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Phytochemical screening of flavonoids with their antioxidant activities from rapeseed (*Brassica napus* L.)

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ABSTRACT

Ten flavone compounds, including three new flavonoid glycosides, were isolated from defatted rapeseed, and their protective antioxidant effect on H_2O_2-induced oxidative damage in human umbilical vein endothelial cells (ECV-304) was investigated. Three new flavonoid glycosides were identified as kaempferol-3-*O*-[(6-*O*-sinapoyl)-β-D-glucopyranosyl-(1→2)-β-D-glucopyranoside]-7-*O*-β-D-glucopyranoside (**8**), kaempferol-3,7-di-*O*-β-D-glucopyranoside-4'-O-(6-O-sinapoyl)-β-D-glucopyranoside (**9**), and kaempferol-3-*O*-[(3-*O*-sinapoyl)-β-D-glucopyranosyl-(1→2)-β-D-glucopyranoside]-7-*O*-β-D-glucopyranoside (**10**). The protective effects of all of the isolated compounds on H_2O_2-induced oxidative damage were assessed, and the activities of superoxide dismutase (SOD) and lactate dehydrogenase (LDH) were measured. All of compounds had a protective effect on H_2O_2-induced oxidative damage in ECV-304 cells and the presence of a substituted sinapoyl group and its position in the structures were used to elucidate the activity differences.

1. Introduction

Rapeseed (*Brassia napus* L.) is the most common source of vegetable oils in Europe. Rapeseed meal, comprised of substantial available natural resources, is a byproduct of the oil removal process. Since Marczak et al. (2003) had examined the protein of the rapeseed meal as a source of new peptides with ACE-inhibitory activity, it appears to be a good source of other bioactive compounds, such as polyphenols, phytosterols, tocopherols from the rapeseed meal, which are important in the prevention and treatment of many disease (Szydlowska-Czerniak et al., 2011). Much work has been performed to find interesting constituents as sources of natural antioxidants from rapeseed meal. Up to now, phytochemical studies led to the isolation of several phenolic compounds and peptides hydrolyzed from rapeseed oil and protein (Harbaum-Piayda et al., 2010). Kuwahara et al., (2004) and Wakamatsu et al., (2005) had reported a phenolic compound, named 4-vinyl-2,6-dimethoxyphenol (canolol), exhibited more potent anti-alkylperoxyl radical activity than well-known antioxidants, such as α-tocopherol, vitamin C, and β-carotene. Although (Durkee and Harborne, 1973) few flavonoid glycosides and glucopyranosyl sinapate from rapeseed were reported, limited information was available on further study of their antioxidant activity (Amarowicz et al., 1995; Amarowicz and Kolodziejczyk, 2001; Amarowicz and Shahidia, 1994).

From this information, we speculated that rapeseed contains other unknown constituents with remarkable antioxidant activity and were prompted to undertake further studies to assist in its medicinal and food applications. Three new flavonoid glycosides were isolated and identified, their antioxidant activity were also discussed.

2. Materials and methods

2.1. General procedures

Compound samples were dissolved in dimethyl sulfoxide (DMSO-d_6). ^{1}H, ^{13}C, and 2D NMR spectra (^{1}H–^{1}H COSY, HSQC, HMBC) were obtained from the analytical center of the Beijing University of Chemical Technology and recorded at 25 °C on a Bruker AV 600 NMR spectrometer operated at a ^{1}H frequency of 600 MHz and ^{13}C frequency of 150 MHz (Bruker BioSpin, Rheinstetten, Germany). Chemical shifts (^{1}H) were expressed in ppm and coupling constants (*J*) were reported in Hz. Thermo Scientific Orbitrap Velos Pro hybrid ion trap Orbitrap mass spectrometer was used for determining the accurate molecular weight of the isolated compounds. Optical rotations were measured using an Atago AP-300 automatic polarimeter (Atago Co., Tokyo, Japan), and UV

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spectra were recorded on a Shimadzu SPD-M20AVP UV–vis photodiode array detector (DAD) (Shimadzu Co., Kyoto, Japan). An LC 3000 preparative liquid chromatography system (Beijing ChuangXin TongHeng Science and Technology Co., Ltd., Beijing, China) was used to carry out separations and purifications. Diaion HP-20 (Mitsubishi Chemical Co., Tokyo, Japan), Sephadex LH-20 (Pharmacia Biotech AB, Uppsala, Sweden), and silica gel 60 (160–200 mesh, Qingdao, Qingdao Chemical Work) were used for column chromatography. Thin-layer chromatography (TLC) was performed on silica gel F-254 plates (Merck), and spots were detected by ultraviolet (UV) illumination. GC analysis was performed on an HP-5 column (30 m, 0.320 mm ID) with the column temperature at 230 °C.

