Phytochemistry Letters 6 (2013) 302–305

Contents lists available at SciVerse ScienceDirect

Phytochemistry Letters

journal homepage: www.elsevier.com/locate/phytol

Isolation of phenolic constituents and characterization of antioxidant markers from sunflower (*Helianthus annuus*) seed extract

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ARTICLE INFO

Article history:
Received 26 December 2012
Received in revised form 14 March 2013
Accepted 18 March 2013
Available online 4 April 2013

Keywords:
Sunflower seed
Helianthus annuus
Compositae
Phenolic constituent
Antioxidant

ABSTRACT

A new compound, benzyl alcohol β-D-apiofuranosyl-(1→6)-β-D-(4-*O*-caffeoyl) glucopyranoside (**1**), was isolated from the seed of sunflower (*Helianthus annuus*), together with eight known phenolic compounds: caffeic acid (**2**), methyl caffeoate (**3**), chlorogenic acid (**4**), 4-*O*-caffeoylquinic acid (**5**), 3-*O*-caffeoylquinic acid (**6**), methyl chlorogenate (**7**), 3,5-di-*O*-caffeoylquinic acid (**8**), and eriodictyol 5-*O*-β-D-glucoside (**9**). Their structures were elucidated on the basis of spectroscopic methods and chemical evidence. The antioxidative effect of the phenolic constituents from the sunflower seeds was also evaluated based on the oxygen-radical absorbance capacity (ORAC), and the fraction containing caffeic acid derivatives showed a high antioxidant potency.

1. Introduction

Sunflower (*Helianthus annuus* L.) is an annual plant native to North America, and one of the most important oilseed crops, being the second largest oilseed crop as a global source of vegetable oil (Canella and Sodini, 1977). Sunflower seeds are widely used in the food and nutraceutical industries because of their high oil and protein contents and other valuable bioactive components. The most abundant fatty acids in sunflower oil are linoleic acid (*ca.* 65%), oleic acid (*ca.* 25%), and palmitic and stearic acids (each *ca.* 5%) (Canella et al., 1982; Economides, 1998; Pereira et al., 2003). Sunflower oil also contains high levels of tocopherols and phytosterols (Rashid et al., 2009). On the other hand, extracts with high antioxidant activity may also be obtained from sunflower seed shells and kernels (De Leonardis et al., 2003). In Japan, natural antioxidant "sunflower seed extract" is defined as an ethanol or hot water extract from the seeds of sunflower, and this additive is characterized as an antioxidant containing isochlorogenic and chlorogenic acids (Notice No. 210, 1996). Sunflower seeds are thus suggested to be rich in polyphenols. This paper describes the isolation and structural characterization of phenolics including a new glycoside from the seed of sunflower. The antioxidant activities of fractions partitioned with solvent and the isolated phenolics were also estimated by an oxygen radical absorbance capacity (ORAC) assay (Ou et al., 2001; Huang et al., 2002).

2. Results and discussion

A homogenate of sunflower seeds in 80% ethanol (EtOH) was extracted with *n*-hexane and ethyl acetate (EtOAc) to give the respective *n*-hexane, EtOAc, and water extracts. The antioxidative activity of each extract was evaluated by ORAC (Fig. 1A). The EtOAc extract, which exhibited significant antioxidant activity, was repeatedly chromatographed over MCI-GEL CHP-20P and/or YMC GEL ODS-AQ with aqueous methanol (MeOH) in a stepwise gradient mode to afford a new compound (**1**), together with caffeic acid (**2**), methyl caffeoate (**3**) (Fujioka et al., 1999), chlorogenic acid (**4**) (Iwai et al., 2004), methyl chlorogenate (**7**) (Deyama et al., 1987; Ge et al., 2007), isochlorogenic acid (3,5-di-*O*-caffeoylquinic acid (**8**)) (Dini et al., 2006), and eriodictyol 5-*O*-β-D-glucoside (**9**) (Gujer et al., 1986). Similar chromatographic separation of the water extract gave **4**, 4-*O*-caffeoylquinic acid (**5**), and 3-*O*-caffeoylquinic acid (**6**) (Iwai et al., 2004). The known compounds **2**–**9** were identified, respectively, by direct comparison with authentic specimens and by comparison of their spectral data with those reported in the literature.

