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New monoterpene glycosides from sunflower seeds and their protective effects against H_2O_2-induced myocardial cell injury

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ABSTRACT

Three new monoterpene glycosides (**1**–**3**) and eleven known compounds (**4**–**14**) were isolated from seeds of *Helianthus annuus* L. (sunflower). Their structures were determined by spectroscopic and chemical methods. All the compounds were isolated from sunflower seeds for the first time. Protective effects of compounds **1**–**14** against H_2O_2-induced H9c2 cardiomyocyte injury were evaluated, and compounds **1** and **2** showed some cell-protective effects. No significant DPPH radical scavenging activity was observed for compounds **1**–**14**.

1. Introduction

Helianthus annuus L. (sunflower) is an important oilseed crop (Sackston, 1981). Due to its great capability of adaptation to different climatic and soil conditions (Villamide & San Juan, 1998), the crop is cultivated worldwide, and the total production of seeds was 44.7 million metric tons in 2013 according to the Food and Agriculture Organization (FAO) of the United Nations. Along with rape seed, soybean, peanut, and palm oil, sunflower oil is one of the most important vegetable oils and is widely used in the food and nutraceutical industries (Schmidt, 2013).

Sunflower seeds have excellent nutritional properties. The quality of its edible oil ranks it as one of the best vegetable oils among the cultivated plant oils. Up to 90% of the fatty acids in conventional sunflower oils are typically unsaturated fatty acids, namely oleic and linoleic, palmitic, stearic (British Pharmacopoeia, 2005), and minor amounts of myristic, myristoleic, palmitoleic, arachidic, behenic and other fatty acids account for the remaining 10% (Skoric, Jocic, Sakac, & Lecic, 2008). Sunflower seeds, containing high amounts of proteins and significant contents of tocopherols (British Pharmacopoeia, 2005), copper (USDA, 2012), zinc (USDA, 2012), crude fiber (Skrbic & Filipcev, 2008), sesquiterpenes (Macias et al., 2002), diterpenes (Suo et al., 2007), triterpenes (Ukiya et al., 2007), and phenols (Žilić et al., 2010), are used to prepare breads, biscuits and snack foods. Kernels of sunflower seeds are eaten raw or roasted as a rich source of protein and vitamins B, D, E and K. Other studies have shown its benefits in the reduction of cardio-vascular diseases (Zhu, Liu, Xia, & Ma, 2003). In addition to being used as food, sunflower seeds were reported to be used for medicinal purpose. The Indians of North America use the seeds for the treatments of cold, cough, and the ailments related to throat and lung (Putt, 1978). In Venezuela, the flowers and seeds are used in folk medicine for treating cancer (Hartwell, 1982).

Sunflower seeds are a rich and renewable natural resource with high economic value. The seeds are primarily utilized for production of vegetable oil. However, the pressed cake (or the residue resulting from oil extraction), a by-product from the oil production, is underutilized (Weisz, Carle, & Kammerer, 2013). In order to fully utilize this rich resource, more investigations on the chemical constituents in sunflower seeds are needed. In addition to proteins, other chemicals such as phenolic compounds, organic acids, phospholipids, tocopherols, and phytosterols have been found in the seeds (Amakura, Yoshimura, Yamakami, & Yoshida, 2013; Baydar

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& Erbas, 2005; Martinez-Force, Alvarez-Ortega, Cantisan, & Garces, 1998; Rashid, Anwar, & Arif, 2009; Weisz, Kammerer, & Carle, 2009). Recent studies reported more than twenty compounds identified from the seeds, and some of the compounds were found for the first time from *Helianthus* genus (Amakura et al., 2013; Fei, Chen, Li, Xu, & Yang, 2014). In this paper, we report the isolation and structure identification of three new monoterpene glycosides from sunflower seeds, together with eleven other compounds. In addition, the results about the protective effects of these compounds against cardiomyocytes injury induced by H_2O_2, as well as their DPPH radical scavenging activities are included.

