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Phenolic compounds from *Anaphalis aureo-punctata*

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From the ethanolic extract of the whole plant of *Anaphalis aureo-punctata*, a new acylated flavonoid glycoside 3-*O*-kaempferol-3-*O*-acetyl-6-*O*-(*p*-coumaroyl)- β -D-glucopyranoside (**1**), and five known phenolic compounds were isolated. Their structures were established by spectral methods (UV, IR, MS, 1D, 2D-NMR). The flavonoid glycosides, **1**, **2** and **3** showed markedly inhibited oxidative DNA strand breaks induced by Fenton reaction and NADH/PMS in a concentration-dependent manner.

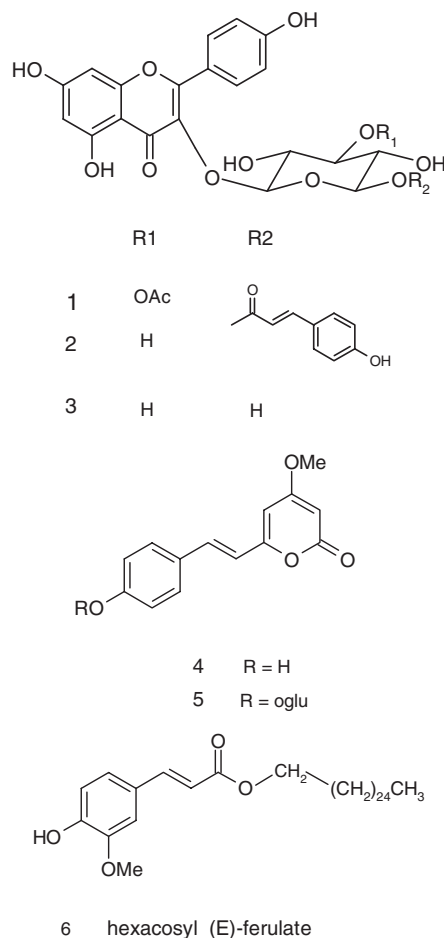
1. Introduction

The genus *Anaphalis* (Compositae) consists of about 80 species distributed throughout the world, 50 species of them growing in China. Some of them have been used as traditional folk medicine [1]. Among them, *Anaphalis morrisoni-cola* HAY showed significant antitumor activity [2]. A phytochemical study of the plant *A. aureo-punctata* has not been reported so far. We report herein the six compounds **1**–**6** isolated from the whole plant of this species as well as the inhibited oxidative DNA strand breaks induced by Fenton reaction and NADH/PMS properties of compounds **1**–**3**.

2. Investigations, results and discussion

The alcoholic extract of the air-dried and powered whole plants of *A. aureo-punctata* was partitioned between water and ethyl acetate. The EtOAc-soluble part was concentrated and chromatographed over silica gel, to yield 3-*O*-kaempferol-3-*O*-acetyl-6-*O*-(*p*-coumaroyl)- β -D-glucopyranoside (**1**), tiliroside (**2**) [3], astragalin (**3**) [3], 4'-hydroxydehydrokaempferol (**4**) [4, 5], 2*H*-pyran-2-one, 6-[2-(4-(β -D-glucopyranosyloxy)phenyl)ethenyl]-4-methoxy-(*E*) (**5**) and hexacosyl (*E*)-ferulate (**6**) [6]. The structures of the known compounds **2**–**4**, and **6** were confirmed by comparing their corresponding properties (melting point, MS, IR, ¹H NMR and ¹³C NMR) with the reported values in the literature or comparing with authentic samples. The spectral data (¹³C NMR) of **5**, however, is reported here for the first time.

