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ORIGINAL ARTICLE

New diacyl flavonoid derivatives from the Egyptian plant *Blepharis edulis* (Forssk.) Pers.

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Abstract In continuation of our phytochemical investigation of *Blepharis edulis* (Forssk.) Pers., family Acanthaceae; two new diacyl flavone derivatives: apigenin-7-*O*-(2″-acetyl-6″-*E*-*p*-coumaroyl-*β*-D-glucopyranoside) (**1**) and apigenin-7-*O*-(4″-acetyl-6″-*E*-*p*-coumaroyl-*β*-D-glucopyranoside) (**2**), along with eight known compounds: apigenin-7-*O*-(3″-acetyl-6″-*E*-*p*-coumaroyl-*β*-D-glucopyranoside) (**3**), naringenin-7-*O*-(3″-acetyl-6″-*E*-*p*-coumaroyl-*β*-D-glucopyranoside) (**4**), apigenin-7-*O*-(6″-*E*-*p*-coumaroyl-*β*-D-glucopyranoside) (**5**), naringenin-7-*O*-(6″-*E*-*p*-coumaroyl-*β*-D-glucopyranoside) (**6**), acacetin (**7**), naringenin (**8**), vanillic acid (**9**) and 4-hydroxybenzoic acid (**10**) were isolated and unambiguously identified from the ether fraction of the total extract of the wild Egyptian species *B. edulis* (Forssk.), family Acanthaceae. Identification and structure elucidation of the isolated compounds were carried out and confirmed through full data obtained from different 1D and 2D NMR experiments and mass spectral analyses. Compounds **1**–**9** were isolated for the first time from the species. Compounds **1** and **2** are new natural products isolated herein for the first time in nature.

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1. Introduction

Blepharis (Acanthaceae) is an Afro-asiatic genus comprising 129 species which occurs in arid and semi-arid habitats.[1] Some of the species belonging to *Blepharis* have been previously investigated from different points of view including the pharmacological and phytochemical studies.[2–4] *Blepharis edulis* Pers. is traditionally used for gastrointestinal, respiratory and inflammatory disorders.[5] It is used in folk medicine to treat asthma, cough, fever, inflammation of throat. It is applied locally to heal fastering wounds and ulcers. It is appetizer, astringent to bowels.[5] The leaves and seeds are reported to be eaten. The herb forms a good fodder for sheep and camels. It has been identified as Uchchata – aphrodisiac drug in ayurvedic. The leaves commonly sold in Indian market, are reported to be useful in wounds, ulcers, nasal hemorrhages, asthma, throat inflammation, purgative, disorders of liver and spleen.[6] The root is considered diuretic and beneficial in urinary discharges and dysmenorrhea.[6] The seeds are considered to be diuretic, expectorant, deobstruent and useful in strangury and conjunctivitis.[7] *B. edulis* seeds are used as food to increase sperm count and as aphrodisiac.[7] Although *B. edulis* was subjected to many biological investigations, the few

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chemical investigations of *B. edulis* result in the identification of benzoxazolone,[8] blepharin,[8] and the presence of galactose and fructose.[9] Other research workers found dl allantoin, saponin and catechol tannin.[7] In our previous research four phenylethanoid derivatives were isolated and identified from Egyptian plant *B. edulis* Pers. and antioxidant effects of different plant extracts were also measured.[10] The ether fraction of the plant extract showed antioxidant activity more than other fractions.[10] In the present paper we report isolation and unambiguous structure elucidation of 10 phenolic compounds from the ether fraction of *B. edulis* extract. Compounds **1**–**9** were isolated herein for the first time from the species. Compounds **1** and **2** are new natural products isolated for the first time in nature (see Fig. 1).

2. Materials and methods

2.1. Plant material

The arial parts of the plant *B. edulis* (Acanthaceae) were collected in 2002 from Sinai district near Mount Catherine also known as Gebel Katherina, located in Saint Katherine city, in Southern provinces of Sinai Peninsula in Egypt and identified by Prof. Dr. Batanouny, K.H., Department of Botany, Faculty of Science; Cairo University, Egypt. Corresponding specimens deposited at the herbarium of the pharmacognosy department, Faculty of Pharmacy, Al-Azhar University.

