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Chemical constituents of Jordanian propolis

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Seventeen compounds, including a new lanostane triterpenoid, 24(*Z*)-1β-3β-dihydroxyeupha-7,24-dien-26-oic acid, have been isolated from the methanolic extracts of two samples of Jordanian propolis collected from two different places with different dominant flora. The structures of the isolated compounds were elucidated by spectral methods including IR, UV, MS and 1- and 2-D NMR.

Keywords: flavonoids; lanostane triterpenoids; 24(*Z*)-1β-3β-dihydroxyeupha-7,24-dien-26-oic acid; oak propolis; pine propolis

1. Introduction

Propolis is a resinous product collected by bees (*Apis mellifera*) from tree exudates, mainly resins of leaf bud mixed with beeswax to form a sealing material in their honeycombs (Isla, Guzman, Moreno, Koo, & Park, 2005). Plants secrete the resin to coat the young shoots and buds in order to protect them from the adverse effects of bad weather and from attack of bacteria, fungi, moulds and viruses. Bees collect these substances, pack them on their hind legs, and bring them back to the hive to cover cracks and reduce the size of the hive entrance, thus protecting them from the attack of large insects like moths, butterflies, beetles, etc. Bees also use the propolis to cover the inside of the hive and mix it with beeswax to protect the colony from pathogenic microorganisms (Sahinler & Kaftanoglu, 2005).

Propolis has been used in folk medicine since ancient times due to its many biological properties, such as antimicrobial, anti-inflammatory and antioxidant activities (Orsi, Sforcin, Funari, & Bankova, 2005). Propolis is also heavily used in cosmetics (Sahinler & Kaftanoglu, 2005). Previous studies of propolis are numerous, and have shown that the chemical composition of propolis depends upon the vegetation of the area from where it has been collected, and on climatic, seasonal and geographical differences (Banskota et al., 2000). The most important classes of compounds found in propolis are: phenolic compounds, flavonoids, terpenoids, lipids, wax and sugars (Kedzai & Holderna-Kedzia, 1991). The chemical

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composition of Jordanian propolis has not been investigated before. The antimicrobial effects of Jordanian propolis collected from pine trees and oak trees have previously been investigated. The results indicated that pine propolis was more potent than oak propolis against different tested microorganisms including resistant strains of bacteria (Abu Fares, 2008).

In an effort to understand the chemical composition of propolis of Jordanian origin, a thorough investigation of the chemical constituents of two types of Jordanian propolis (pine propolis and oak propolis) has been carried out.

2. Results and discussion

A total of 17 compounds have been isolated from two types of Jordanian propolis, namely pine propolis and oak propolis. All of the compounds were identified by studying their spectral properties and comparison with literature data.

The pine propolis afforded 16 compounds. These are: the aromatic ester cinnamyl cinnamate (**1**) (Hu et al., 2005), the fatty acid tetracosanoic acid (**2**) (Zhang et al., 2004), the flavonoids (Figure 1) tectochrysin (**3**) (Agrawal, 1989; Isla et al., 2005), pinocembrin (**5**) (Isla et al., 2005), pinobanksin-3-*O*-acetate (**6**) (Fang, Su, & Cheng, 1988), 3-methylethergalangin (**7**) (Shin, Han, & Kim, 2003), chrysin (**8**) (Chen, Games, & Jones, 2003), galangin (**9**) (Facundo & Morais, 2003), genkwanin (**10**) (Agrawal, 1989), alpinone (**12**) (Christov, Trusheva, Popova, Bankova, & Bertrand, 2006), naringenin (**13**) (Ibrahim, Galal, Ahmed, & Mossa, 2003) and apigenin (**14**) (Hasan, 2004). The sesquiterpenoid cryptomeridiol (**15**) (Ragasa, Co, & Rideout, 2005), the diterpenoid agathadiol (**11**) (Feliciano et al., 1988), and the triterpenoids (Figure 1) 24-(*Z*)-3-oxolanosta-1,7,24-trien-26-oic acid (**4**) (Ansari &