2.2. Materials, solvents and chemicals

The defatted rapeseed meal was supplied by Guizhou Great Wall Oil Co., Ltd. (Guiyang, Guizhou). All organic solvents used for crude sample preparation were of analytical grade and supplied by Beijing Chemical Factory (Beijing, China). HPLC-grade methanol and acetonitrile (Merck KGaA, Darmstadt, Germany) were supplied by Beijing Chemical Factory (Beijing, China). Deuterated solvents were supplied by J&K Scientific Ltd., (Beijing, China). Water was purified by a Milli-Q water purification system (Millipore, Milford, MA, USA). Dulbecco's modified Eagle's medium F12 (DMEM-F12) was obtained from Gibco (Grand Island, NY, USA, batch no: 8119292). Reagent kits for measurement of superoxide dismutase (SOD), with batch No. 20140108, and lactate dehydrogenase (LDH), with batch No. 20140307, were provided by Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

2.3. Extraction and isolation

The milled rapeseed meal (20 kg) was defatted three times with *n*-hexane and subjected to extraction at reflux with a 60% aqueous ethanol solution for three times. The obtained extract was then

Table 1
^{1}H[a]- and ^{13}C[b]-NMR data of compounds **8**,**9** and **10**.

Position	8		9		10	
	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
2		156.3		156.1		156.2
3		133.0		134.0		133.0
4		177.5		177.7		177.6
5		160.8		160.9		160.8
6	6.37 (*d*, 1.9)	99.2	6.46 (*br.* s)	99.4	6.43 (*d*, 2.0)	99.3
7		162.6		162.9		162.7
8	6.62 (*d*, 1.9)	94.2	6.70 (*br.* s)	94.4	6.80 (*d*, 2.0)	94.3
9		155.8		156.1		155.9
10		105.3		105.8		105.6
1′		120.3		123.6		120.6
2′	8.01 (*d*, 8.8)	131.0	8.10 (*d*, 8.8)	130.7	8.09 (*d*, 8.8)	131.0
3′	6.89 (*d*, 8.8)	115.4	7.18 (*d*, 8.8)	115.6	6.94 (*d*, 8.8)	115.4
4′		160.6		159.1		160.3
5′	6.89 (*d*, 8.8)	115.4	7.18 (*d*, 8.8)	115.6	6.94 (*d*, 8.8)	115.4
6′	8.01 (*d*, 8.8)	131.0	8.10 (*d*, 8.8)	130.7	8.09 (*d*, 8.8)	131.0
5-OH	12.51 (*br.* s)		12.55 (*br.* s)		12.65 (*br.* s)	
1″	5.60 (*d*, 7.2)	97.9	5.48 (*d*, 7.5)	100.7	5.75 (*d*, 7.3)	97.8
2″	3.43 (o[c])	83.2	3.16 (o)	74.2	3.55 (o)	81.9
3″	3.32 (m)	76.4	3.22 (m)	76.5	3.30 (m)	76.8
4″	3.25 (o)	69.6	3.16 (o)	69.5	3.10 (o)	69.7
5″	3.06 (o)	77.1	3.41 (o)	77.2	3.11 (o)	77.5
6″	3.70,3.75 (each, o)	60.4	3.67, 3.45 (each, o)	60.6	3.51,3.26 (each, o)	60.5
1‴	4.69 (*d*, 7.6)	104.4	5.06 (*d*, 7.2)	99.7	4.81 (*d*, 7.8)	103.6
2‴	3.32 (*d*, 8.4)	74.5	3.26 (o)	73.2	3.29 (o)	72.3
3‴	3.42 (o)	76.2	3.42 (o)	76.2	4.96 (t, 9.4)	77.4
4‴	3.24 (*d*, 8.1)	69.6	3.08 (o)	69.9	3.45 (m)	67.6
5‴	3.50 (o)	73.9	3.27 (o)	77.7	3.51 (o)	76.7
6‴	4.30 (m), 4.23 (*d*, 10.7)	63.3	3.58, 3.35 (each, o)	60.6	3.63, 3.55 (each, o)	60.4
1⁗	5.03 (*d*, 7.6)	99.7	5.14 (*d*, 7.5)	99.8	5.08 (*d*, 7.4)	99.7
2⁗	3.26 (o)	73.1	3.32 (o)	73.1	3.26 (o)	73.1
3⁗	3.17 (o)	77.1	3.17 (o)	76.2	3.30 (o)	76.4
4⁗	3.27 (o)	69.6	3.35 (o)	69.4	3.17 (o)	69.6
5⁗	3.07 (o)	77.5	3.34 (o)	73.7	3.45 (o)	77.2
6⁗	3.44,3.52 (each, o)	60.6	4.35,4.29 (each, o)	62.9	3.71,3.47 (each, o)	60.6
Sinapoyl						
1		166.5		166.6		166.2
2	6.24 (*d*, 15.8)	114.1	6.54 (*d*, 15.8)	114.5	6.95 (*d*, 15.8)	115.4
3	7.37 (*d*, 15.8)	145.5	7.55 (*d*, 15.8)	145.6	7.56 (*d*, 15.8)	145.0
1′	123.9	124.1	124.5			
2′	6.77 (s)	106.0	6.99 (s)	106.2	7.03 (s)	106.1
3′	147.9	148.0	148.0			
4′		138.7		138.6		138.1
5′		147.9		148.0		148.0
6′		106.0		106.2		106.1
OCH_3		56.0		56.0		56.1

