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ORIGINAL ARTICLE

Flavonoids from Algerian propolis

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Abstract The investigation of propolis collected from Jijel, located in the northern-east part from Algeria afforded five flavones: pectolinarigenin (**1**), pilosin (**2**), ladanein (**3**), Chrysin (**4**) and apigenin (**5**). The structures were elucidated by spectroscopic analysis, including mass spectrometry, 1D and 2D NMR.

1. Introduction

Propolis is a natural substance collected by bees from buds and exudates of plants and trees. Bees use this product to protect their hives from enemies. Propolis is used in folk medicines in many regions of the world (Ghisalberti, 1979). It has been reported to have various biological activities such as antibacterial, antiviral, anti-inflammatory and anticancer (Ammaros et al., 1994; Almeida and Menezes, 2002; Kimoto et al., 1998; Kujumgiev et al., 1999). Recent research has highlighted that propolis prevents such illnesses such as heart disease, diabetes and cancer (Burdock, 2000).

The chemical composition of propolis depends upon the vegetation of the collection site (Bankova et al., 2001; Marcucci, 1995). For example, Propolis from Europe contains many kinds of flavonoids and phenolic esters. In contrast, the major components in Brazilian propolis were terpenoids and prenylated derivatives of *p*-coumaric acids (Marcucci and Bankova, 1999; Tazawa et al., 1999).

In previous studies we have demonstrated that Algerian propolis prevents hepatic toxicity of some cancer therapy (Lahouel et al., 2004). Hence, it is worthy of consideration to carry out a chemical study dealing with the chemical composition of Algerian propolis collected from Jijel.

2. Experimental

2.1. Material

Propolis was collected from the north-east of Algeria (Jijel) in 2006 by scraping the "bee glue" of walls, frames and entrance of the hive.

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3. Extraction and isolation

Propolis (500 g) was extracted with CH_2Cl_2:MeOH (1:1). The extract was concentrated to dryness, the residue was then extracted with MeOH:H_2O (70:30 v/v) and concentrated under reduced pressure. The extract (70:30 v/v) was dissolved in boiling water, stored in the cold and filtered after 24 h. The filtrate was extracted successively with EtOAc to yield (2.7 g) and *n*-BuOH to yield (8.3 g).

Two-dimensional paper chromatography using 15% AcOH and BAW (*n*-BuOH:AcOH:H_2O 4:1:5 upper phase) as solvents shows that the MeOH–CH_2Cl_2 and EtOAc extracts contain different compounds representing flavonoids and phenolic acids.

The CH_2Cl_2:MeOH (1:1) extract (10 g) was fractionated by silica gel CC eluted with *n*-hexane, followed by a gradient of *n*-hexane–CH_2Cl_2 up to 100% CH_2Cl_2 and CH_2Cl_2–MeOH up to 15% MeOH, 28 fractions were collected and analyzed by TLC. Fractions 13, 14 and 15 were concentrated and yellow precipitates were obtained. Recrystallisation of fraction 13 in $CHCl_3$ yielded a mixture of compounds **1** and **2** as yellow crystals (Fig. 1). Due to the small quantity of the mixture, we have not attempted to separate both compounds in order to avoid any loss of material. However, NMR data were discernable since they furnished different intensities of the signals for both compounds. Recrystallisation of fractions 14 and 15 in MeOH yielded compounds **3** and **4** (Fig. 1).

EtOAc extract was subjected to column chromatography on silica gel eluting with a gradient of CH_2Cl_2–MeOH with increasing polarity; 47 fractions were collected and analyzed by TLC. Fraction 5 was separated on thin layer chromatography using CH_2Cl_2:EtOAc (9:1) as an eluting system to offer compound **5** (Fig. 1). Purification was carried out using MeOH over Sephadex LH20. Compounds **1** and **2** were identified by spectroscopic techniques (UV–visible, ^{1}H NMR, ^{13}C NMR, Dept, COSY, HMQC and HMBC), while compound **3** and **4** were identified by UV–visible, ^{1}H NMR and ^{13}C NMR. Compound **5** was identified by UV–visible and ^{1}H NMR and compared with the reported data (Livinenko et al., 1969; Maisashvili et al., 2009).