Compound **1** was obtained as a yellow crystalline powder, m. p. >162 °C (dec.), [α]_D²¹ –53.0 (c 2.3, CH₃OH). The HR-ESIMS gave a [M+Na]⁺ at m/z 659.1369 (Calcd 659.1317), which corresponds to a molecular formula C₃₂H₂₈O₁₄. The ¹³C NMR spectrum and the DEPT experiment (1 × CH₃, 1 × CH₂, 17 × CH and 13 × C) allowed the establishment of the molecular formula as C₃₂H₂₈O₁₄. The IR spectrum (KBr) indicated the presence of hydroxyl (3270 cm^{–1}), ester carbonyl (1717 cm^{–1}), γ -pyrone (1655 cm^{–1}, 1606 cm^{–1}). The 3H singlet at δ 2.03 in the ¹H NMR, together with its multiple-bond connectivity to the carbonyl carbon signal



at δ 169.8 (Fig. 2) in HMBC experiment was in agreement with the resonances of an acetyl group. In the ¹H NMR 2H AX system at δ 6.10 (d, J = 16.0 Hz, H- β) and δ 7.37 (d, J = 16.0 Hz, H- α) due to trans-double together with the 4H AA'XX' system at δ 7.38 (d, H-2''', 6''') and δ 6.80 (d, H-3''', 5''') as *p*-substituted aromatic ring groups were deduced the presence of an acyl group as

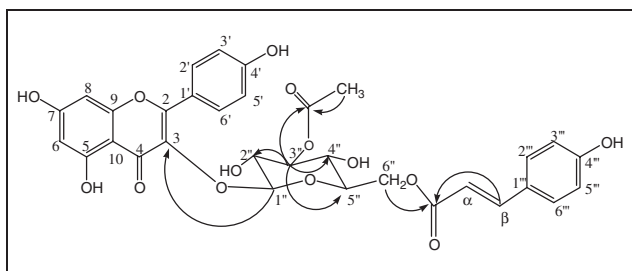


Fig. 1: The Key correlation of **1** in HMBC (H→C)

p-coumaroyl, and in ^1H NMR spectrum, there is an anomeric proton of glucopyranoside (δ 5.53 d, J = 7.8 Hz). That data together with ^{13}C NMR signals at δ 101.0 (CH), 73.5 (CH), 70.9 (CH), 71.5 (CH), 74.2 (CH), 62.0 (CH_2), indicated that compound **1** has a β -glucosyl moiety. The ^1H NMR signals for *p*-substituted aromatic protons (δ 8.00 and 6.89), *m*-coupled aromatic protons (δ 6.17 and 6.39), together with ^{13}C NMR signals indicated compound **1** is a 3-*O*-kaempferol (*p*-coumaroyl) glycoside [7].

The HMBC cross peak at δ 5.53/133.0 (H-1''/C-3) revealed the connection point between the sugar moiety and aglycone to be at C-3. To assign the points of attachment of the two acyl groups, their respective carbonyl signals were used as entry points in the HMBC spectrum. The correlations from the acetyl protons (δ 2.03) and H-3'' (δ 4.71) to the acetyl carbonyl (δ 169.8), from H- β (δ 7.37) and H-6'' (δ 4.00) to the coumaroyl carbonyl (δ 166.0) suggested the acetoxy group at C-3'' and the *p*-coumaroyloxy group at C-6'' (Fig. 1) [8]. Consequently, the structure of **1** was established as 3-*O*-kaempferol-3-*O*-acetyl-6-*O*-(*p*-coumaroyl)- β -D-glucopyranoside.

It is now commonly recognized that oxygen-derived species (e.g., hydroxyl radical, superoxide anion radical, and peroxy radicals), generated from cellular respiration (mitochondrial electron transport), inflammatory reactions, cell injury or as consequence of exogenous exposure to toxins such as air pollutants, play a critical role in the development of a variety of chronic health problems, such as cancer, aging, atherosclerosis and other degenerative diseases [9–12]. Reactive oxygen species may attack DNA bases or deoxyribose residues to produce damaged bases or strand breaks when present in excess, leading to mutagenesis and cancer. Plant and food derived antioxidants, such as green tea polyphenols, resveratrol in grape skin and α -carotene are increasingly found beneficial in protecting against these diseases [13, 14], hence antioxidant therapy has become an attractive therapeutic strategy [15].