[a] ^{1}H NMR data were obtained at 600 MHz with dimethyl sulfoxide-d_6 (25 °C). Chemical shifts are shown in δ values relative to solvent peak. Multiplicity and coupling constant (s) in Hz are in parentheses.
[b] ^{13}C NMR data were obtained at 150 MHz with dimethyl sulfoxide-d_6 (25 °C). Chemical shifts are shown in δ values relative to solvent peak.
[c] Overlapped signals were indicated by "o".

concentrated under reduced pressure in a rotary evaporator to remove the ethanol, yielding the crude sample extract. The residue was dissolved in water and extracted with ethyl acetate for three times, yielding the EtOAc extract (190 g). The water solution was separated by commercially available AB-8 column chromatography using H_2O as the initial eluent, followed by 30%, 60%, and 90% aqueous ethanol. Each solution was evaporated in vacuo to yield the H_2O fraction (523 g), 30% fraction (180 g), 60% fraction (200 g) and 90% fraction (9.6 g). The EtOAc extract was pulverized and chromatographed on a silica gel column (column volume 1500 mL) using varying ratios of petroleum ether–ethyl acetate (PE/EtOAc), PE–acetone and chloroform–methanol ($CHCl_3$/MeOH) as the eluting solvents to afford 1–135 fractions.

Fractions 72-83 were further subjected to Sephadex LH-20 column chromatography elution with 90% aqueous methanol to give compound **1** (60 mg). Fractions 115–127 were chromatographed on an ODS C18 gel column (20 mm × 250 mm, 5 μm) and eluted with MeOH–H_2O (6:4) to afford compound **2** (35 mg), compound **3** (15 mg) and compound **4** (27 mg). The 60% fraction from the macroporous resin was preliminarily separated on an MCI gel column to yield 10%, 30%, 45%, 60% and 100% subfractions. The 30% aqueous methanol subfraction was separated on a Sephadex LH-20 column chromatography to yield a crude sample of compounds **5** (21 mg), **6** (13 mg), **7** (120 mg) and **8** (55 mg) and further purified on a semi-preparative HPLC eluted with H_2O–acetonitrile (91:9). Similarly, compounds **9** (32 mg) and **10** (19 mg) were purified by semi-preparative scale HPLC eluted with H_2O-acetonitrile (89:11) from the 45% subfraction.