2.2. General procedures

1D NMR spectra including ^{1}H NMR, ^{13}C NMR, distortionless enhancement by polarization transfer (DEPT) and 2D NMR spectra including correlation spectroscopy (COSY), heteronuclear multiple quantum coherence (HMQC) and heteronuclear multiple bond connectivity, (HMBC) were recorded for the isolated compounds using deuterated dimethylsulfoxide (DMSO-d_6) and deuterated methanol (CD_3-OD) as NMR solvent, and tetramethylsilane (TMS) as internal standard, on a Bruker ARX 500 NMR spectrometer. ESI-MS spectra recorded on Finningan MAT TSQ-7000 mass spectrometer coupled with HPLC. MALDI-MS spectra were obtained on a Perseptive Biosystems Voyager-DE PRO MALDI-TOF mass spectrometer and on a Bruker Esquire 2000 LC–MS system equipped with an electrospray source. Sample application for MALDI-MS was carried out directly on sample plates with a mixture of 1 μL of matrix (saturated 2,5-dihydroxybenzoic acid in 50% acetonitrile, 0.3%TFA) and 1 μL of a 50% MeOH solution containing about 0.2 μg of the sample, (these MALDI-MS were carried out for confirmation of the molecular weight of the new compounds **1** and **2**). UV spectra were measured in methanol on a Perkin–Elmer UV/vis lambda spectrophotometer. Solvents were distilled prior to use, and spectral grade solvents were used for spectroscopic measurements. TLC was performed on TLC plates pre-coated with silica gel F254 (Merck, Darmstadt, Germany). For HPLC analysis, samples were injected into an HPLC system with a photodiode array detector (Dionex, Munich, Germany). The separation column (125 × 4 mm, i.d.) was prefilled with C18 (Knauer, Berlin, Germany). The plotting of the peaks was guided by UV–vis photodiode array detector operating in four different wave lengths 235, 254, 280 and 340 nm. The solvent gradients used started with 10:90 [MeOH 100%: nanopure H_2O (adjusted to pH 2 with *O*-phosphoric acid)], the commencing methanol ratio was equilibrated for 5 min at 10%, then gradiently increased up to 100% after 35 min., then washing with 100% MeOH for additional 10 min. The flow rate of the mobile phase was at 1 ml/min. Semipreparative HPLC was performed on the HPLC system (Merck, Darmstadt, Germany) coupled with UV detector L7400 (UV detection at 235 nm), the separation column (250 × 8 mm) prepacked with Eurosphere C18 (Knauer, Berlin, Germany).

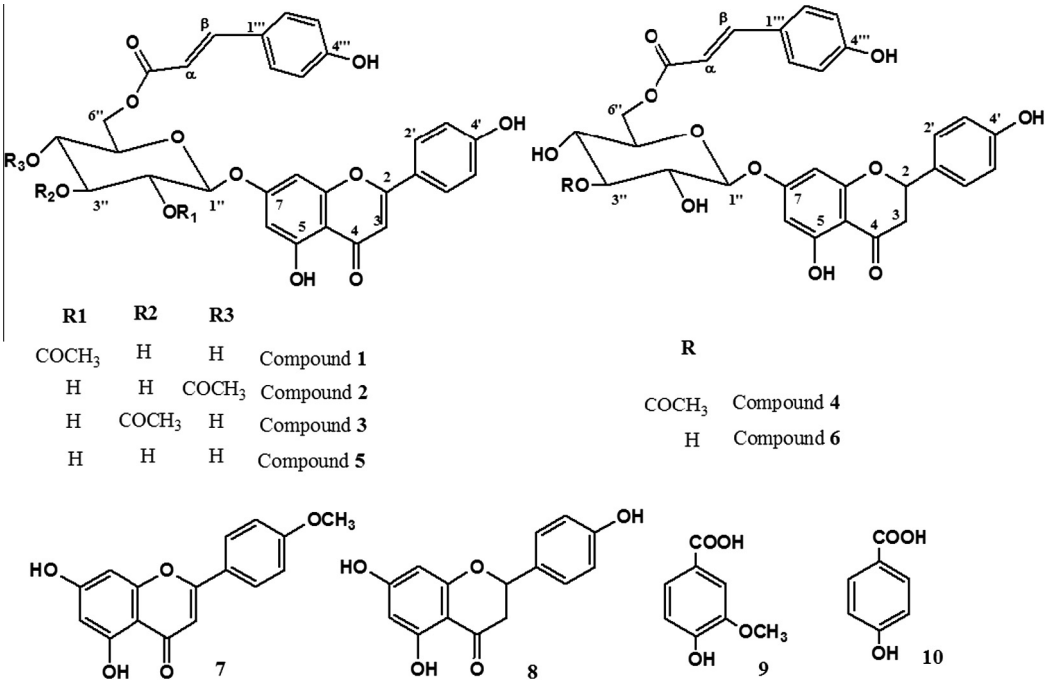

Figure 1 Chemical structures of compounds (**1**–**10**).

The compounds were eluted with the solvent system of MeOH/H_2O, at a flow rate of 5 ml/min.

2.3. Extraction and isolation procedure

The aerial part (500 g) was extracted with 70% cold methanol. The total alcohol extract (47 g) was subjected to vacuum liquid chromatography using silica gel (Si gel) for column, where petroleum ether (7.5 g), diethylether (6.08 g), ethylacetate (4.3 g), methanol (10.9 g) and aqueous (14.3 g) fractions were obtained. Diethylether subfraction (6.08 g), was chromatographed using Si gel for column and n-hexane:ethylacetate (EtOAc) as mobile phase in different ratios [100:0–50:50] where 250 fractions (10 ml each) were obtained [fraction Be1–250 respectively]. All fractions and subfractions were monitored by analytical HPLC. Fractions Be206–211 were pulled together and concentrated where a white amorphous substance was precipitated out, the supernatant was subjected to semipreparative HPLC separation using RP column and MeOH–H_2O as a mobile gradient system where compounds **5** (22.7 mg), **3** (25.0 mg), and mixture of **1** and **2** were obtained respectively. The mixture containing compounds **1** and **2** was rechromatographed using semipreparative HPLC column again where compound **2** (4.5 mg) and **1** (8.9 mg) were obtained. The precipitated substance was purified using Si gel column to afford compound **3** (370.6 mg). Fractions Be201–205 were pulled together, concentrated where yellowish white substance was precipitated out, this substance was collected and purified to afford compound **4** (341.0 mg), the supernatant was subjected to semipreparative HPLC separation using RP column and MeOH–H_2O as mobile gradient system where compounds **6** (14.6 mg) and **4** (15.2 mg) were obtained respectively. Fraction Be111–127 (obtained, from the ether fraction, by mobile phase n-hexane: ethylacetate 9:1) were pulled together and concentrated, where a colorless substance was crystallized out; the mother liquor was further chromatographed using Si gel for column and (n-hexane: ethylacetate 9:1) as mobile phase where 20 fractions were obtained [fraction Be127-1 to Be127-20]; from which fraction Be127-6 was rechromatographed to afford subfractions Be127-6-1 to Be127-6-28. Fraction Be127-6-12 was further purified using semipreparative HPLC column using reversed phase (RP) Si gel to afford compound **7** (11.2 mg), while Fraction Be127-6-4 was treated similarly to afford compound **8** (17.1 mg). The white substance obtained after concentration of fractions Be111–127 was re-crystallized from hot methanol to afford compound **9** (25.2 mg). Fraction Be127-6-2 was purified using column chromatography packed with reversed phase (RP) Si gel to afford compound **10** (10.6 mg). The isolated compounds (**1–10**) were monitored by analytical HPLC and the characteristic UV spectra.