3.1. Compound *1*, $C_{17}H_{14}O_6$; mp 210–211 °C

UV (λ_{max} in MeOH): gives bands at 321 and 267 nm for band I and II, addition of NaOH; 385, 329, 267 and $AlCl_3$: 338, 299; and HCl: 340, 299; and NaOAc: 331, 272; while H_3BO_3: 331, 272. Mass spectrum EI/MS *m/z* (**rel. int**): 314 $[M]^+$ (100), 299 [M-Me] (70), 271 [M-Me-CO] (76), 183 $[A_1+H]^+$ (10), 167 $[A_1-Me]^+$ (90), 133 $[B_1+H]^+$ (40).

^{1}H NMR spectrum (300 MHz, DMSO-d_6), δ (ppm): δ 13.01 (1H, s, 5-OH), 10.67 (1H, s, 7-OH), 8 (2H, d, J = 8.9 Hz, H-2′ and H-6′), 7.08 (2H, d, J = 8.9 Hz, H-3′ and H-5′), 6.83 (1H, s, H-3), 6.59 (1H, s, H-8), 3.84 (3H, s, OMe), 3.75 (3H, s, OMe).

^{13}C NMR (75 MHz, DMSO-d_6), δ_c (ppm): 182.60 (C-4), 163.30 (C-2), 160.93 (C-4′), 15.23 (C-7), 152.15 (C-5), 151.9 (C-9), 131.29 (C-6), 128.75 (C-2′ and C-6′), 123.70 (C-1′), 11.48 (C-3′ and C-5′), 104.10 (C-10), 103.9 (C-3), 94.25 (C-8), 60.40 (6-OMe), 55.80 (4-OMe).

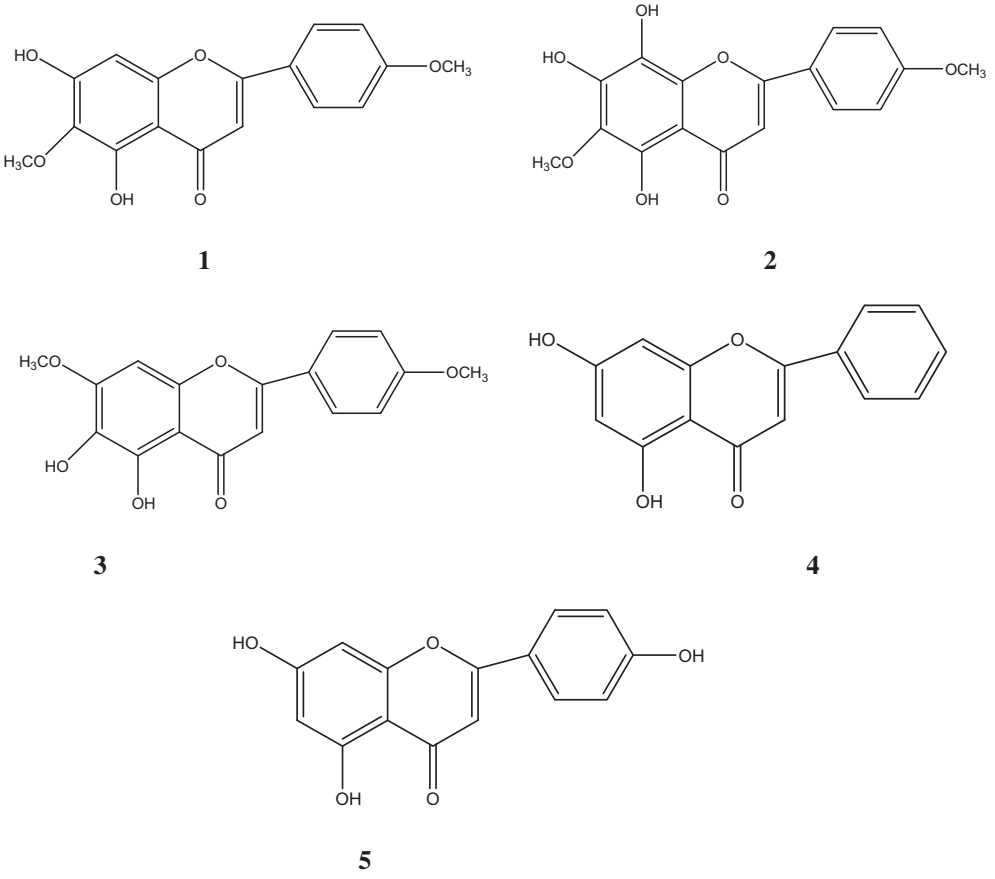

Figure 1 Chemical structures of compounds **1**–**5** from Algerian propolis.