Compound **1** was isolated as a brown amorphous powder. Its molecular formula was assigned as $C_{27}H_{32}O_{13}$ from its HR-ESI-MS (*m*/*z* 563.1741 $[M-H]^-$; calcd. for $C_{27}H_{32}O_{13}$-H: 563.1765) and ^{13}C-NMR (27 ^{13}C signals) spectra. The UV spectrum showed absorption maxima at 207, 248sh, 289, and 330 nm, respectively.

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http://dx.doi.org/10.1016/j.phytol.2013.03.013

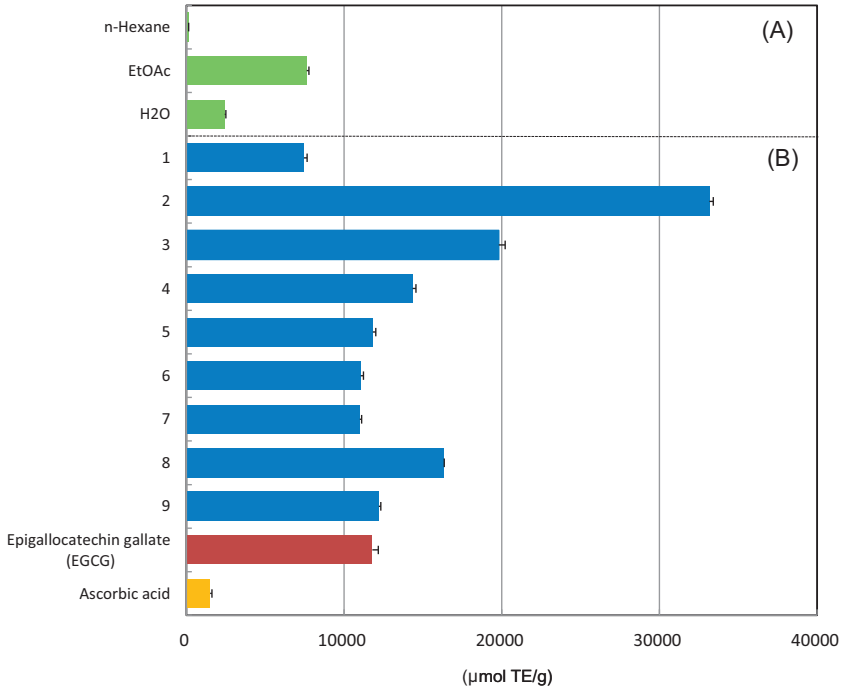

Fig. 1. ORAC values of each fraction (A) and isolated compounds **1**–**9** (B).

