Analytical Methods

The specific chemical profile of Mediterranean propolis from Malta

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A B S T R A C T

Seventeen Maltese propolis samples were studied by GC–MS after silylation. They exhibited the typical Mediterranean chemical profile, rich in diterpene compounds (18–92% of TIC, GC–MS): 32 individual diterpenes were identified; 22 of them were present in each specimen. The other abundant compound group was that of sugars and sugar derivatives. In some samples, however, another compound group was observed (0–12% of TIC, GC–MS): the corresponding mass spectra were consistent with mono- and sesquerpenyl esters of substituted benzoic acids. Two new propolis constituents of this group, daucane diterpene esters of hydroxybenzoic acids, were isolated. Their origin is suggested to be Ferula communis, as they are taxonomic markers for this species. All propolis samples were active against Staphylococcus aureus but only those with high concentrations of terpenyl esters showed antifungal activity against Candida albicans. The present results confirm that Mediterranean propolis is a valuable natural product with potential to improve human health.

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1. Introduction

Propolis, the plant-derived resinous material found in beehives, has been used as a medicine since ancient times. In the past decade, it has gained popularity as a constituent of healthy foods and drinks to improve human health and enhance natural immunity. Its diverse biological activities, such as antibacterial, antiviral, anti-inflammatory, immunomodulating, cytotoxic, antioxidant, and anti-inflammatory, have been well documented in numerous research papers (Ahn et al., 2007; Bankova, 2005; Banskota, Tezuka, & Kadota, 2001; Seidel, Peyfoon, Watson, & Fearnley, 2008; Sforcin, 2007; Tosi, Ré, Ortégara, & Cazzoli, 2007). A well-recognised peculiarity of propolis is its chemical variability, due to fact that (in different phyto-geographical regions) the bees choose different plant sources of resin (Bankova, 2009). For this reason, there are different propolis types, according to their plant origin, and they are characterised by their distinct chemical profiles (Bankova, Popova, & Trusheva, 2006). Different propolis chemical profiles are associated with the presence of specific compound types, such as polyphenols, terpenoids, prenylated acetylenes, isoflavonoids (Cuesta-Rubio et al., 2007; Kumazawa, Hamasaka, & Nakayama, 2004; Velikova et al., 2000). Their contents could be responsible for the biological activities of propolis varieties; therefore, the study of samples from areas where propolis has never been studied before could reveal new propolis types and new propolis constituents of important biological activity.

Recent studies have revealed a new type of European propolis: Mediterranean propolis, distinguished by its high concentration of diterpenoids (Trusheva et al., 2003; Popova, Chinou, Marekov & Bankova, 2009; Popova, Graikou, Chinou, & Bankova, 2010). This propolis type was found in the south of Greece, in Sicily and in some Croatian Adriatic islands. Because of the significant antibacterial activity of diterpenes detected in Mediterranean propolis, it is important to find out whether other Mediterranean propolis is of the same or similar chemical type. In this paper we report the study of the chemical profile of propolis samples from Malta using GC–MS analysis. They were also screened for activity against Staphylococcus aureus 209, Escherichia coli WF + and Candida albicans 562 by the agar cup method.

2. Materials and methods

2.1. Reagents and chemicals

Ethanol analytical grade was purchased from Alkaloid, Skopje, Macedonia; light petroleum and ethyl acetate were from Labscan, Gliwice, Poland. BSTFA, pyridine, Silica gel Alufolien F254 and silica gel 60 F254 glass plates were purchased from Merck, Darmstadt, Germany.
2. Propolis samples

Propolis samples were collected from apiaries at 17 different locations in Malta and the island of Gozo in 2008. The exact locations of origin are listed in Table 1.

2.3. Extraction and sample preparation

Propolis, grated after cooling, was extracted for 24 h with 70% ethanol (1:10, w/v) at room temperature. The extract was evaporated to dryness in vacuo. About 5 mg of the residue were mixed with 50 μl of dry pyridine and 75 μl of 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.