2. Materials and methods

2.1. Materials and chemicals

Melting points of the isolates were determined using the XT5 micro-melting-point system (Beijing Optical Instrument Factory, Beijing, China). Specific optical rotation values were determined using a 241 polarimeter (Perkin–Elmer Inc., Waltham, MA, USA). IR spectra were recorded on a 983 G spectrometer (Perkin–Elmer Inc., Waltham, MA, USA). ^{1}H NMR, ^{13}C NMR, and 2D NMR spectra were obtained from an Inova 500 spectrometer (Varian Inc., Palo Alto, CA, USA) in C_5D_5N using tetramethylsilane (TMS) as the internal standard. HR-ESI-MS spectra were determined on a Q-TOF2 spectrometer (Micromass Corp., London, UK). EI-MS was determined on a Micromass Zabspec spectrometer (Micromass Corp., London, UK). High performance liquid chromatography (HPLC) analysis and purification were performed with an Agilent Zorbax SB-C18 semipreparative HPLC column (250 × 9.4 mm i.d., 5 μm, Agilent Corp. Palo Alto, CA, USA) on a Shimadzu HPLC system composed of a LC-20AT pump with an SPD-20A detector (Shimadzu Corp., Kyoto, Japan), the flow rate was 2 mL/min, and the wavelength for detection was 203 nm. Medium pressure liquid chromatography (MPLC) purification was performed on a Büchi Flash Chromatography system composed of a C-650 pump with a flash column (460 mm × 26 mm i.d., Büchi Corp., Flawil, Switzerland). GC analysis was conducted on a GC-14C (Shimadzu Corp., Kyoto, Japan) instrument with a flame ionization detector and the analytical conditions were as follows: DB-5 column (i.d. 0.25 mm, length 30 m; Suzhou Huitong Chromatography Technology Co., Ltd., Suzhou, China), column temperature at 210 °C; injector temperature at 270 °C; and detector temperature at 300 °C. The samples (1 μL) were injected manually into the column.

Silica gel (200–300 mesh) for column chromatography and precoated silica gel TLC plates were purchased from Qingdao Marine Chemical Factory. ODS for MPLC was purchased from Merck KGaA (Darmstadt, Germany). Sephadex LH-20 for column chromatography was purchased from GE Healthcare Corp. (Beijing, China). Compounds on TLC were colored by spraying 10% sulfuric acid alcohol solution and heating. Vitamin E was purchased from J&K Scientific Ltd. (Beijing, China). 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), 1,1-diphenyl-2-picrylhydrazyl (DPPH) and phosphate buffered saline (PBS) were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), mitochondrial dehydrogenase and fetal bovine serum (FBS) were purchased from Gibco™ (Grand Island, NY, USA). Rat H9c2 cardiomyocytes were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). 96-Well plates were obtained from Corning Costar (Corning Costar, Cambridge, MA, USA). CD_3OD and $CDCl_3$ were obtained from Merck (Darmstadt, Germany). Methanol (MeOH) for HPLC analysis and purification was HPLC grade and purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Standard (+)-campholenol and (−)-myrtenol were obtained from Jiangxi Bencaotiangong Technology Co., Ltd. (Nanchang, China). All of the other chemicals and reagents were of analytical grade and purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

2.2. Plant materials

The sunflower seeds (*H. annuus* L.) were purchased from a farmers' market in Qiqihar city (Heilongjiang province, China) in September 2011 and authenticated by Prof. Xiaoran Li (Department of Pharmacognosy, College of Pharmaceutical Science, Soochow University) using morphological identification. A voucher specimen (No. 10-09-08-01) was deposited in the herbarium of the College of Pharmaceutical Science at Soochow University.