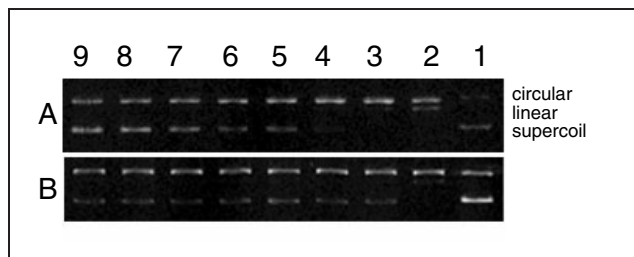


Fig. 2: Inhibition effect of antioxidants against $\text{Fe}^{2+}/\text{H}_2\text{O}_2$ -induced pBR322 DNA strand breaks. The reaction mixture contained 100 ng pBR322 DNA in phosphate buffer saline (PBS, pH 7.4). DNA damage was initiated, as monitored by agarose gel electrophoresis of ethidium bromide-stained pBR322 DNA, by added 160 μM of FeSO_4 and 400 μM of H_2O_2 , incubated under air at 37 °C for 30 min. (A) Lane 1: control; Lane 2: $\text{FeSO}_4/\text{H}_2\text{O}_2$; Lane 3–7: **2** (0.625–40 μM and $\text{FeSO}_4/\text{H}_2\text{O}_2$). (B) [Antioxidants] = 1.25 μM . Lane 1: control; Lane 2: $\text{FeSO}_4/\text{H}_2\text{O}_2$; Lane 3: BHT; Lane 4: Trolox; Lane 5: resveratrol; Lane 6: **1**; Lane 7: **3**; Lane 8: **2**; Lane 9: quercetin.

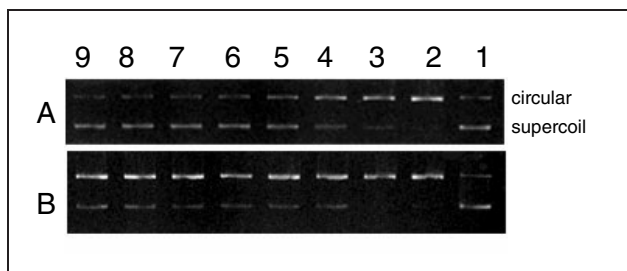


Fig. 3: Effects of antioxidants on NADH/PMS-induced pBR322 DNA strand breaks. The reaction mixture contained 100 ng pBR322 DNA in phosphate buffer saline (PBS, pH 7.4). DNA damage was initiated, as monitored by agarose gel electrophoresis of ethidium bromide-stained pBR322 DNA, by added 1.2 mM of NADH and 0.24 mM of PMS, incubated under air at 37 °C for 60 min. (A) Lane 1: control; Lane 2: NADH/PMS; Lane 3–7: **2** (2.5–160 μM) and NADH/PMS. (B) [Antioxidants] = 5 μM . Lane 1: control; Lane 2: NADH/PMS; Lane 3: BHT; Lane 4: Trolox; Lane 5: resveratrol; Lane 6: **1**; Lane 7: **3**; Lane 8: **2**; Lane 9: quercetin.

In the present study, the method we used to detect DNA strand breakage was based on the differential mobility of different forms of plasmid pBR322 DNA [16]. DNA exists in the three forms: supercoiled, nicked circular and linear. In untreated plasmid pBR322 DNA we found a major band, corresponding to the intact supercoiled forms, a minor band, corresponding to circular form, indicative of single-strand break. We examined the effects of the antioxidants from *A. aureo-punctata* on oxidative DNA strand breaks induced by Fenton reaction and NADH/PMS. Our results showed that both Fenton reaction and NADH/PMS could cause DNA strand breaks in plasmid DNA. Addition of the compounds and other antioxidants such as BHT, trolox and resveratrol markedly inhibited oxidative DNA strand breaks induced by Fenton reaction and NADH/PMS in a concentration-dependent manner. The results showed the compounds **1–3** might protect DNA against oxidative damage.