2.4. 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 ionisation voltage 70 eV.

Mass spectra of unidentified compounds: Compound with RT 43.2 min, m/z (%): 341 (9), 205 (100), 147 (33), 133 (18), 130 (23), 117 (14), 73 (40). Compound with RT 45.9 min, from Cox, Yamamoto, Otto, & Simoneit, 2007. The semiquantification and using reference literature spectra where possible (mainly structures were proposed on the basis of their general fragmentation). The cases of lack of the corresponding reference compounds, the semiquantification of the main compounds was carried out by internal normalisation with the area of each compound. The addition of individual areas of the compounds corresponds to 100% area.

2.6. Analytical TLC

Analytical TLC was performed on TLC Alufolien Kieselgel Merck F 254, mobile phase light petroleum–ethyl acetate 7:3. Visualisation of the spots: UV light 254 and 366 nm, spraying with vanillin-sulphuric acid reagent (5:95 w/v vanillin: methanol solution, freshly mixed with a 5:95 v/v sulphuric acid: methanol solution) and heating at 110 °C.

2.7. Isolation of individual compounds

Individual compounds were isolated from the ethanol extract of sample M16. Eighty-eight milligram of this extract were subjected to preparative TLC (silica gel 60 F254 glass plates, (Merck, 20 × 20 cm, 0.25 mm), mobile phase light petroleum–ethyl acetate 9:2; detection under UV light 254 and 366 nm), and 1 mg of 2-acetoxy-6-p-methoxybenzoyl jaeschkeanadiol 1 (RF 0.28) and 2.5 mg of 2-acetoxy-6-p-hydroxybenzoyl jaeschkeanadiol 2 (RF 0.60) were isolated. NMR spectra of compounds 1 and 2 were taken in CDC31 at 600 MHz for protons and 150 MHz for 13C on an AVANCE AV600 II+ NMR Bruker spectrometer.

2.8. Antimicrobial tests

For investigation of the antibacterial activity, the agar cup method (Spoonier & Sykes, 1972) was used with test strains Staphylococcus aureus 209, E. coli WF + and Candida albicans 562 (obtained from the Bulgarian Type Culture Collection, institute for State Control of Drugs, Sofia). An inhibitory zone, with a diameter less than 10 mm, corresponds to lack of activity (10 mm is the diameter of the cup). A test solution (with concentration 4 mg/ml in ethanol) was prepared for every extract and isolated compound; 0.1 ml of this test solution (containing 0.4 mg of each substance) was applied to every cup (cup volume 0.1 ml). Control experiments with solvent showed that it does not have any activity. Streptomycin and nystatin were used as positive controls.

2.9. Statistics

For correlation analysis, Pearson’s correlation coefficients were applied. Statistica 5 was used.

3. Results and discussion

The yields of dry propolis extracts of Maltese propolis specimens are represented in Table 1. The silylated ethanol extracts were analysed by GC–MS. The main classes of compounds identified and their abundances are listed in Table 2. GC–MS analysis was chosen for chemical profiling because of the high resolving power of capillary GC and the valuable structural information provided by the EIMS, especially for phenolics and terpenes (Cuesta-Rubio et al., 2007; Kalogeropoulos, Konteles, Troullidou, Mourtzinos, & Karathanos, 2009; Popova et al., 2010), despite the disadvantages of the silylation procedure.