2.3. Extraction and isolation

Sun-dried seeds (20 kg) were crushed into fine powder and extracted twice in MeOH (200 L). The solvent was removed under reduced pressure. The resulting residue (150 g) was dissolved in distilled water and fractionated successively with petroleum ether, chloroform, and *n*-butyl alcohol. The chloroform fraction (34 g) was dissolved in chloroform, loaded onto a silica gel column, and eluted with $CHCl_3$–MeOH (80:20, 60:40, 40:60, 0:100; 4.0 L each) under reduced pressure. The $CHCl_3$–MeOH (60:40) eluate (6.2 g) was subjected to separation on a MPLC/ODS column and eluted with MeOH–H_2O (40:60, 60:40, 80:20, and 90:10; 1000 mL each) at 20 mL/min to provide five fractions. Fraction 1 (196 mg) was separated by Sephadex LH-20 gel column chromatography (100 cm × 3 cm i.d.) eluting with MeOH to give compounds **6** (43 mg) and **10** (32 mg). Compounds **7** (31 mg, t_R 13.27 min), **8** (7.2 mg, t_R 29.35 min) and **9** (6.4 mg, t_R 37.15 min) were obtained from fraction 2 (163 mg) after separating by semi-preparative RP-HPLC eluting with MeOH–H_2O (67:33) at 2.0 mL/min. Compounds **4** (15 mg, t_R 37.14 min), **3** (13 mg, t_R 14.61 min), **2** (15 mg, t_R 16.00 min), **5** (15 mg, t_R 26.61 min), and **1** (9.2 mg, t_R 32.70 min) were obtained from fraction 3 (120 mg) after the purification using semi-preparative RP-HPLC with MeOH–H_2O (56:44) for elution. Fraction 4 (90 mg), which was subjected to separation by semi-preparative RP-HPLC and eluted with MeOH–H_2O (86:14), yielded compounds **11** (32 mg, t_R 12.42 min) and **12** (19 mg, t_R 15.62 min). Compounds **13** (51 mg, t_R 28.16 min) and **14** (13 mg, t_R 37.43 min) were obtained from fraction 5 (102 mg) after purification by using semi-preparative RP-HPLC with MeOH–H_2O (89:11) as the eluent.

2.3.1. (+)-Campholenol-10-O-β-D-glucopyranoside (**1**)

(4S)-2,2,3-trimethyl-3-cyclopentene-1-ethanol-10-O-β-D-glucopyranoside. white powder; $[\alpha]_D^{25}$ −36 (c = 0.015, MeOH); ^{1}H NMR (500 MHz, MeOD) and ^{13}C NMR (125 MHz, MeOD) spectroscopic data are shown in Table 1; HR-ESI-MS (negative ion mode) m/z 315.1813 ([M−H]$^-$, calcd for $C_{16}H_{28}O_6$; 315.1808).

2.3.2. (+)-Campholenol-10-O-β-D-apiofuranosyl-(1 → 6)-β-D-glucopyranoside (**2**)

(4S)-2,2,3-trimethyl-3-cyclopentene-1-ethanol-10-O-β-D-apiofuranosyl-(1 → 6)-β-D-glucopyranoside. white powder; $[\alpha]_D^{25}$ −55 (c = 0.016, MeOH); ^{1}H NMR (500 MHz, MeOD) and ^{13}C NMR (125 MHz, MeOD) spectroscopic data are shown in Table 1; HR-ESI-MS (negative ion mode) m/z 447.2236 ([M−H]$^-$, calcd for $C_{21}H_{36}O_{10}$; 447.2230).

Table 1
NMR spectroscopic data of **1**, **2** and **3** (in MeOD).

	1		**2**		**3**	
	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
1	148.1		148.1		43.1	2.22 m
2	121.4	5.21 s	121.4	5.21 s	144.8	
3	35.0	2.29 m	35.0	2.30 m	119.8	5.57 m
		1.84 m		1.85 m		
4	46.7	1.85 m	46.7	1.86 m	30.8	2.28 m
5	46.4		46.4		40.8	2.08 m
6	11.4	1.59 s	11.4	1.59 s	37.5	
7	24.8	0.98 s	24.8	0.99 s	31.1	1.20 m
						2.42 m
8	18.7	0.77 s	18.7	0.78 s	25.2	1.30 s
9	29.9	1.54 m	29.9	1.54 m	20.2	0.87 s
		1.84 m		1.84 m		
10	69.3	3.53 m	69.3	3.53 m	71.5	4.00 dd (2.0, 12.0)
		3.97 m		3.94 m		4.18 dd (1.5, 12.0)
Glc-1′	103.1	4.25 d (8.0[a])	103.2	4.23 d (8.0)	102.0	4.27 d (7.5)
2′	73.7	3.17 m	73.7	3.16 m	73.7	3.18 m
3′	76.7	3.35 m	76.6	3.33 m	76.6	3.32 m
4′	70.2	3.28 m	70.3	3.26 m	70.6	3.28 m
5′	76.5	3.27 m	75.4	3.39 m	75.1	3.38 m
6′	61.3	3.66 m	67.2	3.59 m	66.6	3.60 m
		3.85 m		3.97 m		3.98 m
Apio-1″			109.5	4.99 d (3.0)	109.8	4.96 d (5.0)
2″			76.6	3.89 d (3.0)	81.1	4.01 d (5.0)
3″			79.2		80.9	
4″			73.6	3.76 d (12.0)	74.5	3.82 d (12.0)
				3.95 d (12.0)		3.99 d (12.0)
5″			64.2	3.57 d (12.0)	62.8	3.65 d (12.5)
				3.66 d (12.0)		3.73 d (12.5)

[a] Data in parentheses are *J* values (in Hz).