3. Experimental

3.1. Equipment

Optical rotation was measured on a Jasco DIP-180 instrument. Melting points were determined on a Yanagimoto micro-melting point apparatus and are uncorrected. The UV spectra were obtained on a Hitachi 200–20 spectrophotometer, and IR spectra were recorded on a Shimadzu UV-260 spectrophotometer. ^1H , ^{13}C NMR and 2D NMR spectra were scanned on a Bruker AM 400 FT-NMR spectrometer with TMS as internal standard. FABMS and EIMS data were obtained on a VG-ZAB-HS spectrometer at 70 eV. CC was carried out using silica gel (200–300 mesh). Sephadex LH-20 and TLC were conducted on silica gel. Spots were detected on the TLC under UV light (254 nm) or by heating after spraying with 5% H_2SO_4 .

3.2. Plant material

The whole plant of *A. aureo-punctata* used in this experiment was collected from the Gansu Province of China in August 2000. The plant was identified by adjunct Prof. Yong-Hong Zhang, Faculty of Pharmacy, Lanzhou Medical College of P. R. of China. A specimen has been deposited at the Laboratory of Biomedicine, Faculty of Pharmacy, Lanzhou Medical College of P. R. China.

3.3. Extraction and isolation

The air-dried whole plant of *A. aureo-punctata* (3.7 kg) was powdered and extracted three times (each 7 days) with EtOH at RT. The extract was concentrated under reduced pressure. The residue (150 g) was suspended in H_2O (1 L) and successively partitioned by petroleum ether (3×1 L, 20 g), EtOAc (3×1 L, 34 g) and BuOH (3×1 L, 32 g). The EtOAc extract (34 g) was obtained and subjected to CC over silica gel (500 g, 200–300 mesh) column with a pet. ether- Me_2CO gradient. It was separated into 7 crude fractions (fractions A–G). Fraction G (1.6 g, pet. ether-acetone 1:1) was chromatographed on prep. silica gel TLC, eluting with CHCl_3 -MeOH (7:1) to give **1** (20 mg), **2** (50 mg), **3** (13 mg) and **5** (12 mg).

Table: ^1H (400 MHz) and ^{13}C (100.6 MHz)NMR data of compound 1 (DMSO- d_6 , TMS, δ ppm)

No.	δ_{H} (J Hz)	δ_{C}	DEPT	No.	δ_{H} (J Hz)	δ_{C}	DEPT
2		156.8	C	3''	4.71 d (9.2, 9.0)	70.9	CH
3		133.0	C	4''	3.55 d (9.0, 9.0)	71.5	CH
4		177.4	C	5''	3.70 m	74.2	CH
5		161.3	C	6''	4.00 d (5.0)	62.0	CH ₂
6	6.17 d (1.8)	98.9	CH	coumaroyl			
7		164.3	C	COO		166.0	C
8	6.39 d (1.8)	93.8	CH	α	6.10 d (16.0)	113.5	CH
9		156.5	C	β	7.37 d (16.0)	144.9	CH
10		104.0	C	1'''		125.0	C
1'		120.8	C	2''', 6'''	7.38 d (8.4)	130.2	CH
2' 6'	8.00 d (8.8)	130.9	CH	3''', 5'''	6.80 d (8.4)	115.9	CH
3' 5'	6.89 d (8.8)	115.2	CH	4'''		159.9	C
4'		160.1	C	3''-Acetyl			
glu				COO		169.8	C
1''	5.53 d (7.8)	101.0	CH	CH ₃	2.03 s	20.9	CH ₃
2''	3.37 d (7.8, 9.2)	73.5	CH				

1 was purified by a Sephadex LH-20 column eluted with MeOH. Fraction E (2.0 g, pet. ether-acetone 3:1) was subjected to silica gel CC (petroleum-EtOAc, 1:1) to yield 4 (22 mg). Fraction B (0.8 g, pet. ether-acetone 10:1) was repeatedly rechromatographed over silica gel and eluted with CHCl_3 -EtOAc (6:1) to yield 6.