Kaempferol-3-*O*-[(6-*O*-sinapoyl)-β-D-glucopyranosyl-(1 → 2)-β-D-glucopyranoside]-7-*O*-β-D-glucopyranoside (**8**). Pale yellow powder; $[\alpha]_D^{20}$ −2.7 (c 0.100, MeOH); UV λ_{max} (MeOH): 225, 267, 328 nm; ^{1}H and ^{13}C NMR data: see Table 1; ESI/Orbit Trap MS (*m*/*z*): $[M+Na]^+$ 1001.2541 calcd. for $C_{44}H_{50}O_{25}Na$: found, 1001.2559.

Kaempferol-3,7-di-*O*-β-D-glucopyranoside-4′-*O*-(6-O-sinapoyl)-β-D-glucopyranoside (**9**). Pale yellow powder; $[\alpha]_D^{20}$ −3.2 (c 0.100, MeOH); UV λ_{max} (MeOH): 243, 267, 324 nm; ^{1}H and ^{13}C NMR data: see Table 1; ESI/Orbit Trap MS (*m*/*z*): $[M+Na]^+$ 1001.2541 calcd for $C_{44}H_{50}O_{25}Na$: found, 1001.2532.

Kaempferol-3-*O*-[(3-*O*-sinapoyl)-β-D-glucopyranosyl-(1 → 2)-β-D-glucopyranoside]-7-*O*-β-D-glucopyranoside (**10**). Pale yellow powder; $[\alpha]_D^{20}$ −5.1 (c 0.100, MeOH); UV λ_{max} (MeOH): 234, 266, 367 nm; ^{1}H and ^{13}C NMR data: see Table 1; ESI/Orbit Trap MS (*m*/*z*): $[M+Na]^+$ 1001.2541 calcd. for $C_{44}H_{50}O_{25}Na$: found, 1001.2551.

2.4. Acid hydrolysis of compounds *8*, *9* and *10*

Compounds **8**, **9** and **10**, 2 mg each in a mixture of 3.0 M trifluoroacetic acid (2 mL), were separately hydrolyzed for 3 h at 110 °C. In each case, the reaction mixture was extracted with ethyl

