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Abstract

A new sulfonyl flavonol glucoside, quercetin 6′-[propanoic acid-(2-acetoxy-3-sulfonyl)]-3′-*O*-β-D-glucopyranoside (**1**) was isolated from the aerial parts of *Cotula anthemoides* L. in addition to fifteen known compounds (**2-16**). The structure elucidation of these compounds was based on analyses of spectroscopic data including 1D-, 2D-NMR and HR-ESI-MS techniques and by comparing their NMR data with those reported in the literature. These compounds were evaluated for their DPPH radical scavenging and tyrosinase inhibitory activity. Compound **6** showed a high DPPH radical scavenging with EC_{50} value of 9.1 ± 0.4. Compound **11**, **9** and **1** exhibited a mild tyrosinase inhibitory activity with IC_{50} values of 85 ± 0.8, 95 ± 1.5 and 100 ± 0.5, respectively.

Keywords: *Cotula anthemoides*, Asteraceae, antioxidant activity, tyrosinase inhibitory activity.

1. Introduction

The genus *Cotula* (Asteraceae) consists of approximately 80 species, among them, three are distributed in Algeria (Ozenda, 1958). Plants of this genus are used in traditional medicine for their anti-inflammatory, analgesic, antiseptic properties (Jana et al., 1992) and as aromatic and digestive substance in tea (Bellakhdar, 1997). In addition, it has been reported as antipyretic (Larhsini et al., 2002), bacteriostatic (Jana et al., 1992), antiprotozoal (Markouk et al., 1999) and anticorrosive (Benmenine et al., 2011). Several flavonoids (Ahmed et al., 1987; Mahran et al., 1976), alkaloids (Mahjoub et al., 2012), sesquiterpene lactones (Jakupovitch et al., 1988; Metwalli et al., 1986) and coumarins (Greger et al., 1985) were isolated from this genus. *Cotula anthemoides* L commonly known as '*Babunaj'* in Algeria is used for colic and as a remedy for head and chest colds (Abhay and Tripathi, 2011). Previous phytochemical investigation on *C. anthemoides* led to the isolation of coumarin derivatives: 6-methoxy-2-oxo-2H-chromene-8-carboxylic acid methyl ester , α-pinene, ursolic acid and gibberellic acid (Showkat, 2012), 5-hydroxy-6,3',4'-trimethoxyisoflavone-7-*O*-β-D-glucopyranosyl(1→6)-α-L-rhamnopyranoside and 5,7-dihydroxy-6,8-dimethyl-3-methoxyflavone-7-*O*-β-D-galactopyranosyl-(1→2)-α-L-rhamnopyranoside (Yadava and Barsainya, 1998). The present work describes the isolation and structural identification of sixteen compounds from *C. anthemoides* that grows wild in Algerian Sahara; fourteen are reported here for the first time from the aerial parts of *Cotula anthemoides* L. In addition, the radical scavenging ability (DPPH assay) and the tyrosinase inhibitory activity of all compounds were investigated for the first time, from this plant.

2. Results and discussion

The *n*-BuOH extract of the aerial parts of *C. anthemoides* was separated by combined chromatographic methods to obtain a new sulfonyl flavonol glucoside (**1**) (Fig. 1), in addition to fifteen known compounds (**2-16)** which were elucidated as luteolin-7-*O*-β-D-glucopyranoside (**2**) (Furkan et al., 2012), vicenin 2 (**3**) (Yinrong and Yeap foo, 2000), luteolin-7-*O*-β-D-glucuronopyranoside (**4**) (Tsukasa et al., 2011), apigenin-7-*O*-β-D-glucuronopyranoside (**5**) (Xiao et al., 2006), 6-hydroxyluteolin-7-*O*-β-D-glucopyranoside (**6**) (Yinrong and Yeap foo, 2000), isoetin-5′-*O*-β-D-glucopyranoside (**7**) (Alberto et al., 1988), tachioside (**8**) (Shogo et al.,

1987), pseudolaroside A (**9**) (Peng et al., 2006), junipediol A 8-*O*-β-D-glucopyranoside (**10**) (Gilles et al., 1997; Arunee et al., 2014), vanillic acid-4-*O*-β-D-glucopyranoside (**11**) (Akiyo et al., 1995), 2-hydroxy-3-phenylpropionamide (**12**) (Zhi-gang et al., 2012), protocatechuic acid (**13**) (Hui et al, 1998), uridine (**14**) (Yalçin et al., 2003), tuberonic acid β-D-glucopyranoside (**15**) (Baoliang et al., 1993) and rosmarinic acid (Gohari et al., 2009) (Fig.S1) . Their structural assignments were made by HR-ESI-MS, 1D-, and 2D-NMR analyses and by comparison with spectral data from the literature values. To the best of our knowledge, compounds **3-16** were isolated for the first time from the genus *Cotula*.