The ^{1}H- and ^{13}C-NMR spectra of **1** exhibited the signals characteristic of *trans*-caffeoyl and benzyl moieties, as follows. The ^{1}H NMR spectrum exhibited AB-type proton signals due to a benzylic methylene group at δ 4.68 and 4.91 (each d, J = 12 Hz, H-7) and multiplets due to a phenyl group at δ 7.25–7.43 (5H) as well as those assignable to a caffeoyl group [δ 6.28 (d, J = 16 Hz, H-8′), 6.77 (d, J = 8.5 Hz, H-5′), 6.95 (dd, J = 2, 8.5 Hz, H-6′), 7.05 (d, J = 2 Hz, H-2′), and 7.68 (dd, J = 16 Hz, H-7′)]. These aromatic units were also supported by 16 carbon signals due to the caffeoyl [δ 114.8, 115.2, 116.5, 123.1, 127.7, 146.8, 147.6, 149.8, 168.4 (C-1′-9′)] and benzyl group [δ 71.9, 128.8, 129.3 (4C), 138.9 (C-1–7)] in the ^{13}C NMR spectrum. The presence of two sugar units in **1** was shown by two anomeric proton signals at δ 4.40 (d, J = 8 Hz, Glc H-1) and 4.95 (d, J = 2 Hz, Api H-1) and others assigned by the ^{1}H–^{1}H shift correlation spectrum (Table 1). The sugar residues were presumed to be hexose and branched pentose, as revealed by the ^{13}C NMR spectrum exhibiting 11 aliphatic carbon signals which included a quaternary (δ 80.6, Api C-3) and three methylene carbon resonances (δ 68.6, 65.7 and 75.1). D-Glucose and D-apiose as the sugar units in **1** were confirmed by acid hydrolysis followed by RP-HPLC analysis for derivatives prepared by reaction with L-cysteine methyl ester and *O*-tolyl isothyocyanate according to the previously reported method (Tanaka et al., 2007). The linking position of each unit was determined by cross-peaks among glucose H-1 (δ 4.40)/C-7 (δ 71.9) of the benzyl group, glucose H-4 (δ 4.87)/C-9′ (δ 168.4) of the caffeoyl group, and apiose H-1 (δ 4.95)/glucose C-6 (δ 68.6) in HMBC (Fig. 2). β-Glycosidic linkage in the glucose core was evidenced by large coupling constants (J = 8 Hz). The configuration at the anomeric center of the apiose was also determined as β on the basis of the comparison of the ^{13}C-NMR data for **1** with those for α- and β-D-apiofuranoside (Kitagawa et al., 1989), and the $J_{H\text{-}1,\ H\text{-}2}$ coupling constant consistent with reported data of β-D-apiofuranoside (Liu et al., 2010). Therefore, **1** was established as benzyl alcohol β-D-apiofranosyl-(1→6)-β-D-(4-*O*-caffeoyl) glucopyranoside.

To clarify the relationship between the polarity of the ingredients and antioxidative activity, the ORAC values were estimated for each fraction obtained by partitioning with organic solvent for the sunflower seed extracts (Fig. 1A). As a result, the EtOAc fraction exhibited a marked activity which is considered to be responsible for **4** and **8** as the main component in the extract (Fig. 3). The antioxidant activity of isolated compounds **1**–**9** was also evaluated (Fig. 1B). All of them showed potent antioxidative activity with ORAC values of *ca.* 10,000 μmol TE/g or above. It is particularly notable that the potency of **2** was about three times more potent than that of epigallocatechin gallate (EGCG), a typical tea catechin. These results suggest that the antioxidant activity of

Table 1
^{1}H-(500 MHz) and ^{13}C-NMR(126 MHz) data of compound **1** measured in MeOH-d_4.

Position	δ_C	δ_H (J in Hz)
1	138.9	–
2	129.3	7.25-7.43[a]
3	129.3	7.25-7.43[a]
4	128.8	7.25-7.43[a]
5	129.3	7.25-7.43[a]
6	129.3	7.25-7.43[a]
7	71.9	4.68 (d, J = 12), 4.91 (d, J = 12)
1′	127.7	–
2′	115.2	7.05 (d, J = 2)
3′	146.8	–
4′	149.8	–
5′	116.5	6.77 (d, J = 8.5)
6′	123.1	6.95 (dd, J = 2, 8.5)
7′	147.6	7.68 (d, J = 16)
8′	114.8	6.28 (d, J = 16)
9′	168.4	–
Glucose (Glc)-1	103.2	4.40 (d, J = 8)
2	75.3	3.35 (dd, J = 8, 9.5)
3	75.8	3.60 (t, J = 9.5
4	72.8	4.87 (t, J = 9.5)
5	74.9	3.66 (m)
6	68.6	3.53 (dd, J = 6.5, 11), 3.73 (dd, J = 2.5, 11)
Apiose (Api)-1	111.1	4.95 (d, J = 2)
2	78.1	3.89 (d, J = 2)
3	80.6	–
4	65.7	3.53 (d, J = 9.5), 3.74 (d, J = 9.5)
5	75.1	3.56 (2H, s)

[a] Overlapped signals.