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Compound **1** appeared purple on TLC. Methanol spectra of **1** showed two major absorption peaks at 321 and 267 nm. The peaks are characteristics of flavones skeleton. The UV spectral behavior with diagnostic reagents indicated the presence of free 5 and 7 hydroxyl groups. ^{13}C NMR spectrum showed 17 carbon signals including two methoxy carbons (55.80 and 60.40 ppm), 14 aromatic carbons and a carbonyl carbon (182.60 ppm), indicative of flavones structure of compound **1**.

EI–MS spectrum showed *m/z* 314 as base ion and fragment ions of 167 and 133, which could be obtained by **retro Diels–Alder reaction** (rDA) Lee et al., 1994 of a flavone with one methoxyl group and two hydroxyl groups on A ring (*m/z* 167) and one methoxyl group on B ring (*m/z* 133).

The ^1H NMR spectrum of **1** showed an A_2X_2 system at δ 7.08 and 8 ppm corresponding to 7.08 (2H, d, J = 8.9 Hz, H-3′/H-5′) and H-2′, H-6′ (δ = 8 ppm, J = 8.9 Hz), two singlets of aromatic protons (6.59 and 6.83 ppm) that could be assigned for the two protons of C-8 and C-3 of a flavone compound and two hydroxyl groups protons (10.67 and 13.01 ppm). The latter proton is assignable as hydroxyl group with a hydrogen bond and could be assigned as a hydroxyl group on C-5 position of the flavone compounds. In order to clarify the position of the methoxyl groups and to assign all the chemical shifts of the carbon, we carried out HSQC and HMBC experiments. The chemical shifts of all the protonated carbons were assigned firmly based on the cross peaks found in HSQC. Based on C–H long range correlation found in the HMBC experiment, all the carbons were assigned as shown in Fig. 2.

The HSQC and HMBC correlation between C10-H8, C6-H8 and C6- the methoxyl protons and between C7-H8 and C9-H8 assigned the substitution on C-6 by the methoxyl group (δ_H = 3.84 ppm, δ_C = 60.4 ppm). The substitution on C-4′ by the second methoxy group (δ_H = 3.75 ppm, δ_C = 60.8 ppm) was deduced by the HMBC and HSQC spectrums, which showed the correlation between C4′-H3′ and C4′-methoxyl protons.

Compound **1** was identified as 5,7-dihydroxy-6,4′-dimethoxyflavone (pectolinarigenin) (Imre et al., 1977).

3.2. Compound **2**

UV (λ_{max} in MeOH): gives bands at 321 and 267 nm for band I and II, addition of NaOH: 385, 329, 267; and $AlCl_3$: 338, 299; and HCl: 340, 299; and NaOAc: 331, 272; while H_3BO_3: 331, 272. Mass spectrum EI/MS *m/z* (rel. int): 330 $[M]^+$ (100), 316 $[M\text{-}Me+H]^+$ (46), 287 [M-Me−CO] (90), 199 $[A_1+H]^+$ (50), 155 [A_1-CO-Me] (20), 133 $[B_1]^+$ (32).

^1H NMR spectrum (300 MHz, DMSO-d_6), δ (ppm): δ 12.8 (1H, s, 5-OH), 8.02 (2H, d, J = 8.9 Hz, H-2′ and H-6′), 7.08 (2H, d, J = 8.9 Hz, H-3′ and H-5’), 6.83 (1H, s, H-3), 3.84 (3H, s, OMe), 3.75 (3H, s, OMe).