2.4. Acid hydrolysis of the glycosides and identification of the sugar moieties

Compounds **1**–**6** (1 mg each) were dissolved separately in 1/2 NaOH (5 ml), refluxed for 1 h, the deacylated flavonoid glucosides were then refluxed with 1/2 N HCl (5 ml) for 2 h. and the sugar of each sample was identified through TLC comparison with authentic sugar samples on pre-coated Si gel (developing solvent CH_2Cl_2:MeOH:H_2O, 10:5:1) where all sample sugars showed the same *Rf* value as β-D-glucose (*Rf* 0.47).

3. Results

3.1. Compound *1*

It was isolated as yellowish white amorphous powder, (8.9 mg). It has HPLC R_t 29.49 min. UV spectrum showed absorption maxima at λ_{max} MeOH: 207.0, 269.1 and 317.7 nm. ESI-MS *m/z*: 643 (45%) $[M+Na]^+$, 621(100%) $[M+H]^+$, 271(40%) $[aglycone+H]^+$. **^{1}H NMR** (DMSO-d_6, 500 MHz) chemical shifts for **1** were: Aglycone: 6.84 (1H, s, H-3), 6.43 (1H, d, J = 2.2 Hz, H-6), 6.76 (1H, d, J = 2.2 Hz, H-8), 7.95 (2H, d, J = 8.8 Hz, H-2′and H-6′), 6.91 (2H, d, J = 8.8 Hz, H-3′ and H-5′); Glucosyl moiety: 5.47 (1H, d, J = 8.2 Hz, H-1″), 4.85 (1H, dd, J = 8.2, 9.5 Hz, H-2″), 3.56 (1H, t, J = 9.5 Hz, H-3″), 3.37 (1H, t, J = 9.5 Hz, H-4″), 3.93 (1H, ddd, J = 1.9, 7.3, 9.3 Hz, H-5″), 4.51 (1H, dd, J = 1.9, 12.0 Hz, H-6″a), 4.17 (1H, dd, J = 7.3, 12.0 Hz, H-6″b); Coumaroyl moiety: 6.35 (1H, d, J = 15.7 Hz, H-α), 7.49 (1H, d, J = 15.7 Hz, H-β), 7.40 (2H, d, J = 8.5 Hz, H-2‴ and H-6‴), 6.68 (2H, d, J = 8.5 Hz, H-3‴ and H-5‴); Acetyl moiety: 2.04 (3H, s, CH_3COO). **^{13}C NMR** (DMSO-d_6, 125 MHz) chemical shifts for **1**: Aglycone: 161.9 (C-2), 103.0 (C-3), 182.0 (C-4), 162.0 (C-5), 99.2 (C-6), 164.3 (C-7), 94.9 (C-8), 156.8 (C-9), 105.6 (C-10), 120.9 (C-1′), 130.1 (C-2′), 115.6 (C-3′), 159.8 (C-4′), 115.6 (C-5′), 130.1 (C-6′). Glucosyl moiety: 96.9 (C-1″), 73.0 (C-2″), 73.2 (C-3″), 69.9 (C-4″), 74.2 (C-5″), 63.1 (C-6″). Coumaroyl moiety: 166.4 (C═O), 113.7 (α-CH), 145.1 (β-CH), 124.9 (C-1‴), 130.1 (C-2‴), 115.6(C-3‴), 161.3 (C-4‴), 115.6(C-5‴), 130.1 (C-6‴). Acetyl group: 20.9 (CH_3), 169.3 (COO).

