	–	–	–	1196
44. *n*-Decanal	4.8	3.4	6.2	2.8	10.3	1202
45. *trans*-Carveol	–	0.2	1.0	–	–	1217
46. Carvacrol methyl ether	–	1.5	0.9	0.4	0.4	1245
47. Geraniol	0.4	–	–	–	–	1253
48. Nonanoic acid	0.5	–	–	–	–	1271
49. Bornyl acetate	1.6	1.6	1.8	0.9	1.4	1289
50. α-Terpinyl acetate	–	–	0.7	2.3	–	1349
51. α-Cubebene	–	–	0.2	–	–	1351
52. α-Longipinene	2.8	–	–	–	–	1353
53. Cyclosativene	0.4	–	–	–	–	1371
54. α-Ylangene	1.6	–	–	0.8	–	1375
55. α-Copaene	2.4	0.2	0.2	0.9	0.3	1377
56. β-Bourbonene	0.3	–	–	–	–	1388
57. β-Cubebene	–	0.1	–	–	–	1388
58. Sativene	0.8	–	–	–	–	1392
59. Junipene	11.7	2.1	1.5	1.7	–	1408
60. Dodecanal	–	–	–	–	2.4	1409
61. *E*-caryophyllene	0.9	–	0.4	–		1419
62. α-Humulene	–	–	0.1	0.3	0.3	1455
63. allo-Aromadendrene	–	–	0.2	1.7	0.2	1460

(*continued on next page*)

Table 1 (*continued*)

Compounds	Chalkidiki	Andros	Agrinio	Arta	Preveza	RI
64. *ar*-Curcumene	–	0.1	–	0.8	0.5	1481
65. Acetovanillone	2.8	–	–	1.4	0.6	1483
66. γ-Curcumene	–	–	–	0.4	–	1483
67. γ-Elemene	0.5	–	–	–	–	1490
68. α-Zingiberene	–	–	–	0.2	–	1494
69. α-Muurolene	4.7	–	2.7	5.0	1.5	1500
70. γ-Cadinene	0.9	–	1.5	3.7	1.5	1514
71. δ-Cadinene	3.7	0.3	4.8	8.4	3.3	1523
72. α-Cadinene	–	–	1.0	0.4	–	1539
73. α-Calacorene	0.4	–	0.2	0.6	–	1546
74. Spathulenol	1.7	0.4	–	0.2	–	1578
75. Caryophyllene oxide	–	–	–	0.1	0.4	1583
76. Globulol	–	0.1	–		–	1585
77. Hexadecen-1	–	–	–	4.1	1.5	1590
78. Guaiol	–	–	5.0	–	–	1601
79. Cedrol	–	4.3	4.7	–	6.3	1601
80. Unknown	–	–	–	–	3.8	1606
81. Cedrol <epi>	0.3	–	–	–	–	1619
82. γ-Eudesmol	–	–	4.0	3.1	1.8	1632
83. *epi*-α-Muurolol	0.7	–	–	–	–	1642
84. α-Muurolol	0.3	–	–	–	–	1646
85. β-Eudesmol	0.7	–	–	4.7	3.2	1651
86. α-Cadinol	–	–	–	3.8	–	1654
87. α-Eudesmol	–	–	12.1	3.0	3.7	1654
88. Unknown	1.4	–	3.2	1.3	–	1666
89. Unknown	1.2	–	–	–	–	1668
90. Benzyl benzoate	–	–	–	–	0.5	1760
91. Nonadecane	–	–	–	–	0.2	1900
92. Cembrene	–	–	–	–	1.5	1939
93. Manoyl oxide	7.1	0.1	0.3	0.4	1.8	1998
94. Manool	–	0.6	–	0.9	5.2	2056
95. Heneicosane	0.1	0.2	0.2	0.1	0.2	2101
96. Tricosane	1.1	0.8	0.9	0.7	1.3	2301
97. *trans*-Totarol	2.5	0.1	2.3	0.6	3.2	2314
Total	84.0	93.3	92.7	95.5	89.1	

The components are listed in the order of their elution on the DB-5 column.

constituents. The major constituents of the essential oil of Arta region (sample D) were α-pinene (18.2%), δ-cadinene (8.4%) and α-muurolene (5.0%). The major constituents of the essential oil of Preveza region (sample E) were α-pinene (10.9%), *n*-decanal (10.3%), cedrol (6.3%), *n*-nonanal (5.4%) and manool (5.2%).