2.3.3. (−)-Myrtenol-10-O-α-D-apiofuranosyl-(1 → 6)-β-D-glucopyranoside (**3**)

(1R)-6,6-dimethylbicyclo[3.1.1]hept-2-ene-2-methanol-10-O-α-D-apiofuranosyl-(1 → 6)-β-D-glucopyranoside. white powder; $[\alpha]_D^{25}$ +12 (*c* = 0.013, MeOH); ^{1}H NMR (500 MHz, MeOD) and ^{13}C NMR (125 MHz, MeOD) spectroscopic data are shown in Table 1; HR-ESI-MS (negative ion mode) *m/z* 445.2079 ($[M-H]^-$, calcd for $C_{21}H_{34}O_{10}$; 445.2074).

2.4. Acid hydrolysis of compounds **1**–**3**

The acid hydrolysis was used to identify the type and configuration of sugar moiety and aglycone (Gao, Zhang, Liu, Cai, & Yang, 2011). A solution of each monoterpene glycoside (5 mg) was dissolved in 2 N HCl (2 mL) and heated at 90 °C for 2 h. After cooling, the reaction mixture was neutralized with silver carbonate (10 mg), and evaporated to dryness under N_2 overnight. The reaction mixture was extracted with chloroform and H_2O, and both layers were concentrated to dryness. The chloroform extract was purified by Sephadex LH-20 gel column chromatography eluting with $CHCl_3$–MeOH, (1:1). The purified compound (aglycone) was then analyzed to determine its chemical structure by comparing its retention time with that of the standard in the GC analysis and by comparing the specific optical rotation value with that of the standard. The residue from the aqueous layer was dissolved in anhydrous pyridine (1 mL) followed by the addition of 2 mg of L-cysteine methyl ester hydrochloride (0.2 mL). After heating at 60 °C for 2 h, the solvent was evaporated under N_2, and 0.2 mL of (trimethylsilyl) imidazole dissolved in H_2O was added. Then the mixture was heated at 60 °C for another 2 h and the dried reactant was partitioned with *n*-hexane and water (0.1 mL, each). The organic layer was used for GC analysis, and the following sugar units in compounds **1**–**3** were identified by comparison with authentic samples: D-glucose (t_R 11.49 min), L-glucose (t_R 11.10 min), D-apiose (t_R 9.15 min), L-apiose (t_R 9.41 min).

2.5. Assay for testing the protective effects against myocardial cell injury induced by H_2O_2

Rat H9c2 cardiomyocytes were seeded into 96-well flat microtiter plates at a density of 1×10^5 per well and cultured in DMEM supplemented with 10% FBS, 100 U/mL of penicillin, and 100 μg/mL of streptomycin at 37 °C in a humidified atmosphere of 5% CO_2, and allowed to adhere for 24 h before the tested compounds (compounds **1**–**14**) or positive control (vitamin E) were introduced. The tested compounds with five different concentrations (12.5–200 μM) were added and following 12 h of incubation, 200 μM H_2O_2 was added to each well for 24 h (Konorev, Kennedy, & Kalyanaraman, 1999). The cells in the control groups were treated with the same volume of PBS. Cell viability was evaluated by MTT assay, based on the reduction of MTT by the mitochondrial dehydrogenase of intact cells to a purple formazan product. Absorbance was read on an ELISA plate reader (Biorad iMark™ Microplate Reader, Bio-Rad Laboratories, Inc., Hercules, CA, USA) at 540 nm to measure the quantity of formazan. The percentage cell viability was calculated as a ratio of optical density (OD) value of sample to the OD value of a control (Li et al., 2014). All experiments were repeated three times.