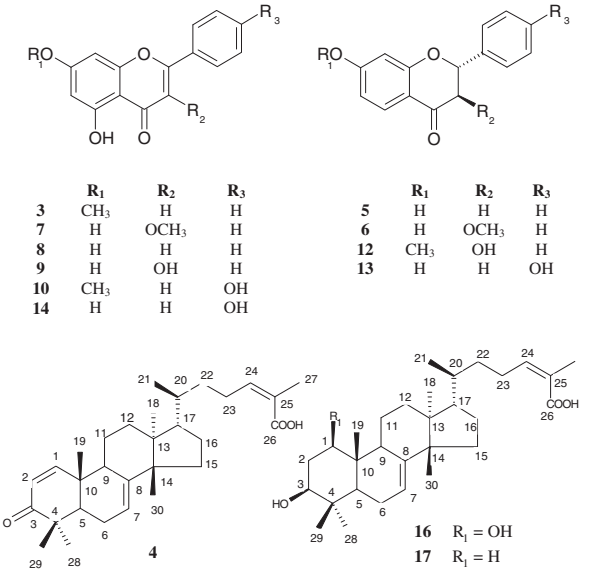

Figure 1. The structures of flavonoids and triterpenoids isolated from Jordanian propolis.

Ali, 1996) and the new compound 24(*Z*)-1β,3β-dihydroxyeupha-7,24-dien-26-oic acid (**16**) were also isolated.

The oak propolis afforded six compounds, five of which were isolated from pine propolis. These are: the fatty acid **2**, the flavonoids **5** and **7** and the triterpenoids **4** and **16**. The sixth compound was the known triterpenoid 24(*Z*)-3β-hydroxyeupha-7,24-dien-26-oic acid (**17**) (Deng, Starck, Sun, Sabat, & Hecht, 2000).

The new compound 24(*Z*)-1β, 3β-dihydroxyeupha-7,24-dien-26-oic acid (**16)** was obtained as a white solid (m.p. 229–230°C). Its EIMS showed a molecular ion peak *m/z* 472 corresponding to the molecular formula $C_{30}H_{48}O_4$ (HREIMS *m/z* 472.7086 Calcd for 472.7087). The spectrum showed prominent peaks at *m/z* 457 and 421 corresponding to the ions$[M-CH_3]^+$and $[M-CH_3-H_2O]^+$, respectively, and a base peak at *m/z* 439 corresponding to the ion $[M-CH_3-2H_2O]^+$ which indicated the presence of two hydroxyl groups in the molecule. The IR spectrum confirmed the presence of the OH groups (3380 cm^{-1}) and a carbonyl group (1680 cm^{-1}).

The ^{1}H-NMR spectrum of **16** was typical of a lanostane triterpene (Venkatraman, Thombare, & Sabata, 1994; Weis & Seebacher, 2002). It displayed signals for five quaternary methyls at δ_H 0.62, 0.68, 0.70, 0.79 and 0.90, one secondary methyl δ_H at 0.81 (d, $J = 6.1$ Hz, Me-21) and one vinylic methyl at δ_H 1.75. The spectrum also showed signals for two vinylic protons at δ_H 5.17 (t, $J = 7.1$ Hz) and δ_H 5.84 (br s) assignable to H-7 and H-24, respectively. An important feature of the ^{1}H-NMR spectrum was the two signals at δ_H 3.01 (dd, $J = 11.5$ and 2.9 Hz) and δ_H 3.27 (dd, $J = 10.8$ and 3.7 Hz) for two hydroxymethine protons confirming the presence of two hydroxyl groups in this compound.)

The ^{13}C-NMR and DEPT spectra confirmed that compound **16** was a tetracyclic triterpenoid consisting of seven methyls, eight methylenes, eight methines including two vinylic (δ_C 117.9 and 142.3) and two hydroxylated (δ_C 74.5 and 75.5) methines, and seven quaternary carbons including two olefinic (δ_C 127.7 and 146.5) and one carboxyl (δ_C 169.5) carbons.