It is very interesting that α-pinene was identified as the major constituent in four of the five samples (7.9–45.8%). This compound has been reported till now only as a trace among the volatiles in few European (Borčić et al., 1996; Greenaway et al., 1991) and tropical propolis samples (Bankova, Christov, Kujumgiev, Marcucci, & Popov, 1995; Bankova, Christov, & Tejera, 1998; Bankova et al., 1999). Besides, α-pinene has never been appeared among the major compounds, as in our study. According to Petri et al. (1988) the propolis from the temperate zone can be separated to two types, based on the presence of substantial amounts of β-eudesmol or benzyl benzoate. The samples of Greek propolis presented a distinct profile, characterized by uniformly elevated concentration of α-pinene and not of the aforementioned compounds.

Additionally, it should be noted that several compounds with substantial concentration in most samples, such as *trans*-β-terpineol, junipene, manool or manoyl oxide, have never been previously reported as propolis constituents.

Concerning the comparison between the studied samples, it is noteworthy that 16 among the total constituents existed in all samples, while 10 more compounds were present in four of the five samples. These results show that there is a relative similarity among the five samples from various regions of Greece although several constituents (α-fenchene, myrcene, α-cadinol, guaiol, etc.) were exclusively identified in some samples. The total profile of the volatile constituents of all studied samples, reveals the predominance of terpenoids against aromatic compounds. These data are in accordance of our previous work (Melliou & Chinou, 2004) about the elevated level of terpenoids in Greek propolis.

The volatiles of all samples were also studied for their antimicrobial activity against four Gram-negative, two Gram-positive bacterial strains and three human-pathogen fungi (*C. albicans, C. tropicalis, C. glabrata*). The results of

Table 2
Antimicrobial activities (zones of inhibition/ MIC mg/mL, $n = 3$) of the studied propolis samples and theirs main component α-pinene

	Staphylococcus aureus	*S. epidermidis*	*Pseudomonas aeruginosa*	*Enterobacter cloacae*	*Klebsiella pneumoniae*	*Escherichia coli*	*Candida albicans*	*C. tropicalis*	*C. glabrata*
Chalkidiki (sample A)	6.80	5.60	5.70	5.80	5.30	4.90	5.70	4.30	2.40
Andros (sample B)	4.10	4.90	5.20	5.30	4.80	4.20	5.80	4.90	0.90
Agrinio (sample C)	5.30	7.20	6.90	7.90	6.90	3.80	5.40	5.10	1.40
Arta (sample D)	6.70	6.50	5.90	3.10	5.60	3.40	5.20	4.20	0.50
Preveza (sample E)	6.50	6.40	7.10	7.90	7.80	3.90	5.90	5.50	1.90
α-Pinene	6.50	6.50	5.80	4.10	8.50	3.20	4.60	4.70	2.90
Itraconazole	–	–	–	–	–	–	1×10^{-3}	0.1×10^{-3}	1×10^{-3}
5-Flucytocine		–	–	–	–	–	0.1×10^{-3}	1×10^{-3}	10×10^{-3}
Netilmicin	4×10^{-3}	4×10^{-3}	8.8×10^{-3}	8×10^{-3}	8×10^{-3}	10×10^{-3}	–	–	–
AMCA	3×10^{-3}	3×10^{-3}	3.1×10^{-3}	4.2×10^{-3}	4.8×10^{-3}	5×10^{-3}	–	–	–

these tests (Table 2) showed interesting antimicrobial activity.

Among the tested microorganisms, the highest activity was observed against fungi. The strongest fungicidal activity was exhibited by the oil coming from sample D (MIC values 0.50–5.20 mg/ml). The same sample showed the highest activity against *E. cloaceae* and *E. coli* (MIC values 3.10 mg/ml, 3.40 mg/ml, respectively). The oil coming from sample B presented as the most active against *S. aureus*, *S. epidermidis*, *P. aeruginosa* and *K. pneumoniae*, with MIC values ranging from 4.10 to 5.30 mg/ml, mostly due to its higher content to α-pinene (45.8%), which is well known to possess similar antimicrobial activity (Magiatis, Melliou, Skaltsounis, Chinou, & Mitaku, 1999).