2.6. DPPH radical scavenging capacity assay

DPPH free radical scavenging activity of compounds **1**–**14** were measured in triplicate following a previously described method (Hatano, Kagawa, Yasuhara, & Okuda, 1988). Briefly, a solution of 0.5 mM methylated DPPH was prepared. An aliquot (100 μL) of tested compound at final concentrations (5–200 μM) in methanol was mixed with 100 μL of methylated DPPH radical solution and incubated in the dark for 30 min. Vitamin E served as a positive control. A freshly prepared DPPH solution exhibited a deep purple color with an absorption maximum at 517 nm. The DPPH free radical scavenging rate in percent (*I* %) was calculated in following way:

$$I\% = \left(100 \times \left[1 - \left(A_{sample} - A_{blank}\right)/A_{Control}\right]\right)$$

$A_{Control}$ is the absorbance of the control reaction (containing all reagents except the test compound), A_{sample} is the absorbance of the test compound, and A_{blank} is the absorbance of a blank sample.

2.7. Statistical analysis

The results were expressed as the mean ± SD. Differences were evaluated with one way ANOVA. The *post hoc* test was done with the student's Dunnett test. *P* values less than 0.05 were considered statistically significant. Statistical calculations were performed using the SPSS 16.0 for Windows software package.

3. Results and discussion

3.1. Phytochemical investigation

The MeOH extract of dried seeds of *Helianthus annuus* L. was separated by successively partitioning with petroleum ether, chloroform, *n*-butyl alcohol, and by repeated silica gel and ODS column chromatography to afford three new monoterpene glycosides (**1**–**3**) (Fig. 1), as well as eleven known compounds, which were identified as (−)-myrtenol-10-O-*β*-D-glucopyranoside (**4**) (Kawahara, Fujii, Ida, & Akita, 2006), (−)-myrtenol-10-O-*β*-D-apiofuranosyl-(1 → 6)-*β*-D-glucopyranoside (**5**) (Kikuchi et al., 2012), 1,4-butanolide (**6**) (Yu & Yang, 2005), succinic acid (**7**) (Cui et al., 1995), erucic acid (**8**) (Ji et al., 2007), hexadecanoic-2,3-dihydroxypropyl ester (**9**) (Wang, Zhu, Sheng, Chen, & Yang, 2011), 9-octadeconic-2,3-dihydroxypropylester (**10**) (Zhu, Zhu, Zhu, Wen, & Liu, 2011), 9,12,13-trihydroxy-10,15-octadecadienoic acid (**11**) (Li, Kuang, Okada, & Okuyama, 2003), 9,12,13-trihydroxy-10,15-octadecadienoic acid methyl ester (**12**) (Suemune, Harabe, & Sakai, 1988), 9,12,13-trihydroxy-10-octadecaenoic acid (**13**) (Li et al., 2003), and 9,12,13-trihydroxy-10-octadecaenoic acid methyl ester (**14**) (Suemune, Harabe, & Sakai, 1988), based on their spectroscopic data and comparisons with the reference values and authentic samples.

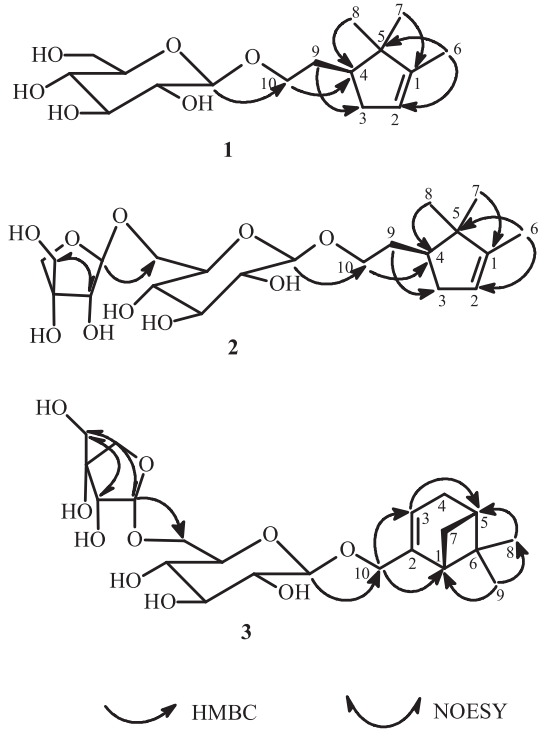

Fig. 1. Structures of compounds **1**–**3** and their key HMBC and NOESY correlations.