^{13}C NMR (75 MHz, DMSO-$_{d6}$), δ_c (ppm): 182.60 (C-4), 162.74 (C-2), 160.93 (C-4′), 153.18 (C-7), 151.9 (C-9), 146.84 (C-5), 136.7 (C-6), 128.7 (C-2′ and C-6′), 123.70 (C-1′), 115.02 (C-3′ and C-5′), 103.9 (C-10), 102.2 (C-3), 129.7 (C-8), 60.45 (6-OMe), 55.99 (4′-OMe) (Bankova et al., 2001).

EI–MS spectrum of **2** showed *m/z* 330 as base ion and fragment ions of 199 and 133 which could be obtained by **retro Diels–Alder reaction** (rDA) (Lee et al., 1994) of a flavone with one methoxyl group and three hydroxyl groups on A ring (*m/z* 199) and one methoxyl group on B ring (*m/z* 133).

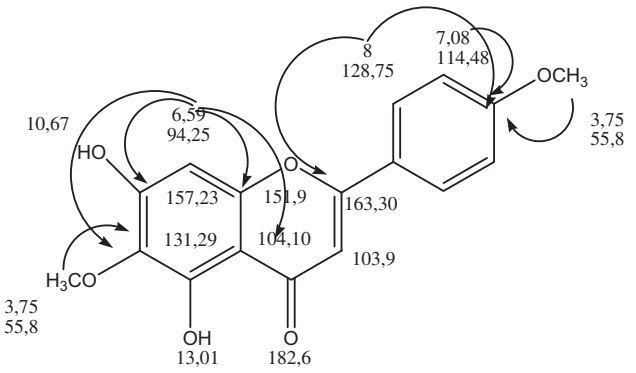

Figure 2 C–H long range correlation found in HMBC.

The ^1H NMR and ^{13}C NMR spectra of 2 showed similar signals of compound **1** except for the absence of the H-8 signal and the presence of the signal at δ = 129.7 ppm, that confirmed the substitution of hydroxyl at C-8. **Compound 2** was identified as **5, 7, 8-trihydroxy-6,4′-dimethoxyflavone (pilosin)** (Christine et al., 2004).

3.3. Compound **3**, $C_{17}H_{14}O_6$; mp 215–217 °C

UV (λ_{max} in MeOH): gives bands at 331 and 277 nm for band I and II, addition of NaOH: 363, 275; and $AlCl_3$: 351, 294, 261; and HCl: 352, 301, 260; and NaOAc: 365, 276; while H_3BO_3: 334, 278. Mass spectrum EI/MS *m/z* (**rel. int**): 314 $[M]^+$ (100), 296 $[M\text{-}H_2O]$ (84), 268 $[M\text{-}H_2O\text{-}CO]$ (24), 182 $[A_1]^+$ (15), 152 $[A_1\text{-}OMe+H]^+$ (23), 139 [153-Me+H] (15), 133 $[B_1]^+$ (33).

^1H NMR spectrum (250 MHz, $CDCl_3$), δ (ppm): 12.88 (1H, s, 5-OH), 10.94 (1H, s, 6-OH), 8.03 (2H,d, J = 8.9 Hz, H-2′ and H-6′), 7.10 (2H, d, J = 9 Hz, H-3′ and H-5’), 6.91(1H, s, H-3), 6.8 (1H, s, H-8), 3.90 (3H, s, OMe), 3.84 (3H, s, OMe).

^{13}C NMR (62.5 MHz, DMSO-$_{d6}$), δ_c (ppm): 182.54 (C-4), 163.83 (C-2), 162.70 (C-4’), 157.73 (C-7), 152.84 (C-9), 151.88 (C-5), 131.78 (C-6), 128.69 (C-2′ and C-6′), 123.19 (C-1′), 114.98 (C-3′ and C-5′), 104.53 (C-10), 103.38 (C-3), 94.72 (C-8), 60.40 (7-OMe), 55.94 (4′-OMe).