Significant was the δ values of the methyl signals at C-13 and C-14 (δ_C 22.2 and 27.6, respectively), which were comparable to the δ values of the corresponding signals in similar known lanostanes with the same stereochemistry ($\delta_C \sim 22$ and 27.5, respectively), thus supporting the lanostane skeleton of compound **16** (Kamo, Asanoma, Shibata & Hirota, 2003; Lin & Shiao, 1989; Makino, Motegi, & Fujimoto, 2004).

The ^{1}H- and ^{13}C-NMR spectra of compound **16** and of the known lanostane **17** were very similar, except that the C-1 methylene of 17 was replaced by a hydroxylated methine in 16. The upfield shift of Me-19 (δ_C 8.1) relative to C-1 unsubstituted analogues ($\delta_C \approx 13$) (Lin & Shiao, 1989) further supported there was a hydroxyl group substituted at C-1, and the coupling constants of H-1/H-2a ($J = 10.8$ Hz) and H-3/H-2a ($J = 11.5$ Hz) suggested that both hydroxyls of C-1 and C-3 were of β-orientation. Moreover, the upfield shift of H-24 (δ_H 5.48), relative to the *E*-analogues ($\delta_H \approx 6.9$) (Deng et al., 2000) implied that vinilyic group ($\Delta^{24,25}$) was *Z* conformation.

The structure of compound **16** was further confirmed by 2-D NMR experiments (COSY, HMQC and HMBC). The location of the hydroxyl groups to C-3 (δ_C 74.5). and C-1 (δ_C 75.5) was confirmed by the HMBC correlations of Me-28 and Me-29 to C-3 and the correlation of Me-19 to C-1. Moreover, the location of the double bond

in ring B ($\Delta^{7,8}$) was confirmed by the HMBC correlation between H-7 and each of C-5 and C-9, and that of the side chain double bond ($\Delta^{24,25}$) by the COSY correlation between H-24 (δ_H 5.84) and Me-27 and the HMBC correlation between Me-27 and each of C-24, C-25 and the carboxyl carbon (C-26).

It is noteworthy that, with the exception of the sesquiterpenoid **15** and the new triterpenoid **16**, all the compounds isolated form the Jordanian pine propolis have previously been isolated from propolis, and with the exception of compounds **7** and **16**, all of these compounds have been reported before from the genus *Pinus*. Additionally, this is the first report of the lanostane triterpenoid **17** from propolis, and although this type of triterpenoids exists in the genus *Quercu*s, the lanostanes **4**, **16** and **17** have not been reported before from this genus.

This study of the two compositions of the two types of Jordanian propolis shows that both types contain flavonoids and triterpenoids but pine propolis is richer in flavonoids. It has been reported that Jordanian pine propolis has a higher antimicrobial activity than Jordanian oak propolis (Darwish, Abu Fares, Abu Zarga & Nazer, 2009). This could be explained on the basis of the different compositions of the two types of propolis and could be related to the higher abundance of flavonoids in pine propolis relative to the oak propolis. This is in accord with previous studies which showed that the antimicrobial activity of propolis is mainly due to the presence of flavonoids (Choi et al., 2006; Kosalec, Pepeljnjak, Bakmaz, & Vladimir-Knezevic, 2005; Velikova et al., 2000).

3. Experimental

3.1. *General*

The UV spectra were recorded on SpectroScan 80D double beam UV–vis spectrophotometer. Mass spectra were recorded using Finnigan MAT TSQ-70, JMS 600 H or MAT-112 S spectrometers at 70 eV; ion source temperature at 200°C. The ^{1}H-NMR spectra were recorded on a Bruker DPX-300 MHz spectrometer with TMS as internal standard. ^{13}C-NMR spectra were recorded at 75.5 MHz. The IR spectra were recorded on Thermo-Nicolet Nexus 870 FT-IR spectrophotometer. Melting points were measured using Fisher-Johns melting point apparatus and are uncorrected. TLC was performed on precoated silica gel G/UV_{254}, 0.25 or 0.5 mm in thickness (Macherey–Nagel). Compounds were examined under UV light and spraying with sulphuric acid, anisaldehyde spray reagent followed by heating at 120°C.