3.2. Compound *2*

It was isolated as yellowish white amorphous powder, (4.5 mg). Its HPLC R_t 29.18 min. UV spectrum showed absorption maxima at λ_{max} MeOH: 206.2, 268.9 and 318.3 nm. ESI-MS *m/z*: 643 (65%) $[M+Na]^+$, 621 (100%) $[M+H]^+$, 271 (40%) $[Aglycone+H]^+$. **^{1}H NMR** (DMSO-d_6, 500 MHz) chemical shifts for **2** were: Aglycone: 6.84 (1H, s, H-3), 6.49 (1H, d, J = 1.9 Hz, H-6), 6.82 (1H, d, J = 1.9 Hz, H-8), 7.95 (2H, d, J = 8.8 Hz, H-2′ and H-6′), 6.92 (2H, d, J = 8.8 Hz, H-3′ and H-5′); Glucosyl moiety: 5.28 (1H, d, J = 7.9 Hz, H-1″), 3.42 (1H, dd, J = 8.8, 7.9 Hz, H-2″), 3.58 (1H, dd, J = 8.8, 9.5 Hz, H-3″), 4.73 (1H, t, J = 9.5 Hz, H-4″), 4.17 (1H, m, H-5″), 4.18 (1H, m, H-6″a), 4.16 (1H, m, H-6″b); Coumaroyl moiety: 6.30 (1H, d, J = 16.1 Hz, H-α), 7.47 (1H, d, J = 16.1 Hz, H-β), 7.38 (2H, d, J = 8.5 Hz, H-2‴ and H-6‴), 6.68 (2H, d, J = 8.5 Hz, H-3‴ and H-5‴); Acetyl moiety: 2.1 (3H, s, CH_3COO). [m: means multiplicities of more than one proton signal are overlapped in the same chemical shift and not recognized]. **^{13}C NMR** (DMSO-d_6, 125 MHz) chemical shifts for **2**: Aglycone: 161.4 (C-2), 103.0 (C-3), 182.0 (C-4), 162.5 (C-5), 99.4 (C-6), 164.25 (C-7), 94.7 (C-8), 156.9 (C-9), 105.4 (C-10), 121.0 (C-1′), 128.6 (C-2′), 115.9 (C-3′), 159.9 (C-4′), 115.9 (C-5′), 128.6 (C-6′). Glucosyl moiety: 99.1 (C-1″), 72.5 (C-2″), 73.3 (C-3″), 70.5 (C-4″), 71.2 (C-5″), 62.3 (C-6″). Coumaroyl moiety: 166.2 (C═O), 113.5 (α-CH), 145.0 (β-CH), 124.8 (C-1‴), 128.6 (C-2‴), 115.9 (C-3‴), 161.2 (C-4‴), 115.9 (C-5‴), 128.6 (C-6‴). Acetyl group: 20.8 (CH_3), 169.8 (COO).

3.3. Compound 3

Apigenin-7-*O*-(3″-acetyl-6″-*E*-*p*-coumaroyl-*β*-D-glucopyranoside): It was isolated as white amorphous powder, (396.0 mg). HPLC R_t 28.13 min. UV spectrum showed absorption maxima at λ_{max} MeOH: 206.9, 269.3 and 317.6 nm. ESI-MS *m/z*: 643(40%) $[M+Na]^+$, 621 (100%) $[M+H]^+$, 271 (25%) $[Aglycone+H]^+$. **^{1}H NMR** (DMSO-d_6, 500 MHz) chemical shifts for **3** were: Aglycone: 6.83 (1H, s, H-3), 6.50 (1H, d, J = 1.9 Hz, H-6), 6.84 (1H, d, J = 1.9 Hz, H-8), 7.93 (2H, d, J = 8.8 Hz, H-2′ and H-6′), 6.93 (2H, d, J = 8.8 Hz, H-3′ and H-5′); Glucosyl moiety: 5.32 (1H, d, J = 7.9 Hz, H-1″), 3.51 (1H, m, H-2″), 4.96 (1H, t, J = 9.5 Hz, H-3″), 3.52 (1H, t, J = 9.5 Hz, H-4″), 3.98 (1H, m, H-5″), 4.44 (1H, dd, J = 1.9, 12.0 Hz, H-6″a), 4.20 (1H, dd, J = 6.6, 12.0 Hz, H-6″b); Coumaroyl moiety: 6.35 (1H, d, J = 16.1 Hz, H-α), 7.5 (1H, d, J = 16.1 Hz, H-*β*), 7.37 (2H, d, J = 8.5 Hz, H-2‴ and H-6‴), 6.66 (2H, d, J = 8.5 Hz, H-3‴ and H-5‴); Acetyl moiety: 2.1(3H, s, CH_3COO). **^{13}C NMR** (DMSO-d_6, 125 MHz) chemical shifts for **3**: Aglycone: 159.9 (C-2), 94.8 (C-3), 182.0 (C-4), 162.4 (C-5), 99.5 (C-6), 164.2 (C-7), 103.1 (C-8), 156.9 (C-9), 105.5 (C-10), 121.0 (C-1′), 128.6 (C-2′), 116.0 (C-3′), 161.2 (C-4′), 116.0 (C-5′), 128.6 (C-6′). Glucosyl moiety: 99.0 (C-1″), 67.9 (C-2″), 77.2 (C-3″), 70.9 (C-4″), 73.5 (C-5″), 63.0 (C-6″). Coumaroyl moiety: 166.5 (C═O), 113.7 (α-CH), 145.1 (*β*-CH), 124.9 (C-1‴), 130.2 (C-2‴), 115.7 (C-3‴), 161.4 (C-4‴), 115.7 (C-5‴), 130.2 (C-6‴). Acetyl group: 21.2 (CH_3), 169.9 (COO). Compound **3** was isolated previously from allied species *Blepharis ciliaris* growing in Saudia Arabia[11] and Egypt,[12] but it is the first time for isolation and identification of **3** from *B. edulis*.