4. Conclusion

The present work provides additional data about the European propolis volatiles and reveals the interesting character of the Greek propolis even in its volatiles content. As far as it concerns the antimicrobial activity, it should be noted that the studied samples showed minor differences in their activities independently from their geographic origin or chemical consistency. All these data confirm that bees have the ability to collect from their environment the best agents to protect their hives against bacterial and fungal infections.

References

Adams, R. P. (2001). *Identification of essential oil components by gas chromatography/mass spectometry*. Carol Stream, USA: Allured Publishing Corp.

Bankova, V., Christov, R., Popov, S., Pureb, O., & Bocari, G. (1994). Volatile constituents of propolis. *Zeitschrift für Naturforschung, 49c*, 6–10.

Bankova, V., Christov, R., Kujumgiev, A., Marcucci, M. C., & Popov, S. (1995). Chemical composition and antibacterial activity of Brazilian propolis. *Zeitschrift für Naturforschung, 50c*, 167–172.

Bankova, V., Christov, R., & Tejera, A. D. (1998). Lignans and other constituents of propolis from the Canary islands. *Phytochemistry, 49*, 1411–1415.

Bankova, V., Christov, R., Popov, S., Marcucci, M. C., Tsvetkova, I., & Kujumgiev, A. (1999). Antibacterial activity of essential oils from Brazilian propolis. *Fitoterapia, 70*, 190–193.

Banskota, A. H., Tezuka, Y., Adnyana, I. K., Ishii, E., Midorikawa, K., Matsushige, K., et al. (2001). Hepatoprotective and anti-*Helicobacter pylori* activities of constituents from Brazilian propolis. *Phytomedicine, 8*, 16–23.

Banskota, A. H., Tezuka, Y., & Kadota, S. (2001). Recent progress in pharmacological research of propolis. *Phytotherapy Research, 15*, 561–571.

Borčić, I., Radonić, A., & Grzunov, K. (1996). Comparison of the volatile constituents of propolis gathered in different regions of Croatia. *Flavour and Fragrance Journal, 11*, 311–313.

Burdock, G. A. (1998). Review of the biological properties and toxicity of bee propolis. *Food and Chemical Toxicology, 36*, 347–363.

Ghisalberti, E. (1979). Propolis: a review. *Bee World, 60*, 59–84.

Greenaway, W., May, J., Scaysbrook, T., & Whatley, F. R. (1991). Identification by gas chromatography-mass spectrometry of 150 compounds in propolis. *Zeitschrift für Naturforschung, 46c*, 111–121.

Keskin, N., Hazir, S., Baser, K. H. C., & Kürkçüoglu, M. (2001). Antibacterial activity and chemical composition of Turkish propolis. *Zeitschrift für Naturforschung, 56c*, 111–112.

Magiatis, P., Melliou, E., Skaltsounis, A-L., Chinou, I., & Mitaku, S. (1999). Chemical composition and antimicrobial activity of the essential oils of *Pistacia lentiscus* var. chia. *Planta Medica, 65*, 749–752.

Marcucci, M. C. (1995). Propolis: chemical composition, biological properties and therapeutical activity. *Apidologie, 26*, 83–99.

Massada, Y. (1976). *Analysis of essential oil by gas chromatography and spectrometry*. N. York: Wiley J., & Sons.

Melliou, E., & Chinou, I. (2004). Chemical analysis and antimicrobial activity of Greek propolis. *Planta Medica, 70*, 515–519.

Petri, G., Lembercovics, E., & Folalvari, M. (1988). Examination of differences between propolis (bee glue) produced from different floral environments. In B. M. Lawrence, B. D. Mookhergjee, & B. J. Williams (Eds.), *Flavors and fragrances; A world perspective* (pp. 439–446). Amsterdam: Elsevier.

Van den Dool, H., & Kratz, P. D. (1963). A Generalization of the retention index system including linear temperature programmed gas-liquid partition chromatography. *Journal of Chromatography, 11*, 463–471.