3.2. *Propolis collection*

The propolis samples were collected during the period June 2004 to September 2005, by honey bees (*A. mellifera*), from two locations with two different dominant flora. Pine propolis (160 g) was collected from University of Jordan campus, Amman, Jordan, where pine trees (mainly *Pinus halepensis*) are common. The oak propolis (180 g) was collected from Al-Hashemia region (12 km west of Amman) in which oak trees (mainly *Quercus coccifera*) are most common.

3.3. *Extraction and isolation*

The samples were chopped into small pieces and extracted four times (each time for 2 days using 3 L of methanol). The methanolic extract of each type was evaporated under reduced pressure to give a gummy residue. The pine propolis residue (109 g) was chromatographed on a silica gel 60 (70–230 mesh, Fluka) column eluted with a gradient of MeOH/$CHCl_3$ of increasing polarity to give five fractions (PI–PV). The oak propolis extract (118 g) was chromatographed in a similar manner to give five fractions (OI–OV). The fractions were further purified by a combination of column chromatography (silica gel, 400 mesh, Macherey–Nagel) and TLC using suitable solvent systems.

Fraction PI (37 g) afforded compounds **1** (130 mg), **2** (300 mg), **3** (30 mg), **4** (70 mg), **5** (300 mg), **6** (425 mg) and **7** (20 mg). Fraction PII (15 g) gave compounds **8** (480 mg), **9** (25 mg), **10** (30 mg) and **11** (20 mg). Fraction PIII (14 g) afforded compound **12** (21 mg). Fraction PIV (10 g) afforded compounds **13** (10 mg), **14** (25 mg) and **15** (20 mg). Fraction PV (7 g) afforded compound **16** (75 mg). Fraction OII (39 g) gave compounds **2** (200 mg), **4** (700 mg) and **17** (140 mg). Fraction OIII (22 g) afforded compounds **5** (60 mg) and **8** (300 mg). Fraction OV (13 g) gave compound **16** (5000 mg).

3.4. *Spectral data of compound 16*

IR (KBr) ν_{max} (cm^{-1}) 3558, 3379, 2957, 1680, 1640, 1453, 1382, 1246 and 1057; EIMS *m/z* (relative intensity) 472 (18, M^+, $C_{30}H_{48}O_4$), 457 (33), 439 (54), 421 (100), 313 (22), 253 (19), 225 (22), 201 (74), 173 (38), 161 (40), 145 (74), 121 (45) and 95 (64). HREIMS *m/z* 472.7086 (Calcd for $C_{30}H_{48}O_4$, 472.7087). ^{1}H-NMR (DMSO-d_6) δ 0.62 (3H, s, Me-19), 0.68 (3H, s, Me-29), 0.70 (3H, s, Me-18), 0.79 (3H, s, Me-28), 0.81 (3H, d, Me-21), 0.90 (3H, s, Me-30), 1.75 (3H, br s, Me-27), 3.01 (1H, dd, $J = 11.5$, 2.9 Hz, H-3), 3.27 (1H, dd, $J = 10.8$, 3.7 Hz, H-1), 5.17 (1H, br s, H-7), 5.84 (1 H, t, $J = 7.1$ Hz, H-24), 12.25 (1H, br s, H-26), ^{13}C-NMR (DMSO-d_6) δ 8.1 (C-19), 15.2 (C-29), 18.5 (C-21), 21.1 (C-27), 21.2 (C-11), 22.2 (C-18), 24.2 (C-6), 26.4 (C-23), 27.7 (C-30), 28.0 (C-28), 28.1 (C-16), 34.3 (C-12), 34.5 (C-15), 35.7 (C-22), 36.0 (C-20), 38.8 (C-2), 39.2 (C-4), 40.6 (C-10), 43.1 (C-13), 49.1 (C-5), 49.9 (C-9), 51.2 (C-14), 52.8 (C-17), 74.5 (C-3), 75.6 (C-1), 117.9 (C-7), 127.7 (C-25), 142.3 (C-24), 146.5 (C-8) and 169.5 (C-26).

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