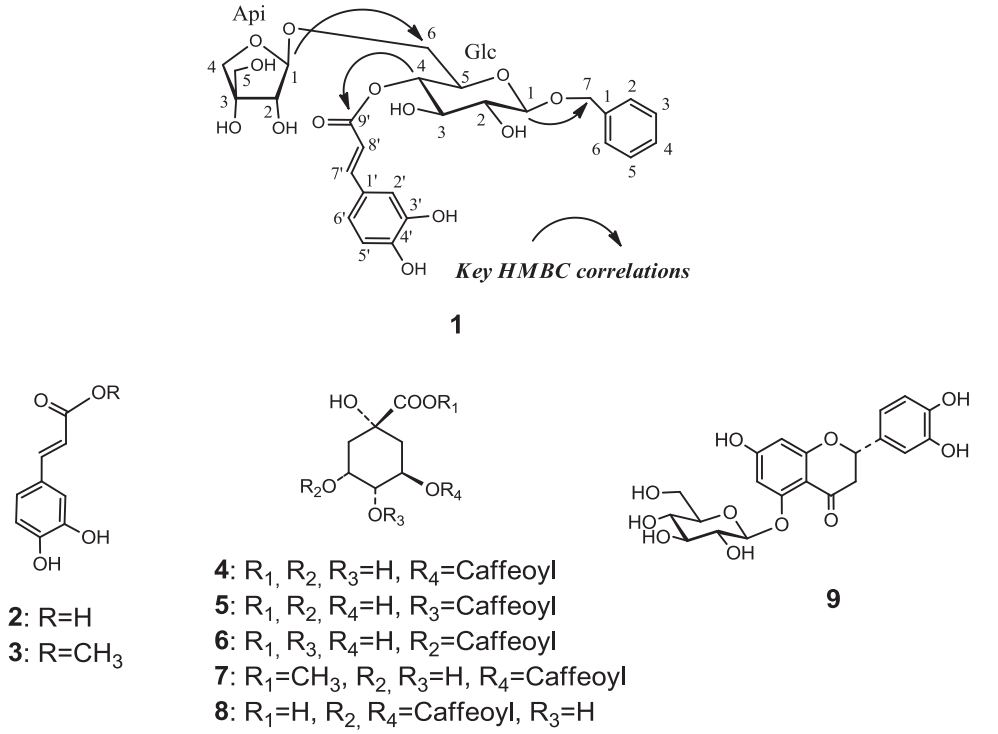

Fig. 2. Structures of compounds **1**–**9** and selected HMBC correlations of **1**.

sunflower seed extract is largely attributed to these caffeic acid derivatives.

3. Experimental

3.1. General

Optical rotations were measured with a JASCO P-1020 digital polarimeter. UV spectra were recorded on a Shimadzu UVmini-1240 (Kyoto, Japan). Electrospray ionization (ESI)-MS and high-resolution (HR) ESI-MS spectra were obtained using a micrOTOF-Q (Bruker Daltonics, Billerica, MA, USA) mass spectrometer with acetonitrile as the solvent. ^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker AVANCE500 instrument (Bruker BioSpin, Billerica, MA, USA) (500 MHz for ^{1}H and 126 MHz for ^{13}C) and chemical shifts are given in ppm values relative to those of the solvents [methanol-d_4 (δ_H 3.30; δ_C 49.0)] on a tetramethylsilane scale. The standard pulse sequences programmed for the instrument (AVANCE 500) were used for each 2D measurement (COSY, HSQC, and HMBC). J_{CH} was set at 8 Hz in HMBC. Column chromatography was carried out with Diaion HP-20, MCI-gel CHP-20P (Mitsubishi Chemical Co., Tokyo, Japan), and YMC gel ODS (YMC Co. Ltd., Kyoto, Japan), respectively. Normal-phase (NP) HPLC conducted on a YMC-Pack SIL A-003 (YMC Co., Ltd.) column (4.6 mm i.d. × 150 mm) developed with *n*-hexane-MeOH-tetrahydrofuran-formic acid (55:33:11:1) containing oxalic acid (450 mg/L) (flow rate: 1.5 mL/min; 280 nm UV detection). Reversed-phase (RP) HPLC conditions were as follows: [Condition 1] column, L-column ODS (5 μm, 150 mm × 2.1 mm i.d.) (Chemicals Evaluation and Research Institute, Tokyo, Japan); mobile phase, solvent A was 5% acetic acid and solvent B was acetonitrile (0–30 min, 0–50% B in A; 30–35 min, 50–85% B in A; 35–40 min, 85–85% B in A); injection volume, 2 μL; column temperature, 40 °C; flow-rate, 0.3 mL/min; detection, 200–400 nm. [Condition 2] column, COSMOSIL Cholester Waters (5 μm, 150 × 2.0 mm i.d.) (Nacalai Tesque, Kyoto, Japan); mobile phase, 10 mM H_3PO_4-10 mM KH_2PO_4-MeOH (37.5:37.5:25); column temperature, 40 °C; flow-rate, 0.2 mL/min; detection, 280 nm. The microplate reader was an Infinite F200 microplate reader (TECAN, Männedorf, Switzerland).