3.4. 3-O-Kaempferol-3-O-acetyl-6-O-(*p*-coumaroyl)- β -D-glucopyranoside (1)

Yellow crystalline powder, showed positive tests with magnesium hydrochloride and ferric chloride test solutions, m.p. > 162 °C (dec.). $[\alpha]_{\text{D}}^{21}$ -53.0 (c 2.3, CH_3OH). HR-ESIMS: $[\text{M}+\text{Na}]^+$ m/z 659.1369 for $\text{C}_{32}\text{H}_{28}\text{O}_{14}$ (Calcd 659.1317). IR (film, cm^{-1}): 3270 (O-H), 1717 (COOR), 1655, 1606 sh (α -pyrone), 1360, 1174, 833. UV (MeOH) λ_{max} nm: 267, 303 sh, 313, 356. ^1H NMR and ^{13}C NMR see Table.

3.5. 2H-Pyran-2-one, 6-[2-(4-(β -D-glucopyranosyloxy)phenyl)ethenyl]-4-methoxy-(E) (5)

Yellow crystalline powder, m.p. 144–145 °C, FAB-MS m/z 429 $[\text{M}+\text{Na}]^+$, 413 $[\text{M}+\text{Li}]^+$ $\text{C}_{20}\text{H}_{22}\text{O}_9$. ^{13}C NMR (DMSO- d_6 , 100 MHz): 56.4 (OCH_3), 88.4 (C-3), 100.1 (C-5), 116.6 (C-3',5'), 117.7 (C-7), 128.9 (C-1'), 129.0 (C-2', 6'), 136.1 (C-8), 158.4 (C-6), 162.7 (C-2), 170.9 (C-4), 158.6 (C-4'), 100.7, 77.1, 76.6, 73.2, 69.7, 60.7, (glucosyl). ^1H NMR (DMSO- d_6 , 400 MHz, TSP) δ 7.58 and 7.04 (4H, AA'XX' system, J = 8.6 Hz, H-2', 6' and 3', 5'), 6.87 and 7.28 (2H, 2d, J = 16.0 Hz, H-7 and H-8), 5.60 and 6.25 (2H, 2d, J = 2.0 Hz, H-3 and H-5), 3.82 (3H, s, OCH_3), 4.92 (1H, d, J = 7.2 Hz, glc-1), 3.68 (2H, d, J = 10.9 Hz, glc-6), 3.69–3.13 (4H, m, sugar protons).

3.6. Assay for oxidative DNA strand breaks

Induction of DNA strand breaks by ROS and inhibition by antioxidants were assessed by measuring the conversion of supercoiled pBR322 plasmid DNA to open circular and linear forms. In the present study, the hydroxyl radical was generated by Fenton reaction and the superoxide anion radical was produced by NADH/PMS and pBR322 DNA (100 ng) was incubated with the indicated concentrations of antioxidants in 1.5 ml microcentrifuge tubes at 37 °C. The samples were mixed with 5 μl of gel loading buffer (0.13%) bromophenol blue and 40% (w/v) sucrose and

immediately loaded in a 1% agarose gel containing 40 mM Tris, 20 mM sodium acetate and 2 mM EDTA, and electrophoresed in a horizontal slab gel apparatus in Tris/acetate/EDTA gel buffer for 1 h. After electrophoresis, the gels were stained with 0.6 $\mu\text{g}/\text{ml}$ solution of ethidium bromide for 30 min, followed by another 30 min destaining in water. The gels were then photographed under UV light.

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