Compound **1** was isolated as a yellow amorphous powder. The positive HR-ESI-MS showed a molecular ion peak m/z 681.0744 $[M+Na]^+$ (calcd for $C_{26}H_{26}O_{18}NaS$, 681.0744) enabling to determine the molecular formula $C_{26}H_{26}O_{18}S$. The UV spectrum revealed absorption bands at 206, 260 and 350 nm. The ^1H and ^{13}C NMR spectra of **1** showed characteristic signals indicating the presence of one sugar unit and one flavonol moiety (Ozbek et al., 2015). Signals arising from one anomeric proton at δ_H 5.07 (1H, d, J = 7.4 Hz) and other protons of sugar moiety in the range of (δ_H 3.5-4.0 ppm) showed the presence of d-glucose with β-configuration on the basis of the large coupling constants between H-1″ to H-5″ (Table 1) (Alabdul Magid et al., 2008). Complete assignments of osidic protons and carbons were determined by the analysis of COSY, HSQC and HMBC experiments. The relative configuration of β-D-glucopyranosyl moiety was further confirmed by analysis of the ROESY correlations from the α-axial protons H-1/H-3 and H-1/H-5. The A-ring of the flavonol was represented by two meta-coupled resonances at δ_H 6.26 (1H, brs) and δ_H 6.38 (1H, brs) assigned to H-6 and H-8, respectively. The ^1H NMR data (see experimental part) exhibited two singlet signals at δ_H 7.43 (1H, s) and δ_H 7.85 (1H, s) assignable to H-5′ and H-2′, respectively, which were correlated in the HSQC spectrum with their aromatic carbons at δ_c 119.1 and δ_c 113.9 ppm, respectively. In addition, one methyl proton appeared at δ_H 1.90 (3H, s) together with a set of three aliphatic protons δ_H 3.45 (1H, dd, J = 14.1, 6.8 Hz), 3.56 (1H, m) and 5.42 (1H, dd, J = 6.5, 4.1 Hz). The ^1H and ^{13}C NMR signals of compound **1** were completely assigned by a combination of HSQC, HMBC and ^1H-^1H COSY experiments. The ^1H-^1H COSY correlation between H_2-3‴ (δ_H 3.45, 3.56) and H-2‴ (δ_H 5.42) and the cross-peaks in the HMBC spectrum (Fig. S17) between H_2-3‴/C-2‴(δ_C 67.1) and C-1‴ (δ_C 171.5), H-2‴/C-1‴, C-3‴ (δ_C 57.2), and C-4‴ (δ_C 171.3) and H_3 (δ_H 1.90)/C-4‴, indicated the presence of propanoic acid-2-acetoxy-3-sulfonyl moiety. The chemical shift of C-3‴ (δ_C 57.2) in addition to the molecular formula indicated a sulfonyl group. The site of linkage of the glucopyranosyl and sulfonyl groups in **1** was determined by cross-peaks observed in the HMBC experiment between H-1″ of glucose (δ_H 5.07) and C-3′ (δ_C 148.9) of quercetin, and between H_2-3‴ (CH_2SO_2) of sulfonyl group and C-6′ (δ_C 133.6) of quercetin. Thus, the structure of compound **1** was characterised as quercetin 6′-[propanoic acid-(2-acetoxy-3-sulfonyl)]-3′-O-β-D-glucopyranoside.

Several biological activities of extracts and essential oils of *Cotula* were reported however, the biological potential of isolated pure compounds has not been investigated. Thus, the isolated compounds **1-16** were tested for their DPPH radical scavenging and anti-tyrosinase activities.

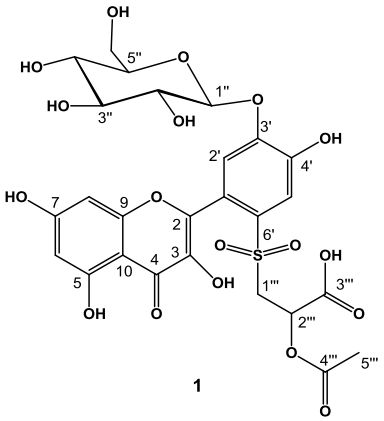

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Figure 1: Chemical structure of compound **1** isolated from *C. anthemoides* L.