Compound **1** was isolated as white powder. The molecular formula of **1** was determined as $C_{16}H_{28}O_6$ on the basis of its HR-ESI-MS (*m/z* 315.1813 ($[M-H]^-$)). The ^1H NMR spectrum showed singlet resonances of three methyl groups at *δ* 1.59 (3H, s, Me-6), 0.98 (3H, s, Me-7), 0.77 (3H, s, Me-8), an olefinic proton signal at *δ* 5.21 (1H, s, H-2) and signals of an oxymethylene group at *δ* 3.97 (1H, m, H-10), 3.53 (1H, m, H-10). The NMR spectroscopic data of compound **1** were similar to those of (+)-campholenol (Chapuis & Brauchli, 1992), except for the presence of additional signals at δ_C 103.5, 76.7, 76.5, 73.7, 70.2 and 61.3, corresponding to one glucopyranosyl group. The assignment of the aglycone structure as (+)-campholenol was further confirmed by the GC analysis of the acid-hydrolysis product of **1** (Zhou, Huang, Song, & Wang, 2012), in which the GC retention time of the aglycone compound from **1** was same as that of the standard (+)-campholenol. Furthermore, the aglycone compound from **1** showed almost the same specific optical rotation value of $[\alpha]_D^{25}$ +20.4 (*c* = 0.005, $CHCl_3$) as that of (+)-campholenol ($[\alpha]_D^{25}$ +18.0) (Chapuis & Brauchli, 1992). The sugar residue obtained from the acid hydrolysis of **1** was identified as D-glucose by the GC analysis. The anomeric proton signal at *δ* 4.25 (1H, d, *J* = 8.0 Hz, H-1 of glucose) showed the correlation to the carbon at δ_C 103.5 (C-1 of glucose) in the HSQC spectrum (Table 1). The glucopyranosyl group was located at C-10 of the aglycone, which was revealed by the HMBC correlation between δ_H 4.25 (H-1 of glucose) and δ_C 69.3 (C-10 of aglycone) (Fig. 1). The *β* configuration of glucopyranosyl group was confirmed on the basis of the large $^3J_{H\text{-}1,H\text{-}2}$ coupling constant. Thus the structure of compound **1** was elucidated as (+) campholenol-10-O-*β*-D-glucopyranoside.

Compound **2** was isolated as white powder. The molecular formula of **2** was determined to be $C_{21}H_{36}O_{10}$ on the basis of its HR-ESI-MS (*m/z* 447.2236 ($[M-H]^-$)). The ^1H NMR spectrum of **2** displayed two anomeric signals at δ_H 4.23 (1H, d, *J* = 8.0 Hz) and 4.99 (1H, d, *J* = 3.0 Hz) suggesting it had two sugar moieties. Based on the ^{13}C NMR and COSY spectra of **2**, the signals at δ_C 64.2, 70.3, 76.6, 79.2, and 109.5 were attributed to a terminal *β*-apiofuranose moiety, and the signals at δ_C 67.2, 70.3, 73.7, 75.4, 76.6, and 103.1 were assigned to a *β*-glucopyranose moiety. Compound **2** showed the same signals at δ_C 148.1, 121.4, 69.3, 46.7, 46.4, 35.0, 29.9, 24.8, 18.7, and 11.4 as compound **1** did, suggesting that it had the same monoterpene aglycone moiety. The assignment of the aglycone structure as (+)-campholenol was further confirmed by the GC analysis of the acid-hydrolysis product of **2**, as well as the specific optical rotation of $[\alpha]_D^{25}$ +19.6 (*c* = 0.004, $CHCl_3$) of the aglycone compound (Chapuis & Brauchli, 1992). In the HMBC spectrum of **2** (Fig. 1), the correlation between H-1 of the glucopyranosyl moiety (δ_H 4.23) and C-10 (δ_C 69.3) of the monoterpene moiety and the correlation between H-1 of the apiofuranosyl moiety (δ_H 4.99) and C-6 of the glucopyranosyl moiety (δ_C 67.2) were observed, revealing the connections of the three moieties. Thus the structure of compound **2** was elucidated as (+)-campholenol-10-O-*β*-D-apiofuranosyl-(1 → 6)-*β*-D-glucopyranoside.