3.4. Compound 4

Naringenin-7-*O*-(3″-acetyl-6″-*E*-*p*-coumaroyl-*β*-D-glucopyranoside): It was isolated as yellowish white amorphous precipitate, (356.2 mg). HPLC R_t 26.81 min. UV spectrum showed absorption maxima at λ_{max} MeOH: 212.7, 225.8, 284.9 and 319.7 nm (sh). ESI-MS *m/z*: 645 (65%) $[M+Na]^+$, 623 (100%) $[M+H]^+$, 273 (20%) $[Aglycone+H]^+$. **^{1}H NMR** (CD_3OD, 500 MHz) chemical shifts for **4** were: Aglycone: 5.17 (1H, dd, J = 3.0, 12.6 Hz, H-2), 2.67 (1H, dd, J = 3.0, 17.0 Hz, H-3a), 3.05 (1H, dd, J = 17.0, 12.6 Hz, H-3b), 6.18 (1H, d, J = 2.2 Hz, H-6), 6.6 (1H, d, J = 2.2 Hz, H-8), 7.2 (2H, d, J = 8.5 Hz, H-2′ and H-6′), 6.82 (2H, d, J = 8.5 Hz, H-3′ and H-5′); Glucosyl moiety: 5.06 (1H, d, J = 7.8 Hz, H-1″), 3.46 (1H, dd, J = 7.8, 9.5 Hz, H-2″), 4.97 (1H, t, J = 9.5 Hz, H-3″), 3.56 (1H, t, J = 9.5 Hz, H-4″), 3.83 (1H, m, H-5″), 4.51 (1H, dd, J = 1.9, 12.0 Hz, H-6″a), 4.28 (1H, dd, J = 6.9, 12.0 Hz, H-6″b); Coumaroyl moiety: 6.28 (1H, d, J = 16.1 Hz, H-α), 7.54 (1H, d, J = 16.1 Hz, H-*β*), 7.36 (2H, d, J = 8.5 Hz, H-2‴and H-6‴), 6.76 (2H, d, J = 8.5 Hz, H-3‴ and H-5‴); Acetyl moiety: 2.1 (3H, s, CH_3COO). **^{13}C NMR** (CD_3OD, 125 MHz) chemical shifts for **4**: Aglycone 80.9 (C-2), 44.5 (C-3), 198.9 (C-4), 164.8(C-5), 98.3 (C-6), 167.0 (C-7), 97.5 (C-8), 165.4 (C-9), 105.4 (C-10), 131.2 (C-1′), 129.4 (C-2′), 116.7 (C-3′), 159.4 (C-4′), 116.7 (C-5′), 129.4 (C-6′). Glucosyl moiety: 101.2 (C-1″), 70.5 (C-2″), 78.9 (C-3″), 73.3 (C-4″), 75.8 (C-5″), 64.8 (C-6″). Coumaroyl moiety: 169.4 (C═O), 115.3 (α-CH), 147.4 (*β*-CH), 127.6 (C-1‴), 131.7 (C-2‴), 117.2 (C-3‴), 161.8 (C-4‴), 117.2 (C-5‴), 131.7 (C-6‴). Acetyl group: 21.5 (CH_3), 172.9 (COO). Compound **4** was isolated previously from *Blepharis ciliaris*.[11]

3.5. Compound 5

Apigenin-7-*O*-(6″-*E*-*p*-coumaroyl-*β*-D-glucopyranoside): It was isolated as yellowish white amorphous solid, (22.7 mg). HPLC R_t 27.74 min. UV spectrum showed absorption maxima at λ_{max} MeOH: 206.6, 269.0 (sh) and 317.8 nm. ESI-MS *m/z*: 601 (40%) $[M+Na]^+$, 579 (100%) $[M+H]^+$, 271 (40%) $[Aglycone+H]^+$. **^{1}H NMR** (500 MHz, DMSO-d_6) chemical shifts for **5** were: Aglycone: 6.81 (1H, s, H-3), 6.45 (1H, d, J = 2.2 Hz, H-6), 6.81 (1H, d, J = 2.2 Hz, H-8), 7.91 (2H, d, J = 8.8 Hz, H-2′ and H-6′), 6.91 (2H, d, J = 8.8 Hz, H-3′ and H-5′); Glucosyl moiety: 5.14 (1H, d, J = 7.5 Hz, H-1″), 3.3 (1H, m, H-2″), 3.3 (1H, m, H-3″), 3.23 (1H, t, J = 9.2 Hz, H-4″), 3.8 (1H, m, H-5″), 4.45 (1H, dd, J = 1.9, 12.0 Hz, H-6″a), 4.15 (1H, dd, J = 6.9, 12.0 Hz, H-6″b); Coumaroyl moiety: 6.31 (1H, d, J = 16.1 Hz, H-α), 7.5 (1H, d, J = 16.1 Hz, H-*β*), 7.35 (2H, d, J = 8.5 Hz, H-2‴ and H-6‴), 6.68 (2H, d, J = 8.5 Hz, H-3‴ and H-5‴). **^{13}C NMR** (DMSO-d_6, 125 MHz) chemical shifts for **5**: Aglycone 162.2 (C-2), 94.1 (C-3), 182.3 (C-4), 161.5 (C-5), 99.8 (C-6), 164.5 (C-7), 103.1 (C-8), 157.8 (C-9), 103.4 (C-10), 121.0 (C-1′), 127.6 (C-2′), 115.8 (C-3′), 161.2 (C-4′), 115.8 (C-5′), 127.6 (C-6′). Glucosyl moiety: 99.5 (C-1″), 75.2 (C-2″), 72.5 (C-3″), 68.1 (C-4″), 73.5 (C-5″), 64.3 (C-6″). Coumaroyl moiety: 167.0 (C═O), 113.8 (α-CH), 144. 5 (*β*-CH), 125.1 (C-1‴), 129.5 (C-2‴), 115.5(C-3‴), 159.5 (C-4‴), 115.5 (C-5‴), 129.5 (C-6‴). Compound **5** was previously isolated from *Blepharis ciliaris*[13] and other plants.[14,15]