The GC–MS analysis revealed that the studied propolis samples possess the typical Mediterranean chemical profile, rich in diterpene compounds. A total of 32 individual diterpenes was identified, 22 of them were present in each one of the 17 samples. Most abundant were the diterpene acids: isocupressic, communic, pimaric and imbricatolic acid, together with totarol and 13-epi-totarol, found in all samples. Among the minor components,
ing of Maltese propolis, together with reference substances for anisates. Based on this fact, we detected the presence of droabietic acid, 2-hydroxyferruginol, 1,6-dihydroxyferruginol, isoa- gatholal, 1,6-jaeschkeanadiol, 1,6-labdadiac acid, 1,6-labda-8(17),12,13-triene, 1,6-ranean type (Popova et al., 2010), and the GC–MS profiles of Greek samples. They were well recognizable because of their UV fluorescence and their specific green colour after spraying with vanillin-sulphuric acid reagent. We succeeded in isolating them, by PTLC, from sample M-16 and identified them as 2-acetoxy-6-p-methoxy-benzyl- jaeschkeanadiol (1) and 2-acetoxy-6-p-hydroxybenzyl- jaeschkeanadiol (2). They were identified by comparison of their spectral characteristics (MS, 1H– and 13C–NMR spectra) with literature data (Lamnaouer, Martin, Molho, & Bodi, 1989). So we were able to use 1 and 2 as reference compounds and to identify them in the mass chromatograms of Maltese propolis. Fig. 1 shows the chemical structures of compounds 1–4. The semiquantitative data about the terpene esters of benzoic acids in the studied specimens is presented in Table 3. The characteristic most abundant MS peaks of the silylated compounds are listed in Table 4.

Compounds 1 and 2 have previously been isolated only from Ferula communis (Lamnaouer et al., 1989) and are here reported, for the first time, in propolis. Sesquiterpene esters of benzoic acids are characteristic secondary metabolites of the genus Ferula. Their presence in Maltese propolis is a clear indication that Ferula species, and most probably Ferula communis, which is typical of the island’s flora (Makhzoumi, 2000), play the role of a propolis plant source in Malta, although not a basic one (Table 2). It is important to note the fact that Ferula was chosen by bees as a secondary propolis source in two different phytogeographic regions: Iran and Malta.

Other constituents of Maltese propolis were aliphatic hydroxy-acids, aromatic and fatty acids, triterpenes (mainly alcohols of the amyrin type) (Table 2). In only one sample, M-15, minor amounts of typical poplar flavonoids (chrysin, pinocembrin chalcone) and poplar caffeic acid esters (pentenyl caffeates) were detected. This might be explained by the occasional presence of an introduced poplar tree.

Having all these data, it was of interest to check if there were any statistically significant correlations between the compound groups in the samples, using Pearson’s correlation. The presence of such correlations might be an indication of whether the particular compound groups originate from the same or different plant sources. A relatively strong negative correlation was observed ($r = -0.750$, $p = 0.0004$) between diterpenes and sugars, while the negative correlation detected between diterpenes and sesquiterpene esters of benzoic acids was only moderate ($r = -0.495$, $p = 0.0043$). The derivation of the diterpenes was suggested by Popova et al. (2009) to be some Coniferous plant, most probably of the Cupressaceae family (e.g. Cupressus sempervirens), while