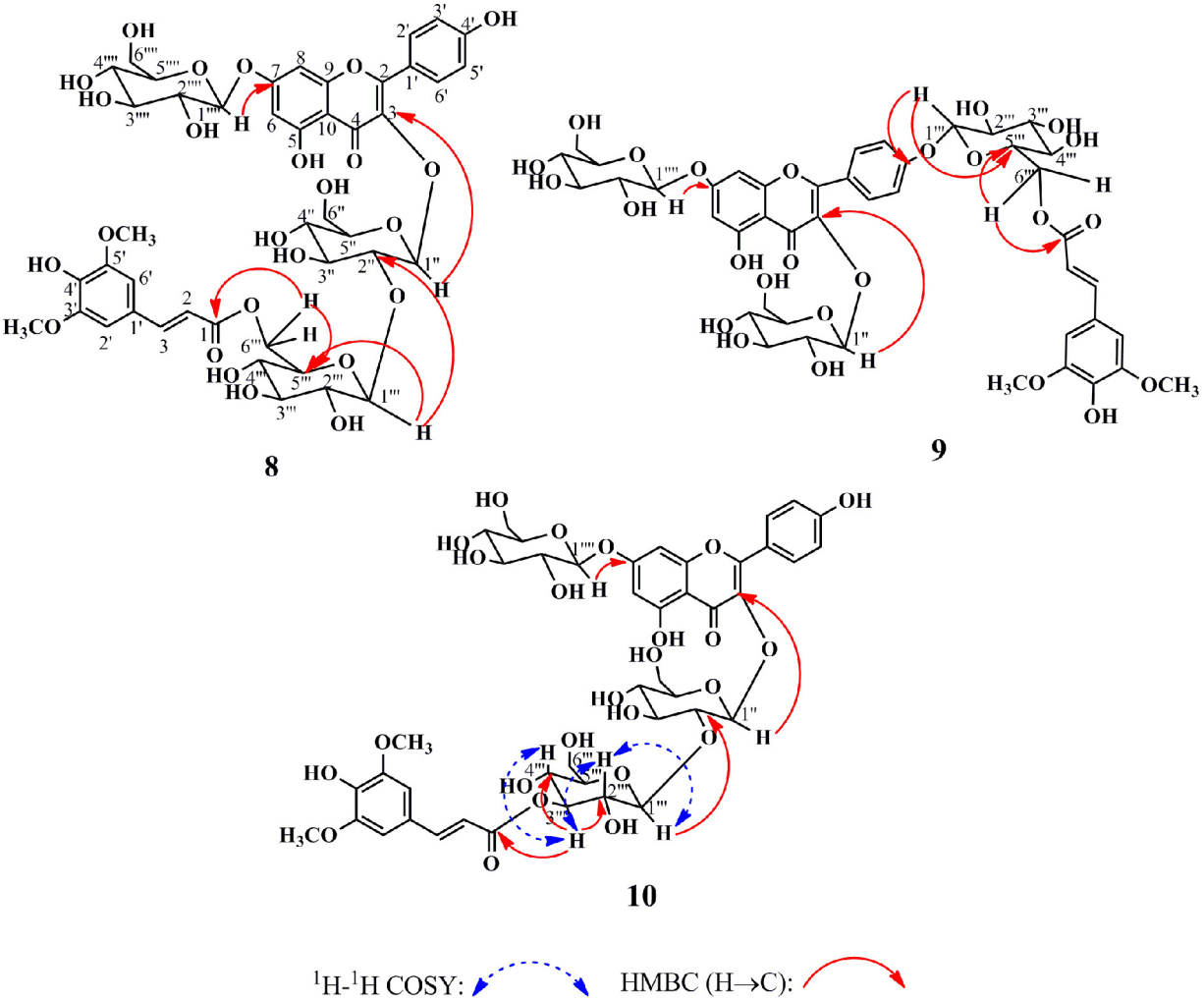

Fig. 2. Structures and significant HMBC and ^{1}H-^{1}H COSY correlations of compounds **8, 9** and **10**.

acetate (3 × 2 mL) and the water soluble mixture was reduced in vacuo to dryness. The residue was dissolved in anhydrous pyridine (100 μL), and 0.06 M L-cysteine methyl ester hydrochloride was added. After heating for 1 h at 60 °C, the reaction mixture was treated with 150 μL of HMDS–TMCS (1:1) and incubated for 0.5 h at 60 °C. Finally, the reaction mixture was centrifuged, and the supernatant was analyzed by gas chromatography. The sugars were identified by comparison of the retention times with those of authentic samples. The derivative of D-glucose was detected at a retention time t_R of 12.04 min.

2.5. Effect of the isolated compounds on hydrogen peroxide-induced oxidative damage in human umbilical vein endothelial cells (ECV-304)

ECV-304 cells were isolated from the vein of normal human umbilical cord as previously reported (Martin et al., 1993). The cells were cultured in DMEM-F12 supplemented with 10% heat-inactivated fetal bovine serum, 100 units/mL penicillin and 100 units/mL streptomycin at 37 °C under 5% CO_2 and 95% air. Cells digested by trypsin and cultured in vitro were transferred to each well of a 24-well microplate with fresh DMEM culture medium and no serum, and randomly divided into three groups: blank control group, model group treated with 100 μg/mL of H_2O_2 for 12 h, and drug group successively treated with H_2O_2 and test compounds for 12 h. After 12 h, the supernatant was collected and used for the detection of SOD and LDH activity using commercially available kits. SOD was detected at 550 nm, while LDH release represents cell viability and was determined at 440 nm. Each experiment was carried out at least six times, and all of the results were expressed as the mean ± S.D. Comparison between experiment groups was performed by ANOVA followed by Tukey's multiple range tests using SPSS 19.0 (SPSS, Inc., Chicago, IL). *P* values less than 0.05 were considered to be significant.