The comparison of EI–MS spectrum of **3** with those of **1** showed the same spectrum. The UV absorption maxima of **3** in MeOH at 331 and 277 nm were typical of flavonoid derivatives. Addition of NaOAc caused a shift in the UV absorption maxima of 49 nm on band I with a decrease in intensity of absorption, confirming the substitution of C-4′ by the methoxyl group. The absence of bathochromic shift of band II in the NaOAc spectrum indicates the absence of free 7-hydroxyl group, this suggest the substitution of C-7 by the second methoxyl group. The addition of acid in $AlCl_3$ containing flavonoid solution produced a bathochromic shift of 21 nm in band I indicating the presence of free hydroxyl group on C-5 and a substitution on C-6. The chemical shift of C-6 (δ_c = 131.78 ppm) confirmed the presence of hydroxyl group on C-6. Compound **3** was identified as **6,7-dihydroxy-7,4′-dimethoxyflavone (ladanein)** (Toth et al., 2007).

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3.4. Compound **4**, $C_{15}H_{10}O_4$; mp 258–268 °C

UV (λ_{max} in MeOH): gives bands at 315 and 269 nm for band I and II; addition of NaOH: 368, 277 and $AlCl_3$: 384, 330, 280, 252; and HCl: 390, 328, 280, 251; and NaOAc: 354, 273; wile H_3BO_3: 320, 269. Mass spectrum EI/MS *m/z* (rel. int): 254 $[M]^+$ (100), 226 [M-CO] (21), 152 $[A_1]^+$ (15), 124 $[A_1\text{-CO}]^+$ (10), 102 $[B_1]^+$ (17).

^{1}H NMR spectrum (250 MHz, DMSO-$_{d6}$), δ (ppm): δ 12.81 (1H, s, 5-OH), 10.92 (1H, s, 7-OH), 8.05 (2H, dd, J = 7.6 and 1.6 Hz, H-2′ and H-6'), 7.57 (3H, m, H-3′, H-4′ and H-5′), 6.93 (1H, s, H-3), 6.50 (1H, d, J = 2.09 Hz, H-8), 6.20 (1H, d, J = 2.09 Hz, H-6) (Chen et al., 2003; Toth et al., 2007).

^{13}C NMR (62,5 MHz, DMSO-$_{d6}$), δ (ppm): 182.31 (C-4), 164.9 (C-7), 163.61 (C-2),161.88 (C-5), 157.9 (C-9), 132.47 (C-4′), 131.13 (C-1′), 129.58 (C-3′ and C-5′), 126.85 (C-2′ and C-6′), 105.10 (C-10), 104.38 (C-3), 99.57 (C-6), 94.57 (C-8).

The UV spectral data with diagnostic reagents indicated the presence of free 5 and 7 hydroxyl groups. The mass spectrum of **4** showed a base ion at *m/z* 254 suggesting the absence of methoxyl group which is confirmed by the ^{1}H NMR and ^{13}C NMR spectra. Compound **4** was identified as **5,7-dihydroxyflavone (chrysin)** (Chen et al., 2003).

3.5. Compound **5**, $C_{15}H_{10}O_5$

UV (λ_{max} in MeOH): gives bands at 333 and 268 nm for band I and II; addition of NaOH: 392, 326, 275; and $AlCl_3$: 382, 347, 302, 275; and HCl: 381, 341, 301, 277; and NaOAc: 387, 308, 275; wile H_3BO_3: 339, 270. Mass spectrum EI/MS *m/z* (rel. int): 270 $[M]^+$ (100), 242 [M-CO] (40), 152 $[A_1]^+$ (75), 124 $[A_1\text{-CO}]^+$ (50), 118 $[B_1]^+$ (50).

^{1}H NMR spectrum (250 MHz, DMSO-$_{d6}$), δ (ppm): δ 13.01 (1H, s, 5-OH), 7.08 (2H, d, J = 8.4 Hz, H-2′ and H-6'), 6.9 (2H, d, J = 9 Hz, H-3'and H-5′), 6.58 (1H, s, H-3), 6.4 (1H, d, J = 1.26 Hz, H-8), 6.20 (1H, d, J = 1.26HZ, H-6) (Maisashvili et al., 2009; Lee et al., 1994). Compound **5** was identified as **5,7,4′-trihydroxyflavone (apigenin)** (Nagao et al., 2002).

All compounds are isolated from Algerian propolis for the first time. Moreover, compounds **1** and **3** were identified from propolis for the first time.

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