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### Table 2

<table>
<thead>
<tr>
<th>Sample</th>
<th>Hydroxy acids</th>
<th>Sugars and sugar derivatives</th>
<th>Aromatic acids</th>
<th>Fatty acids and esters</th>
<th>Diterpenes</th>
<th>Terpenyl esters of substituted benzoic acids</th>
<th>Triterpenes</th>
<th>Others$^b$</th>
<th>Unknown$^c$</th>
<th>Flavonoids &amp; cinnamic acids esters</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>1.2</td>
<td>14.9</td>
<td>0.8</td>
<td>9.4</td>
<td>44.1</td>
<td>5.2</td>
<td>2.7</td>
<td>2.9</td>
<td>10.5</td>
<td>–</td>
</tr>
<tr>
<td>M2</td>
<td>0.4</td>
<td>18.2</td>
<td>1.5</td>
<td>3.3</td>
<td>46.0</td>
<td>5.9</td>
<td>1.2</td>
<td>1.2</td>
<td>6.2</td>
<td>–</td>
</tr>
<tr>
<td>M3</td>
<td>1.2</td>
<td>24.6</td>
<td>1.0</td>
<td>4.6</td>
<td>23.9</td>
<td>2.7</td>
<td>1.9</td>
<td>5.3</td>
<td>5.4</td>
<td>–</td>
</tr>
<tr>
<td>M4</td>
<td>0.9</td>
<td>18.9</td>
<td>&lt;0.1</td>
<td>2.5</td>
<td>55.0</td>
<td>4.8</td>
<td>0.6</td>
<td>2.8</td>
<td>4.2</td>
<td>–</td>
</tr>
<tr>
<td>M5</td>
<td>&lt;0.1</td>
<td>0.8</td>
<td>&lt;0.1</td>
<td>1.5</td>
<td>92.5</td>
<td>0</td>
<td>1.7</td>
<td>1.2</td>
<td>0.9</td>
<td>–</td>
</tr>
<tr>
<td>M6</td>
<td>1.2</td>
<td>25.3</td>
<td>0.7</td>
<td>4.0</td>
<td>34.6</td>
<td>2.2</td>
<td>4.9</td>
<td>4.1</td>
<td>3.1</td>
<td>–</td>
</tr>
<tr>
<td>M7</td>
<td>1.0</td>
<td>15.0</td>
<td>0.2</td>
<td>3.6</td>
<td>60.6</td>
<td>0.2</td>
<td>1.2</td>
<td>4.1</td>
<td>3.9</td>
<td>–</td>
</tr>
<tr>
<td>M8</td>
<td>&lt;0.1</td>
<td>26.2</td>
<td>0.8</td>
<td>0.8</td>
<td>58.2</td>
<td>0.1</td>
<td>&lt;0.1</td>
<td>1.2</td>
<td>0.4</td>
<td>–</td>
</tr>
<tr>
<td>M9</td>
<td>0.8</td>
<td>30.7</td>
<td>0.4</td>
<td>2.5</td>
<td>43.9</td>
<td>1.0</td>
<td>1.8</td>
<td>2.0</td>
<td>2.3</td>
<td>–</td>
</tr>
<tr>
<td>M10</td>
<td>0.7</td>
<td>19.4</td>
<td>0.3</td>
<td>2.2</td>
<td>30.6</td>
<td>1.4</td>
<td>&lt;0.1</td>
<td>4.1</td>
<td>2.0</td>
<td>–</td>
</tr>
<tr>
<td>M11</td>
<td>0.9</td>
<td>23.6</td>
<td>0.3</td>
<td>2.2</td>
<td>53.5</td>
<td>0.2</td>
<td>3.5</td>
<td>3.3</td>
<td>3.2</td>
<td>–</td>
</tr>
<tr>
<td>M12</td>
<td>0.5</td>
<td>18.3</td>
<td>0.4</td>
<td>4.5</td>
<td>46.7</td>
<td>2.2</td>
<td>1.2</td>
<td>4.6</td>
<td>4.5</td>
<td>–</td>
</tr>
<tr>
<td>M13</td>
<td>1.5</td>
<td>22.4</td>
<td>2.7</td>
<td>6.9</td>
<td>26.1</td>
<td>11.8</td>
<td>0.2</td>
<td>5.1</td>
<td>7.1</td>
<td>–</td>
</tr>
<tr>
<td>M14</td>
<td>3.0</td>
<td>37.2</td>
<td>2.4</td>
<td>4.2</td>
<td>20.4</td>
<td>2.9</td>
<td>0.6</td>
<td>8.0</td>
<td>2.3</td>
<td>–</td>
</tr>
<tr>
<td>M15</td>
<td>1.5</td>
<td>29.8</td>
<td>1.5</td>
<td>3.1</td>
<td>18.7</td>
<td>5.5</td>
<td>3.6</td>
<td>4.6</td>
<td>4.8</td>
<td>1.0</td>
</tr>
<tr>
<td>M16</td>
<td>0.5</td>
<td>19.9</td>
<td>1.7</td>
<td>3.4</td>
<td>40.9</td>
<td>7.6</td>
<td>&lt;0.1</td>
<td>2.7</td>
<td>6.7</td>
<td>–</td>
</tr>
<tr>
<td>M17</td>
<td>0.1</td>
<td>17.6</td>
<td>0.4</td>
<td>3.7</td>
<td>59.3</td>
<td>0.2</td>
<td>0.9</td>
<td>2.4</td>
<td>3.5</td>
<td>–</td>
</tr>
</tbody>
</table>