In order to assess the antioxidative properties of **1-16**, the DPPH radical scavenging activity of these compounds was measured. According to the results (Table 2), compounds **1**, **2**, **4-6**, **8**, and **16** have a slightly radical scavenging potential (EC_{50} ranging from 9.1 to 29.9 µM) compared with ascorbic acid, used as positive control (EC_{50} 7.4 µM). The di-OH substitution at 3′ and 4′ in the B ring is particularly important to the antiradical activity of flavonoids (Arora et al.1998). The trend is consistent with the less active flavonoid **5** compared with **4**. The free 3-OH was also found to be important for the antioxidant activity of rosmarinic acid **16** (3-OH) comparing to tachioside **8** (3-OCH_3) (Sawai and Moon, 2000). Comparison of the DPPH radical scavenging activity of **6** with **1**, **2** and **4** showed that the presence of the hydroxyl group at C-6 increased the activity. Compounds **2** and **4** shared a common aglycone which is 3′ and 4′-di-OH substituted in the B ring (luteolin skeleton), the only difference was in the nature of the group linked at C-7 which was β-D-glucopyranose (**2**) and β-D-glucuronopyranose (**4**), suggesting that the glucuronic acid may contribute slightly to the increase of the DPPH radical scavenging activity. Thus, we can conclude that the hindrance effect due to the presence of a hydroxy group at C-6, a β-D-glucose or β-D-glucuronic acid linked at C-7 in a luteolin skeleton is determinant in the scavenging of the DPPH free radical.

The tyrosinase inhibitory activity of isolated pure compounds **1-16** was performed using L-DOPA as substrate and kojic acid, a well-known strong tyrosinase inhibitor, as a positive control (Demirkiran et al. 2013;

Kim et al., 2001; Kim and Uyama, 2005; Xie et al., 2003). The results (Table 2) showed that compounds **1**, **9** and **11** exhibited a mild tyrosinase inhibition. The most active compound was **11** (IC_{50} 85 µM) followed by **9** (IC_{50} 95 µM) and **1** (IC_{50} 100 µM).

Table 1. Antiradical potential and anti-tyrosinase activity of compounds **1-16** isolated from the aerial parts of *C. anthemoides* L.

Compounds	DPPH radical scavenging activity EC_{50} ± S.D.(µM)[a]	Tyrosinase inhibitory activity IC_{50} ± S.D. (mM)[a]
1	22.8 ± 0.5	100 ± 0.5
2	29.9 ± 1.3	-[b]
3	-[b]	-[b]
4	25.1 ± 0.7	-[b]
5	29.5 ± 0.1	-[b]
6	9.1 ± 0.4	-[b]
7	-[b]	-[b]
8	27.6 ± 1.1	-[b]
9	-[b]	95 ± 1.5
10	-[b]	-[b]
11	-[b]	85 ± 0.8
12	-[b]	-[b]
13	-[b]	-[b]
14	-[b]	-[b]
15	-[b]	-[b]
16	12.7 ± 0.3	-[b]
Ascorbic acid[c]	7.4 ± 0.05	-
Kojic Acid[c]		6.4 ± 0.04

[a] Values are presented as mean ± S.D. (n =3).
[b] 50% inhibition not achieved at the concentration of 100µg/ml.
[c] used as a positive control.

In summary, one new sulfonyl flavonol glucopyranoside, along with fifteen known compounds, were isolated from the aerial parts of *C. anthemoides*. The DPPH radical scavenging activity assay showed a high activity for compound **6** compared with ascorbic acid used as a positive control. Compounds **1, 9** and **11** exhibited a mild *in vitro* mushroom tyrosinase inhibition compared with kojic acid, used as a positive control.

3. Experimental

3.1. General experimental procedures

NMR spectra were carried in MeOH-d_4 and DMSO on Bruker Avance DRX III 500 instruments. HR-ESI-MS experiments were performed using a Micromass Q-TOF micro instrument. Biological assays were read on a Fluostar Omega microplate reader (BMG labtech), the amounts for DPPH assay were 100 µL (95 µL DPPH solution and 5 µL sample) and for tyrosinase inhibitory activity are 300 µL (100 µL of sample, 100 µL of mushroom tyrosinase solution and 100 µL of L-dopa). 96-well polystyrene microliter clear plates were used. Thin layer chromatography (TLC) was performed on pre-coated silica-gel 60 F_{254} Merck and compounds were observed under UV light at 254 and 365 nm or visualized by spraying the dried plates with 50% H_2SO_4, followed by heating. CC was carried out on Kieselgel 60 (63-200 mesh) or LiChroprep RP-18 (40-63 mm) Merck. HPLC was performed on a Dionex apparatus equipped with an ASI-100 autosampler, an Ultimate 3000 pump, a diode array detector UVD 340S and Chromeleon software. RP-18 column (Phenomenex 250x15 mm, Luna 5µ) was used for semi-preparative HPLC with binary gradient eluent (H_2O (pH 2.4 with trifluoroacetic acid (TFA)); MeCN) and a flow rate of 4 mL/min; the chromatogram was monitored at 205, 210, 254, and 360 nm. Absorbance (Ab) values in the DPPH free radical scavenging and antityrosinase assay were read on a Fluostar omega microplate reader (BMG labtech).