Compound **3** was isolated as white powder. The molecular formula of compound **3** was determined to be $C_{21}H_{34}O_{10}$ on the basis of its HR-ESI-MS (*m/z* 445.2079, ($[M-H]^-$)). The ^1H NMR spectrum of **3** showed two methyl singlet signals at *δ* 1.30 (3H, s, Me-8) and 0.87 (3H, s, Me-9), one olefinic proton signal at *δ* 5.57 (1H, m, H-3), one oxymethylene signals at *δ* 4.00 (1H, dd, *J* = 2.0, 12.0 Hz, H-10) and 4.18 (1H, dd, *J* = 1.5, 12.0 Hz, H-10) (Table 1). The ^1H and ^{13}C NMR spectra indicated that **3** is a myrtenol derivative by comparing its spectroscopic data with previously published data (Yoshikawa et al., 1997). The ^1H NMR spectrum of **3** displayed two anomeric signals at δ_H 4.27 (1H, d, *J* = 7.5 Hz) and 4.96 (1H,

Table 2
Protective effects of monoterpene glycosides from sunflower on H_2O_2-induced H9c2 cell injury.

Compounds	Viabilities of H9c2 cardiomyocyte (%)					
	0 μM	12.5 μM	25 μM	50 μM	100 μM	200 μM
1	65.72 ± 2.52	67.23 ± 1.69	66.18 ± 3.72	68.52 ± 3.03	73.14 ± 2.55*	75.29 ± 3.38*
2	64.58 ± 1.86	64.14 ± 1.65	65.83 ± 2.55	65.22 ± 2.41	67.36 ± 2.60	72.57 ± 2.86*
3	64.28 ± 2.33	67.41 ± 4.70	65.52 ± 2.58	62.93 ± 3.37	66.62 ± 4.39	63.75 ± 3.56
4	68.43 ± 3.20	66.17 ± 2.62	70.35 ± 5.02	66.48 ± 2.77	66.02 ± 3.34	69.95 ± 3.60
5	67.16 ± 2.67	63.08 ± 4.39	64.74 ± 3.84	68.55 ± 2.48	63.02 ± 1.83	65.72 ± 3.61
6	63.32 ± 1.81	64.59 ± 2.60	68.10 ± 4.39	62.97 ± 4.06	65.31 ± 2.49	67.75 ± 2.94
7	68.94 ± 3.73	67.74 ± 3.70	68.02 ± 6.81	65.41 ± 3.20	63.72 ± 4.06	69.06 ± 4.37
8	70.03 ± 5.16	67.36 ± 3.87	63.60 ± 4.14	66.75 ± 3.12	71.21 ± 4.74	66.95 ± 2.32
9	69.36 ± 4.39	63.92 ± 5.58	69.81 ± 5.53	63.36 ± 1.28	66.42 ± 3.37	69.76 ± 2.94
10	65.63 ± 2.97	64.03 ± 3.40	67.34 ± 3.08	68.09 ± 2.76	66.86 ± 3.25	63.95 ± 2.72
11	69.96 ± 3.84	67.18 ± 2.58	68.93 ± 4.43	65.71 ± 3.12	70.55 ± 3.31	66.73 ± 3.79
12	63.48 ± 3.76	66.50 ± 2.74	68.15 ± 5.32	64.36 ± 4.52	66.93 ± 3.81	64.77 ± 4.18
13	67.26 ± 3.51	65.19 ± 2.63	70.08 ± 1.96	63.35 ± 2.93	68.37 ± 3.64	69.09 ± 5.53
14	69.84 ± 3.51	67.87 ± 2.58	69.14 ± 3.45	67.35 ± 3.02	69.49 ± 4.70	68.31 ± 4.01
Vitamin E[a]	63.56 ± 2.38	65.19 ± 2.03	67.28 ± 1.72	68.52 ± 2.46	75.80 ± 1.83*	81.68 ± 1.66**

[a] Positive control.
* $p < 0.05$.
** $p < 0.01$ vs. 0 μM group.

Table 3
Maximal DPPH radical.

Compounds	Scavenging rate (200 μM)
1	1.26 ± 0.22%
2	6.67 ± 2.32%
3	5.68 ± 2.65%
4	0
5	1.92 ± 0.71%
6	4.72 ± 1.41%
7	1.52 ± 1.74%
8	5.23 ± 0.81%
9	4.28 ± 1.41%
10	5.17 ± 3.72%
11	3.85 ± 3.84%
12	2.79 ± 1.01%
13	3.23 ± 2.41%
14	4.83 ± 1.41%
Vitamin E	93.23 ± 0.72%