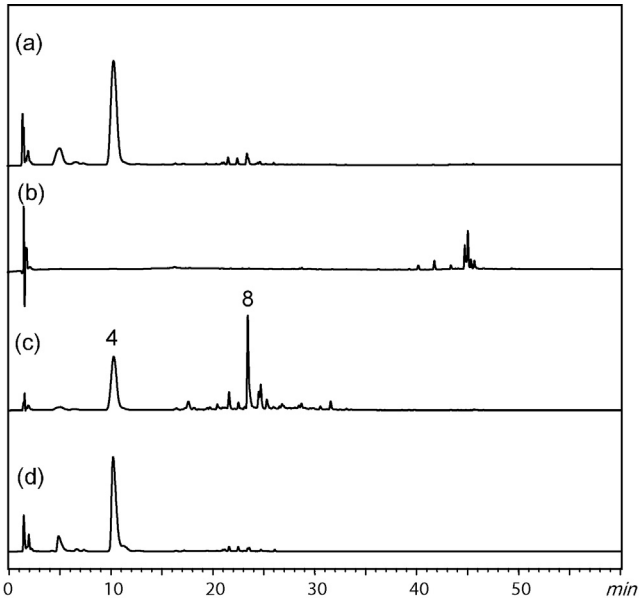

Fig. 3. RP-HPLC profiles of sunflower seed extracts. (a) 50% EtOH extract; (b) 50% EtOH extract-*n*-hexane fraction; (c) 50%EtOH extract-EtOAc fraction; (d) 50%EtOH extract-H_2O fraction. 4, chlorogenic acid; 8, 3,5-di-*O*-caffeoylquinic acid. HPLC conditions are described in condition 1 of "Experimental".

3.2. Samples and reagents

Sunflower seeds (kernels of *H. annuus*) were obtained from Nagaoka Perfumery Co., Ltd. (Osaka, Japan). *N*-Methylmorpholine-*N*-oxide, 2,2′-azobis-(2-amidinopropane) dihydrochloride (AAPH),

L-cysteine methyl ester hydrochloride, and *O*-tolyl isothiocyanate were purchased from Wako Pure Chemical Industries (Osaka, Japan). Fluorescein sodium salt and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were obtained from Sigma-Aldrich (St. Louis, MO, USA). All other reagents were of analytical grade.