3.2. Plant material

The aerial parts of *C. anthemoides* L. were collected in March 2012 from Tinguentourine, Ain Amenas, Eastern Algerian Sahara. The plant has been authenticated by Prof. Gérard De Belair (Annaba University, Algeria) and a voucher specimen (LOST Ca03/12) was deposited at the herbarium of the Laboratory of Therapeutic Substances (LOST), Faculty of Sciences, Université des Frères Mentouri-Constantine-Algeria.

3.3. Extraction and isolation

The powdered dry aerial parts of *C. anthemoides* (1400 g) were macerated at room temperature in methanol 80% (3×7.5 L, 24h). The extract was concentered under low pressure, diluted with H_2O (560 mL), then successively extracted with petroleum ether (PE) (3×300 mL), chloroform (3×300 mL), ethyl acetate (3×300 mL), and *n*-butanol (3×300 mL). After evaporation of the solvents, 0.4g of PE, 1.5 g of $CHCl_3$, 0.55 g of EtOAc and 21.7 g of *n*-BuOH extracts were obtained. The *n*-BuOH extract was subjected to VLC over polyamid SC6 (9 cm x 5 cm) eluted with toluene-methanol with increasing polarity to give 12 fractions (F1-F12 respectively). Fractions F6-7 were combined (1.4 g) and subjected to HPLC preparative using (10-25% MeCN in 60 min) to yield 19 fractions. Frs [4-6] (35.5 mg) were purified by semi-prep. HPLC (5-15% MeCN, in 20 min) affording compounds **14** (R_t 9.9 min, 4 mg) and **8** (R_t 13.8 min, 3 mg). Frs [7-10] (36 mg) were purified by semi-prep. HPLC (5-15% MeCN, in 20 min) yielding compounds **9** (R_t 16.5 min, 7 mg) and **10** (R_t 21.5 min, 4 mg). Frs [11-12] (26.7mg) were purified by semi-prep. HPLC (10-25% MeCN, in 20 min) leading to compound **11** (R_t 6.3 min, 3.2 mg). Frs [26-27] (51 mg) were purified by semi-prep. HPLC (15-35% MeCN, in 20 min) to afford compounds **12** (R_t 8.5 min, 1.6 mg) and **15** (R_t 16.5 min, 4.7 mg). Frs [36-44] (42 mg) were purified by semi-prep. HPLC (10-40% MeCN, in 25 min) yielding compounds **16** (R_t 25.9 min, 4.2 mg). The combined fractions F9-11 (827 mg) were subjected to a preparative HPLC (10-40% MeCN in 60 min) to obtain 35 fractions. Frs [7-13] (50.3 mg) were purified by semi-prep. HPLC (15-35% MeCN, in 20 min) yielding compounds **1** (R_t 13.1 min, 7 mg), **4** (R_t 14.2 min, 5 mg) and **16** (R_t 18.2 min, 1.4 mg). Frs [14-28] (46.2 mg) were purified by semi-prep. HPLC (15-35% MeCN, in 20 min) leading to compounds **3** (R_t 8.0 min, 4.0 mg), **2** (R_t 13.7 min, 3.5 mg) and **5** (R_t 15.1 min, 4.1 mg). Compound **6** (17.1min, 9.2 mg) was purified from

Frs [28-29] by semi-prep. HPLC (15-35% MeCN, in 20 min). Frs [30-32] were purified by semi-prep. HPLC (15-35% MeCN, in 30 min) affording compounds **13** (R_t 6.3 min, 1 mg) and **7** (R_t 26.4 min, 11.2 mg).