3.6. Compound 6

Naringenin-7-*O*-(6″-*E*-*p*-coumaroyl-*β*-D-glucopyranoside): It was isolated as yellowish white amorphous solid, (14.6 mg). HPLC R_t 26.55 min. UV spectrum showed absorption maxima at λ_{max} MeOH: 202.4, 212.8, 225.8 and 263.8 nm (sh). ESI-MS *m/z*: 603 (65%) $[M+Na]^+$, 581 (100%) $[M+H]^+$, 273 (20%) $[Aglycone+H]^+$. **^{1}H NMR** (DMSO-d_6, 500 MHz) chemical shifts for **6** were: Aglycone: 5.45 (1H, dd, J = 2.7, 12.6 Hz, H-2), 2.72 (1H, dd, J = 2.7, 16.9 Hz, H-3a), 3.32 (1H, dd, J = 16.9, 12.6 Hz, H-3b), 6.15 (1H, d, J = 2.2 Hz, H-6), 6.20 (1H, d, J = 2.2 Hz, H-8), 7.30 (2H, d, J = 8.6 Hz, H-2′ and H-6′), 6.77 (2H, d, J = 8.6 Hz, H-3′ and H-5′); Glucosyl moiety: 5.07 (1H, d, J = 7.6 Hz, H-1″), 3.29 (1H, m, H-2″), 3.26 (1H, m, H-3″), 3.24 (1H, m, H-4″), 3.72 (1H, ddd, J = 1.9, 6.9, 8.8 Hz, H-5″), 4.42 (1H, dd, J = 1.9, 11.67 Hz, H-6″a), 4.15 (1H, dd, J = 6.9, 11.67 Hz, H-6″b); Coumaroyl moiety: 6.37 (1H, d, J = 15.76 Hz, H-α), 7.53 (1H, d, J = 15.76 Hz, H-*β*), 7.51 (2H, d, J = 8.8 Hz, H-2‴and H-6‴), 6.81 (2H, d, J = 8.8 Hz, H-3‴ and H-5‴). **^{13}C NMR** (DMSO-d_6, 125 MHz) chemical shifts for **6**: Aglycone 78.6 (C-2), 41.9 (C-3), 197.2 (C-4), 163.0 (C-5), 95.5 (C-6), 165.0 (C-7), 96.3 (C-8), 162.6 (C-9), 103.3 (C-10), 128.4 (C-1′), 128.4 (C-2′), 115.2 (C-3′), 157.7 (C-4′), 115.2 (C-5′), 128.4 (C-6′). Glucosyl moiety: 99.2 (C-1″), 76.7 (C-2″), 72.9 (C-3″), 69.7 (C-4″), 73.8 (C-5″), 63.3 (C-6″). Coumaroyl moiety: 166.4 (C═O), 113.9 (α-CH), 144.9 (*β*-CH), 125.1 (C-1‴), 130.2 (C-2‴), 115.7 (C-3‴), 159.9 (C-4‴), 115.7 (C-5‴), 130.2 (C-6‴). It was also isolated previously from *Blepharis ciliaris*.[13]

3.7. Compound 7

Acacetin (5,7-dihydroxy-4′-methoxyflavones or 4′-*O*-methylapigenin)[16–18]: It was isolated as yellowish amorphous solid, (11.2 mg). HPLC R_t 29.94 min. UV spectrum showed absorption maxima at λ_{max} MeOH: 218.8, 268.2 and 331.8 nm. ESI-MS *m/z*: 285 (100%) $[M+H]^+$. **^{1}H NMR** (DMSO-d_6, 500 MHz) chemical shifts for **7** were: 6.68 (1H, s, H-3), 6.55 (1H, d, J = 1.9 Hz, H-6), 6.25 (1H, d, J = 1.9 Hz, H-8), 8.0 (2H, d, J = 8.8 Hz, H-2′ and H-6′), 7.12 (2H, d, J = 8.8 Hz, H-3′ and H-5′); 3.92 (1H, s, 4′-OCH_3).