$^a$ Two replicates, relative s.d. < 10%.

$^b$ Others include: ethylamine, ethylphosphate, glycerol, butanediol and sitosterol.

$^c$ For mass spectra of the unknown see Materials and methods.

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1 Present in all samples.
the esters 1–4 most probably originate from *Ferula*, as already mentioned. Thus the negative correlation between these groups is expected. On the other hand, the origin of sugars in propolis has received almost no comments in the literature. Small amounts of glucose, fructose and sucrose are believed to originate from nectar or honey, introduced occasionally by the bees (Quan, Khan, Watson, & Fearnley, 2008). The question has been raised as to whether the sugars in propolis come from hydrolysed flavonoid glycosides (Quan et al., 2008). However, there are numerous proofs that bees collect propolis from plant materials that contain flavonoid aglycones but no glycosides. Then again, mucilages were mentioned. Thus the negative correlation between these groups of terpenyl esters of hydroxybenzoic acids. A statistically significant Pearson's correlation ($r = 0.7887$, $p < 0.001$) was found between the amount of the esters and the antifungal activity. These data suggest that the terpenyl hydroxybenzoates play an important role in the antifungal properties of Maltese propolis, while the diterpene constituents are of greater significance for the antibacterial properties.

### Table 3

Terpenyl esters of substituted benzoic acids identified in propolis ethanol extracts by GC–MS (percent TIC, TMS derivatives). $^a$

<table>
<thead>
<tr>
<th>Compound</th>
<th>RT (min)</th>
<th>M1</th>
<th>M2</th>
<th>M3</th>
<th>M4</th>
<th>M5</th>
<th>M6</th>
<th>M7</th>
<th>M8</th>
<th>M9</th>
<th>M10</th>
<th>M11</th>
<th>M12</th>
<th>M13</th>
<th>M14</th>
<th>M15</th>
<th>M16</th>
<th>M17</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>47.2</td>
<td>0.2</td>
<td>–</td>
<td>0.2</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.7</td>
<td>0.6</td>
<td>0.3</td>
<td>0.1</td>
<td>0.4</td>
<td>0.9</td>
<td>Tr.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>48.4</td>
<td>0.4</td>
<td>0.5</td>
<td>–</td>
<td>0.4</td>
<td>0.4</td>
<td>Tr.</td>
<td>Tr.</td>
<td>Tr.</td>
<td>0.1</td>
<td>Tr.</td>
<td>Tr.</td>
<td>1.8</td>
<td>0.3</td>
<td>Tr.</td>
<td>4.8</td>
<td>Tr.</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>44.8</td>
<td>3.4</td>
<td>4.1</td>
<td>2.5</td>
<td>3.9</td>
<td>1.6</td>
<td>0.2</td>
<td>0.1</td>
<td>1.0</td>
<td>1.2</td>
<td>0.2</td>
<td>2.2</td>
<td>8.4</td>
<td>2.3</td>
<td>4.5</td>
<td>2.3</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>46.6</td>
<td>0.4</td>
<td>0.6</td>
<td>0.2</td>
<td>0.4</td>
<td>–</td>
<td>0.2</td>
<td>–</td>
<td>Tr.</td>
<td>0.1</td>
<td>Tr.</td>
<td>Tr.</td>
<td>1.2</td>
<td>0.2</td>
<td>0.8</td>
<td>0.2</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>X</td>
<td>43.7</td>
<td>1.0</td>
<td>0.7</td>
<td>Tr.</td>
<td>0.1</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>Tr.</td>
<td>0.4</td>
<td>0.1</td>
<td>0.2</td>
<td>0.3</td>
<td>–</td>
<td>–</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Two replicates, relative s.d. < 10%.