3.3.1. *Quercetin 6′-[propanoic acid-(2-acetoxy-3-sulfonyl)]-3′-O-β-D-glucopyranoside*

Yellow amorphous powder; $[\alpha]_D^{20}$ + 26.8 (*c* 0.25, MeOH). UV (MeOH) λ_{max} (ab.): 206 (2.1), 260 (1.3), 350 (0.55). ^{1}H NMR (500 MHz, CD_3OD) : δ_H 6.26 (brs, H-6), 6.38 (brs, H-8), 7.85 (s, H-2′), 7.43 (s, H-5′), 5.07 (d, *J* = 7.4 Hz, H-1"), 3.59 (dd, *J* = 8.9,7.4 Hz, H-2"), 3.56 (t, *J* = 8.6 Hz, H-3"), 3.52 (t, *J* = 8.6 Hz, H-4"), 3.60 (m, H-5"), 3.81 (dd, *J* = 12.2,4.8 Hz, H-6"a), 4.00 (dd, *J* = 12.2,2.5 Hz, H-6"b), 5.42 (dd, *J* = 6.5,4.1, H- 2'"), 3.45 (dd, *J* = 14.1,6.8, H- 3'"a), 3.56 (m, H- 3'"b), 1.90 (s, H- 5'").^{13}C NMR (125 MHz, CD_3OD) : δ 145.2 (C-2), 138.9 (C-3), 177.6 (C-4), 162.9 (C-5), 99.8 (C-6), 166.1 (C-7), 94.7 (C-8), 158.4 (C-9), 105.1 (C-10), 125.4 (C-1′), 113.9 (C-2′), 148.9 (C-3′), 151.1 (C-4′), 119.1 (C-5′), 133.6 (C-6′), 103.0 (C-1"), 74.7 (C-2"),77.6 (C-3"), 71.0 (C-4"), 78.3 (C-5"), 62.2 (C-6"), 57.2 (C-1'"), 67.1 (C-2'"), 171.5 (C-3'"), 171.3 (C-4'"), 20.2 (C-5'"). HR-ESI-MS $[M+Na]^+$ *m/z* 681.0744 (calcd for $C_{26}H_{26}O_{18}NaS$, 681.0744).

3.3.2. Acid hydrolysis

A part of the *n*-BuOH extract (300 mg) was refluxed (90 °C) with 10 mL of 2M TFA for 3 h. After extraction with ethyl acetate (3 x 10 mL), the aqueous layer was evaporated to furnish the monosaccharide residue (100 mg). Two sugars were identified as glucose and glucuronic acid by comparison with authentic samples on TLC in MeCOEt:*iso*-PrOH:Me_2CO:H_2O (20:10:7:6). The monosaccharide residue (100 mg) was subjected to a preparative TLC using the same solvent. The optical rotation of each purified sugar was measured and compared with authentic samples to afford D-glucose and D-glucuronic acid.

3.4. Biological activities

3.4.1. DPPH free radical scavenging assay

The scavenging activity of isolated compounds against DPPH was investigated by spectrophotometric methodology, as previously described (Gossan et al., 2015). Briefly, 5 µL of either the standard or sample solutions (dissolved in DMSO) was mixed with 95 µL of DPPH solution (158 µM, dissolved in absolute EtOH). After mixing gently and incubating for 30 min at 37°C, the optical density was measured at λ 515 nm. The percentage of absorbance inhibition at λ 515 nm was calculated using the following equation: % inhibition $[(Ab_{control} - Ab_{sample})/Ab_{control}] \times 100$. DPPH solution in EtOH was used as a control. The curve of the % scavenging activity against the concentration of sample was prepared by the MSExcel based program to obtain the EC_{50}. All the tests were conducted in triplicate. The experimental data were expressed as mean ± standard deviation.

3.4.2. Tyrosinase enzyme assay

Tyrosinase activity inhibition was determined by spectrophotometric methodology, as previously described (Ngoc et al., 2009). 100 µL of each test compound solution (dissolved in 10% DMSO) was added to 96-well plate then mixted with 100 µL of mushroom tyrosinase solution (135 U/ml mushroom tyrosinase in phosphate buffer solution (PBS, pH 6.8)). After pre-incubation at 25 °C for 10 min, 100 µL of L-dopa (0.5 mM, PBS pH 6.8) were added into 96-well plate. The reaction mixture was incubated for another 5 min at 25 °C. The amount of dopachrome in the mixture was determined by the measurement of the absorbance of each well at 475 nm. The inhibitory percentage of tyrosinase was calculated according to the following equation: % inhibition = {[(A - B) - (C - D)]/ (A - B)} × 100. A: Ab at 475 nm without test substance; B: Ab at 475 nm without test substance and tyrosinase; C: Ab at 475 nm with test substance; D: Ab at 475 nm with test substance, but without tyrosinase. Kojic acid was used as positive control agent. All the tests were conducted in triplicate and IC_{50} was determined by interpolation of concentration % inhibition curve obtained by MSExcel based program. The experimental data were expressed as mean ± standard deviation.

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Supporting Information

HR-ESI-MS, ^{1}H and ^{13}C NMR, COSY, HMBC, HSQC and ROESY spectra for compound **1**.

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