3. Result and discussion

3.1. Structural elucidation of compounds *8*, *9* and *10*

Compound **8** was obtained as pale amorphous powder and exhibited a quasi-molecular ion peak at *m*/*z* 1001.2559 $[M+Na]^+$, consistent with a molecular formula of $C_{44}H_{50}O_{25}Na$ from the HR ESI–MS. Acid hydrolysis of the compound afforded D-glucose as the sugar residues, which was confirmed by GC using authentic samples. A typical signal at δ 12.51 ascribable to 5-OH was observed in the ^{1}H NMR spectrum, indicating a free hydroxyl group at the C-5. Signals due to an aromatic AA'BB' system with the expected coupling pattern were also observed, and the assignment of ^{1}H and ^{13}C NMR spectra data, supported by HSQC and HMBC correlations, confirmed the aglycone as kaempferol. Moreover, peaks corresponding to three anomeric protons at δ 5.60 (*d*, 7.2), 5.03 (*d*, 7.6) and 4.69 (*d*, 7.6), with corresponding anomeric carbon signals at δ 97.9, 99.7 and 104.4 based on the HSQC spectrum, suggested a tri-glycoside nature of this compound. The diaxial coupling constants between the anomeric protons and H-2 in the ^{1}H NMR indicated that all of the glucosyl units were β-linked (Agrawal, 1992). In addition, the presence of two methoxy groups at δ 3.72 (each, 3H, s), two phenyl protons at δ 6.77 (each, 1H, s), and the *trans* olefinic protons at δ 6.24 and 7.37 (each, *d*, 15.8) in the ^{1}H NMR clearly revealed the existence of a sinapoyl group (Jung et al., 2009).

In the ^{13}C NMR spectrum, a carbon signal due to C-2″ of a glucose unit shifted downfield at δ 83.2. In comparison with kaempferol-3-glucoside (Agrawal, 1992), it was proved the attachment of an additional glucose unit at this position and suggested a glucopyranosyl-(1 → 2)-β-D-glucopyranoside moiety. This was also further supported by the HMBC correlation peak observed between the anomeric proton (H-1‴, δ 4.69) of a glucose unit and the carbon at δ 83.2 assigned to C-2″ of the kaempferol inner 3-glucosyl moiety. Additionally, according to the HMBC spectrum, the signals of glucosyl anomeric protons at δ 5.60 (H-1″)

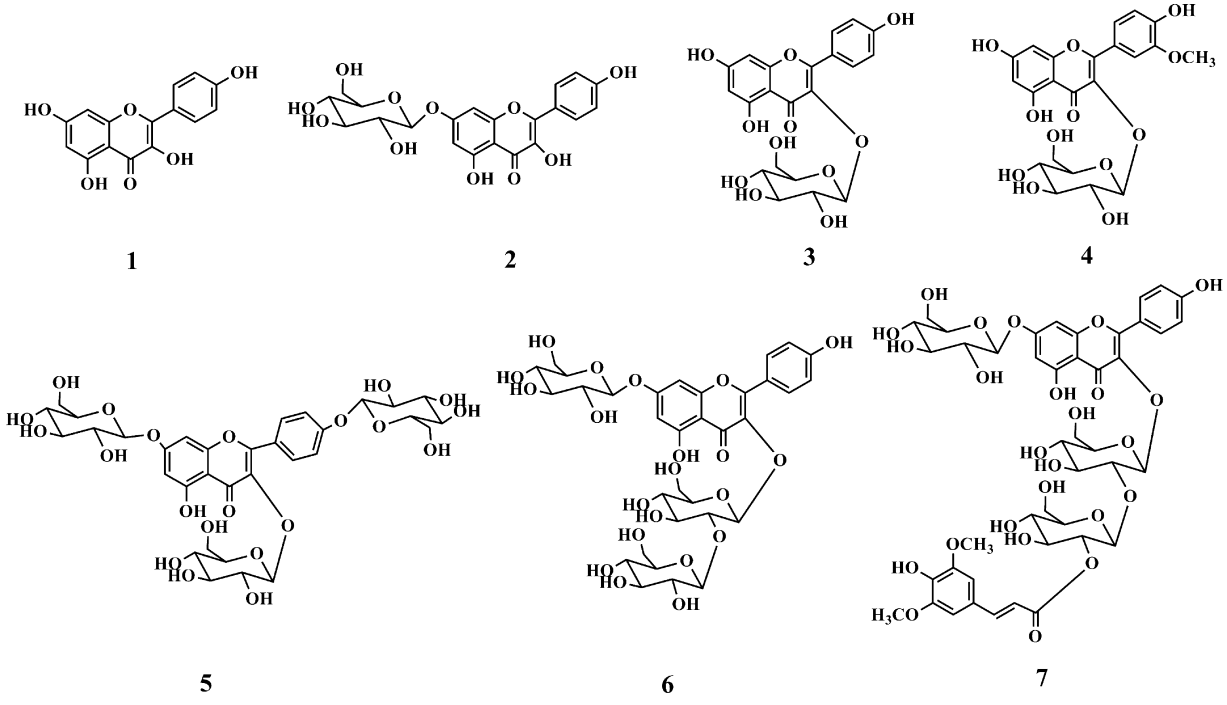

Fig. 1. Structures of the known flavonoids **1**–**7**.