d, J = 5.0 Hz) suggesting it possessed two sugar units. The GC analysis of the sugar residue obtained from the acid hydrolysis of **3** revealed that the two sugars were D-glucose and D-apiofuranose. The glucopyranosyl group was located at C-10 of the aglycone, which was revealed by the HMBC correlation between δ_H 4.27 (H-1 of glucose) and δ_C 71.5 (C-10 of aglycone). Similarly, the apiofuranosyl unit was located at C-6 of the glucose, which was revealed by the HMBC correlation between δ_H 4.96 (H-1 of apiofuranose) and δ_C 66.6 (C-6 of glucose) (Fig. 1). The β-anomeric configuration of the glucuronopyranosyl unit was determined on the basis of the observation of the large $^3J_{H\text{-}1,\ H\text{-}2}$ coupling constant, and the α-anomeric configuration of the apiofuranosyl unit was indicated by NOESY correlations between δ 4.96 (H-1 of apiofuranose) and δ 3.65, 3.73 (H-5 of apiofuranose) (Fig. 1). The NMR data of compound **3** were similar to those of compound **5**, and the main differences arose from the significant down-field shifts of C-2 of D-apiofuranose (+4.5 ppm) at δ 81.1 and C-3 of D-apiofuranose (+1.7 ppm) at δ 80.9, due to a α-D-apiofuranosyl unit in replace of the β-D-apiofuranosyl unit in compound **5**. The specific optical rotation value of the aglycone compound obtained by acid hydrolysis of compound **3** was $[\alpha]_D^{25}$ −43.2 (c = 0.006, $CHCl_3$), almost the same as that of (−)-myrtenol ($[\alpha]_D^{25}$ −47.5), indicating that the aglycone of compound **3** was (−)-myrtenol (Kover, Schottelius, & Hoffmann, 1991). Thus, compound **3** was identified as (−)-myrtenol 10-O-α-D-apiofuranosyl-(1 → 6)-β-D-glucopyranoside.

3.2. Protective effects of the compounds against H_2O_2-induced myocardial cell injury

The intracellular damage induced by reactive oxidative species (ROS) could lead to various diseases such as cardiovascular diseases (Fearon & Faux, 2009). It is believed that oxidative stress plays a pivotal role in the pathogenesis of cardiomyocytes ischemic diseases. Studies showed that antioxidants could mitigate oxidative-stress-mediated cellular injury (Yousuf et al., 2009). Hydrogen peroxide can easily cross cellular membranes, triggering the chain reactions resulting in the damage of cells (Valko et al., 2007). Therefore, H_2O_2 is usually employed in the models for investigating H9c2 cardiomyocyte injury (Hescheler et al., 1991; Yasuoka et al., 2004). In this study, compounds **1–14** were investigated for their protective effects against H_2O_2-induced myocardial cell injury. Compounds **1** and **2** increased the viability of H9c2 induced by H_2O_2 with a dose-dependent manner at the concentration range from 12.5 to 200 μM (Table 2). Vitamin E can incorporate into the membrane lipids of endothelial cells to protect against membrane lipid peroxidation by quenching cytotoxic ROS (Fujimoto, Mizoi, Yoshimoto, & Suzuki, 1984; Yamamoto et al., 1983). We speculated that the active compounds isolated from sunflower might play a similar antioxidant role in the prevention of peroxidative damage. Therefore, the radical scavenging capacity of the isolates were evaluated in the present study. Compounds **1** and **2** might play a similar role according to the results. Further studies are warranted to understand their potential mechanism and to reveal their other bio-activities.

3.3. DPPH radical scavenging capacity assay

Compounds **1–14** were evaluated for antioxidant activity by DPPH radical scavenging capacity assay. As shown in Table 3, the maximal scavenging rates of compounds **1–14** were less than 20% at the concentration of 200 μM. The results suggested that compounds **1–14** had no significant DPPH radical scavenging capacity.

4. Conclusion

Three new monoterpene glycosides (**1–3**) and eleven known compounds were isolated and characterized from sunflower seeds. To our knowledge, it is the first time report about the compounds isolated from sunflower seeds. The protective effects of the isolated

compounds against H_2O_2-induced myocardial cell injury were investigated. Compounds **1** and **2** increased the viability of H9c2 induced by H_2O_2 with a dose-dependent manner at the concentration range from 12.5 to 200 μM. The DPPH radical scavenging capacity assay suggested that protective effects on H_2O_2 induced myocardial cell injury were not directly associated with their radical scavenging capacity.

5. Conflict of interest

The authors declare that there are no conflicts of interest.

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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.foodchem.2015.04.079.

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