3.8. Compound 8

Naringenin[19]: It was isolated as yellowish amorphous solid, (15.1 mg). HPLC R_t 24.28 min. UV spectrum showed absorption maxima at λ_{max} MeOH: 232.9 and 295.1 nm. ESI-MS *m/z*: 273 (100%) $[M+H]^+$. **^{1}H NMR** (DMSO-d_6, 500 MHz) chemical shifts for **8** were: 5.44 (1H, dd, J = 2.8, 12.9 Hz, H-2), 2.66 (1H, dd, J = 2.8, 17.3 Hz, H-3a), 3. 26 (1H, dd, J = 17.3, 12.9 Hz, H-3b), 5.87 (1H, d, J = 1.9 Hz, H-6), 5.88 (1H, d, J = 1.9 Hz, H-8), 7.31 (2H, d, J = 8.5 Hz, H-2′ and H-6′), 6.87 (2H, d, J = 8.5 Hz, H-3′ and H-5′), 9.58 (1H, s, 4′-OH), 10.78 (1H, s, 7-OH), 12.14 (1H, s, 5-OH). **^{13}C NMR** (DMSO-d_6, 125 MHz) chemical shifts for **8**: 78.2 (C-2), 42.3 (C-3), 196.3 (C-4), 162.6 (C-5), 96.1 (C-6), 164.1 (C-7), 94.5 (C-8), 158.3 (C-9), 103.2 (C-10), 128.8 (C-1′), 128.5 (C-2′), 115.2 (C-3′), 158.1 (C-4′), 115.2 (C-5′), 128.5 (C-6′).

3.9. Compound 9

Vanillic acid[20,21]: It was isolated as colorless crystals, (25.2 mg). HPLC R_t 15.59 min. UV spectrum showed absorption maxima at λ_{max} MeOH: 205.4, 218.9, 260.9 and 293.9 nm (sh). EI-MS *m/z*: 168 (100%) [M], 153 (62%), 125 (21%), 97 (29%), 93 (4%), 65 (6%). **^{1}H NMR** (DMSO-d_6, 500 MHz) chemical shifts for **9** were: 7.41 (1H, d, J = 1.9 Hz, H-2), 6.83 (1H, d, J = 8.2 Hz, H-5), 7.43 (1H, dd, J = 1.9 and 8.2 Hz, H-6), 3.8 (2H, s, 3-OCH_3), 9.9 (1H, s, 4-OH), 12.5 (1H, s, COOH). **^{13}C NMR** (DMSO-d_6, 125 MHz) chemical shifts for **9**: 121.6 (C-1), 115.0 (C-2), 151.1 (C-3), 147.2 (C-4), 112.7 (C-5), 123.5 (C-6), 167.2 (COOH), 55.53 (3-OCH_3).

3.10. Compound 10

p-Hydroxybenzoic acid: It was isolated as yellowish amorphous solid, (10.6 mg). HPLC R_t 14.03 min. UV spectrum showed absorption maxima at λ_{max} MeOH: 201.6, 207.5 and 255.3 nm. EI-MS *m/z*: 138 (81.4%) $[M]^+$, 122 (7.9%), 121 (100%), 93 (25.9%), 92 (2.8%), 65 (27.2%). It was identified previously in *B. edulis* extract[22].

4. Discussion

4.1. The isolated compounds

4.1.1. Compound 1

LC–ESI-MS spectrum showed a pseudomolecular ion peak at *m/z* 643 $[M+Na]^+$ which is consistent with the molecular formula $C_{32}H_{28}O_{13}Na$, and pseudomolecular ion peak at *m/z* 621 $[M+H]^+$ which is consistent with the molecular formula $C_{32}H_{29}O_{13}$. ESI-MS/MS (+ve mode) spectrum showed fragment ion peak at *m/z* 474.5 [M-coumaroyl moiety]$^+$ and *m/z* 271 [apigenin aglycone + H]$^+$, while ESI-MS/MS (−ve mode) spectrum showed fragment ion peaks at *m/z* 269 [apigenin aglycone-H]$^{-\cdot}$, *m/z* 473.5 [M−(coumaroyl moiety + H)]$^-$, *m/z* 577.1 [M−(acetyl group + H)]$^-$ in addition to fragment ion peak at *m/z* 619 $[M-H]^-$. This fragmentation pattern of **1** resemble identically that of previously known congener [Apigenin-7-*O*-(3″-acetyl-6″-*E*-*p*-coumaroyl-*β*-D-glucopyranoside)].[11,12] Chemical structure of **1** was determined by utilizing 1D NMR experiments [^{1}H NMR, ^{13}C NMR and DEPT spectra], as well as 2D NMR experiments including COSY, HMBC and HMQC spectra. ^{1}H NMR spectrum showed significant proton signals at δ_H 6.84 (1H, s, H-3), 6.43 (1H, d, J = 2.2 Hz, H-6), 6.76 (1H, d, J = 2.2 Hz, H-8), 7.95 (2H, d, J = 8.8 Hz, H-2′and H-6′) and 6.91 (2H, d, J = 8.8 Hz, H-3′ and H-5′) indicating an apigenin aglycone substructure. The chemical shift values of the hexose proton signals were obtained by follow up COSY correlations starting from the anomeric proton signal at δ_H 5.47 (1H, d, J = 8.2 Hz, H-1″), through signals at δ_H 4.85 (1H, dd, J = 8.2, 9.5 Hz, H-2″), 3.56 (1H, t, J = 9.5 Hz, H-3″), 3.37 (1H, t, J = 9.5 Hz, H-4″), 3.93 (1H, ddd, J = 1.9, 7.3, 9.3 Hz, H-5″) till methylene proton signals 6-Ha and 6-Hb at δ_H 4.51 (1H, dd, J = 1.9, 12.0 Hz, H-6″a) and 4.17 (1H, dd, J = 7.3, 12.0 Hz, H-6″b). The *β*-configuration of the sugar was established through the coupling constant of the anomeric signal (J = 8.2 Hz).[23] Downfield chemical shift values of the sugar proton signals at δ_H 4.85 (1H, dd, J = 8.2, 9.5 Hz, H-2″) and δ_H 4.51 (1H, dd, J = 1.9, 12.0 Hz, H-6″a), 4.17 (1H, dd, J = 7.3, 12.0 Hz, H-6″b) suggest acylation at positions 2″ and 6″ of the sugar moiety. These suggestions were confirmed by HMBC correlations as shown below. Proton signals at δ_H 6.35 (1H, d, J = 15.8 Hz, H-α), 7.49 (1H, d, J = 15.8 Hz, H-*β*), 7.40 (2H, d, J = 8.5 Hz, H-2‴and H-6‴) and 6.68 (2H, d, J = 8.5 Hz, H-3‴ and H-5‴) indicate *p*-coumaroyl moiety with an ″*E*″ configuration. While the methyl proton signal at δ_H 2.04 (3H, s, CH_3) indicates the acetyl moiety. Attachment of the coumaroyl moiety to the hexose was confirmed at position 6″ of the sugar as evident from HMBC correlation between the H-6″a at δ_H 4.51 and H-6″b at δ_H 4.17 to the coumaroyl carbonyl carbon resonating at 166.4 ppm. Also, attachment of the acetyl group to position 2″ of sugar was confirmed through HMBC correlation between the H-2″ at δ_H 4.85 to the carbonyl carbon resonating at 169.3 ppm. The hexose substructure was further confirmed as the *β*-D-glucopyranosyl moiety after acid hydrolysis and comparison with authentic sugar samples. Oxygenated quaternary aromatic carbon resonating at 164.3 ppm shows HMBC correlation with the anomeric proton at δ_H 5.47, and proton (H-6) at 6.43 ppm of ring B indicating the attachment of the sugar moiety to C-7 of apigenin moiety. Therefore compound **1** is unambiguously identified as apigenin-7-*O*-(2″-acetyl-6″-*E*-*p*-coumaroyl-*β*-D-glucopyranoside). To the best of our knowledge it is the first time for isolation and identification of **1** in nature.