$^b$ Unidentified ester of $p$-hydroxybenzoic acid.

### Table 4

Most prominent mass spectral peaks of the TMS ethers of terpenyl esters in Maltese propolis.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Prominent MS peaks</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Acetoxy-6-$p$-methoxybenzoyl jaeschkeanadiol</td>
<td>132 (26), 135 (100), 175 (47),</td>
</tr>
<tr>
<td>2-Acetoxy-6-$p$-hydroxybenzoyl jaeschkeanadiol</td>
<td>132 (35), 175 (35), 193 (100),</td>
</tr>
<tr>
<td>Ferutinin</td>
<td>132 (19), 159 (27), 177 (25), 193 (100),</td>
</tr>
<tr>
<td>Teferin 4</td>
<td>132 (13), 159 (20), 177 (26), 193 (45), 223 (100),</td>
</tr>
<tr>
<td>X (unidentified anisate)</td>
<td>135 (25), 159 (18), 177 (38), 193 (100),</td>
</tr>
</tbody>
</table>

### Table 5

Antimicrobial activities against *S. aureus* and *Candida albicans* (zones of inhibition) of propolis extracts (at 400 µg in the cup).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Zone of inhibition (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S. aureus</td>
</tr>
<tr>
<td>M1</td>
<td>17 ± 1</td>
</tr>
<tr>
<td>M2</td>
<td>22 ± 0</td>
</tr>
<tr>
<td>M3</td>
<td>15 ± 1</td>
</tr>
<tr>
<td>M4</td>
<td>25 ± 1</td>
</tr>
<tr>
<td>M5</td>
<td>27 ± 1</td>
</tr>
<tr>
<td>M6</td>
<td>24 ± 0</td>
</tr>
<tr>
<td>M7</td>
<td>22 ± 0</td>
</tr>
<tr>
<td>M8</td>
<td>23.7 ± 0.6</td>
</tr>
<tr>
<td>M9</td>
<td>23 ± 1</td>
</tr>
<tr>
<td>M10</td>
<td>22 ± 0</td>
</tr>
<tr>
<td>M11</td>
<td>25 ± 1</td>
</tr>
<tr>
<td>M12</td>
<td>24.3 ± 0.6</td>
</tr>
<tr>
<td>M13</td>
<td>22 ± 0</td>
</tr>
<tr>
<td>M14</td>
<td>23 ± 1</td>
</tr>
<tr>
<td>M15</td>
<td>23.7 ± 0.6</td>
</tr>
<tr>
<td>M16</td>
<td>25 ± 1</td>
</tr>
<tr>
<td>M17</td>
<td>22 ± 0</td>
</tr>
<tr>
<td>Bulgarian propolis</td>
<td>20 ± 1</td>
</tr>
<tr>
<td>Streptomycin$^a$</td>
<td>28 ± 1</td>
</tr>
<tr>
<td>Nysratin$^b$</td>
<td>–</td>
</tr>
</tbody>
</table>

Tests were done in triplicate, values are means ± s.d.

$^a$ 100 µg in the cup.

$^b$ 50 I.U.
Mediterranean propolis need further research in order to overcome the problem of standardization, which is an obstacle to wider use of propolis in the food industry.

4. Conclusions

The present results demonstrate that Maltese propolis may be a rich source of antibacterial and antifungal compounds. They also confirm the view that Mediterranean propolis, from different Mediterranean regions, is a valuable natural product with potential to improve human health, and further studies are needed to reveal its chemistry and biological activity in more detail, in order to enable its use in health foods and cosmetics.

References