3.3. Extraction and isolation

The sunflower seeds (800 g) were homogenized in 50% EtOH [EtOH-H_2O (1:1)] (6 L) and the homogenate was filtered. The filtrate was concentrated and extracted with *n*-hexane (2 L) and EtOAc (5 L) to give the respective *n*-hexane (41.6 g), EtOAc (1.0 g), and water (45.1 g) extracts. The EtOAc extract (1.0 g) was chromatographed over MCI-GEL CHP-20P with MeOH-H_2O (0:100→10:90→20:80→30:70→40:60→100:0) in stepwise gradient mode. The fractions showing similar HPLC patterns (condition 1) were combined and further purified by column chromatography over Sephadex LH-20 with EtOH and/or YMC GEL ODS-AQ with aqueous MeOH to afford caffeic acid (**2**) (2.6 mg), methyl caffeoate (**3**) (2.3 mg), chlorogenic acid (**4**) (15.0 mg), methyl chlorogenate (2.3 mg) (**7**), 3,5-di-*O*-caffeoylquinic acid (**8**) (20 mg), eriodictyol 5-*O*-β-D-glucoside (**9**) (3.6 mg), and benzyl alcohol β-D-apiofranosyl-(1→6)-β-D-(4-*O*-caffeoyl) glucopyranoside (**1**) (3.6 mg). The water extract (2 g) was similarly separated by column chromatography over MCI-GEL CHP-20P with aqueous MeOH to give chlorogenic acid (**4**) (15 mg), 3-*O*-caffeoylquinic acid (**6**) (2.0 mg), and 4-*O*-caffeoylquinic acid (**5**) (2.5 mg). These compounds were identified by direct comparison with authentic specimens or by comparison of their spectral data with those reported in the literature. The physical data of the new compound **1** are as follows.

3.3.1. Benzyl alcohol β-D-apiofranosyl-(1→6)-β-D-(4-O-caffeoyl) glucopyranoside (**1**)

A light brown amorphous powder. UV λ_{max} (MeOH) nm (log ε): 207 (4.22), 248sh (3.89), 289 (4.00), 330 (4.12). $[\alpha]^{23}_{D}$ -54° (*c* 0.5, MeOH). ^{1}H NMR (500 MHz, MeOH-d_4) and ^{13}C NMR (126 MHz, MeOH-d_4) data provided in Table 1. HR-ESI-MS *m/z*: 563.1741 ([M–H]$^-$, Calcd. for $C_{27}H_{32}O_{13}$-H: 583.1770).

3.4. Determination of sugar configuration

The sugar configuration was determined using a previously described method (Tanaka et al., 2007). Compound **1** (1.0 mg) was hydrolyzed by heating in 0.5 M HCl (0.2 mL) and neutralized with Amberlite IRA400. After evaporation, the residue was dissolved in pyridine (0.2 mL) containing L-cysteine methyl ester hydrochloride (1.0 mg) and heated at 60 °C for 1 h. *O*-Tolyl isothyocyanate (1.0 mg) in pyridine (0.2 mL) was then added to the mixture and heated at 60 °C for 1 h. The reaction mixture was directly analyzed by RP-HPLC (condition 2). The peaks coincided with those of derivatives of authentic samples, D-glucose and D-apiose, obtained from apiin (7-*O*-[β-D-apio-D-furanosyl-(1→2)-β-D-glucopyranosyl] apigenin) (Yoshida et al., 1989).

3.5. Antioxidant assay

Antioxidant activity was estimated by the ORAC method (Amakura et al., 2009; Yoshimura et al., 2011). Briefly, the ORAC assay was performed in 75 mM phosphate buffer (pH 7.4) with a final reaction volume of 200 μL. Trolox (20 μL) and fluorescein (120 μL; 70 nM, final concentration) solutions were pipetted into each well of a 96-well microplate. The mixture was pre-incubated in a microplate reader for 15 min at 37 °C. A solution of AAPH (60 μL: final concentration, 12 mM) was added rapidly to the microplate, and, after shaking for 15 s, the fluorescence was recorded every minute for 90 min at excitation and emission wavelengths of 485 and 528 nm, respectively. The area under the curve (AUC) was calculated, and the net AUC was calculated by subtracting the AUC of the blank (phosphate buffer only) from that of each sample. ORAC values are expressed as trolox equivalents (μmol TE/g) using the calibration curve generated in each assay.

Acknowledgments

We thank Ms. Kazusa Ouchi and Ayako Yoshimura of Matsuyama University for their technical assistance. This work was supported by a Health Sciences Research Grant from the Ministry of Health, Labour and Welfare, Japan.

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