4.1.2. Compound 2

Although LC–ESI-MS spectra (+ve and –ve modes) showed the same pseudomolecular ion peaks and main fragment ion

peaks as in case of **1**, The significant differences between compound **1** and **2** were the HPLC retention times (29.49 min for **1** and 29.22 min for **2**) indicating that both are not identical; this difference encouraged us to find out the structural differences. The COSY correlations between the proton signals of sugar moiety indicate a sequential difference from those of compound **1**. According to the correlations obtained from COSY spectrum of **2** the sugar proton signals resonate as a continuous spin system at δ_H 5.28 (1H, d, J = 7.9 Hz, H-1″), 3.42 (1H, dd, J = 8.8, 7.9 Hz, H-2″), 3.58 (1H, dd, J = 8.8, 9.5 Hz, H-3″), 4.73 (1H, t, J = 9.5 Hz, H-4″), 4.17 (1H, m, H-5″), 4.18 (1H,m, H-6″a), and 4.16 (1H, m, H-6″b). From these proton resonances, it is clear that H-4″ of the sugar moiety has more down field chemical shift than that of **1**, furthermore, the chemical shift value of H-2″ shows more upfield chemical shift than that of **1**; these differences confirm the attachment of an acyl group to C-4″ instead of C-2″. Attachment of the *E*-*p*-coumaroyl moiety to the hexose was confirmed at position 6″ of the sugar as evident from HMBC correlation between the H-6″a at δ_H 4.18 and H-6″b at δ_H 4.16 to the coumaroyl carbonyl carbon resonating at 166.2 ppm. Also, attachment of the acetyl group to position 4″ of sugar was confirmed through HMBC correlation between the H-4″ at δ_H 4.73 to the carbonyl carbon resonating at 169.8 ppm. After acid hydrolysis and comparison with authentic sugar samples, the hexose substructure was confirmed as the *β*-D-glucopyranosyl moiety. From the above interpretation, compound **2** is unambiguously identified as apigenin-7-*O*-(4″-acetyl-6″-*E*-*p*-coumaroyl-*β*-D-glucopyranoside). To the best of our knowledge it is also the first time for isolation and identification of this new compound in nature.

5. Conclusion

HPLC technology with hyphenated systems (e.g. LC-UV, LC–MS, LC-UV–MS, etc.) opens now a new era of natural product chemistry where the phytochemist can detect, characterize and analyze phytoconstituents directly on line, and with minimum effort and time can easily target and isolate natural product from the sample without tedious isolation of unwanted compounds. In the present publication 10 phenolic compounds were isolated, purified, and full NMR data were reported. The isolated compounds include 4 diacyl flavonoid derivatives, 2 monoacyl flavonoid derivatives, 2 flavonoid aglycones and 2 phenolic acids. Two diacyl flavonoid derivatives (**1** and **2**) were isolated herein for the first time in nature, while compounds **1**–**9** were isolated for the first time from *B. edulis*. Identification of these compounds may explain the positive biological effects of the plant extract and demonstrates from the phytochemical point of view the growing interest in this plant both in folk medicine and natural products research centers.[4–7,22,24]

6. Conflict of interest

No conflict of interest to declare.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bfopcu.2015.02.001.

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