Table 2
Evaluation on LDH and SOD activity in the supernatant of ECV-304 cells of the isolated compounds (mean ± s).

Compounds	Concentration (μg/mL)	LDH (U/L)	SOD (U/mL)
Blank control group	–	121.9 ± 10.1	25.90 ± 1.62
Model control group	–	323.8 ± 6.8[a,#]	19.60 ± 0.73[#]
Kaempferol **(1)**	200	154.5 ± 14.6[**]	24.81 ± 0.42[**]
	100	162.8 ± 6.4[**]	23.90 ± 0.29[**]
Astragalin **(2)**	200	245.9 ± 7.4[**]	22.96 ± 0.34[**]
	100	314.1 ± 11.5	20.44 ± 0.48[*]
Kaempferol-7-*O*-β-D-glucopyranoside **(3)**	200	171.2 ± 2.6[**]	23.91 ± 0.55[**]
	100	276.0 ± 9.0[**]	22.00 ± 0.46[**]
Isorhamnetin-3-*O*-β-D-glucopyranoside **(4)**	200	270.2 ± 6.1[**]	22.65 ± 0.36[**]
	100	317.8 ± 8.7	20.13 ± 0.27[*]
Kaempferol-3,7,4′-tir-*O*-β-D-glucopyranoside **(5)**	200	239.8 ± 3.8[**]	21.80 ± 0.48[**]
	100	293.8 ± 29.3[**]	19.77 ± 0.30
Kaempferol-3-*O*-β-D-glucopyranosyl-(1 → 2)-β-D-glucopyranoside-7-*O*-β-D-glucopyranoside **(6)**	200	198.4 ± 3.2[**]	21.90 ± 0.33[**]
	100	257.9 ± 13.7[**]	20.36 ± 0.28
Kaempferol-3-*O*-[(2-*O*-sinapoyl)-β-D-glucopyranosyl-(1 → 2)-β-D-glucopyranoside]-7-*O*-β-D-glucopyranoside **(7)**	200	125.2 ± 4.9[**]	24.22 ± 0.35[**]
	100	177.7 ± 18.4[**]	23.42 ± 0.73[**]
Kaempferol-3-*O*-[(6-O-sinapoyl)-β-D-glucopyranosyl-(1 → 2)-β-D-glucopyranoside]-7-*O*-β-D-glucopyranoside **(8)**	200	245.2 ± 17.2[**]	21.56 ± 0.42[**]
	100	273.8 ± 16.12[**]	20.02 ± 1.71
Kaempferol-3,7-di-*O*-β-D-glucopyranoside-4′-*O*-(6-*O*-sinapoyl)-β-D-glucopyranoside **(9)**	200	239.8 ± 7.3[**]	19.97 ± 0.77
	100	268.0 ± 8.7[**]	18.80 ± 0.32
Kaempferol-3-*O*-[(3-*O*-sinapoyl)-β-D-glucopyranosyl-(1 → 2)-β-D-glucopyranoside]-7-*O*-β-D-glucopyranoside **(10)**	200	196.6 ± 4.9[**]	21.50 ± 1.51[**]
	100	229.6 ± 11.6[**]	21.98 ± 0.41[**]

[a] Compared with blank control group.
[#] $P < 0.01$, compared with model control group.
[*] $P < 0.05$, compared with model control group.
[**] $P < 0.01$ compared with model control group.

and δ 5.03 (H-1‴) were correlated with C-3 (δ 133.0) and C-7 (δ 162.6) of the aglycone, respectively, indicating the presence of a 3,7-diglucosyl residue. Similarly, the location of the sinapoyl group was confirmed by an HMBC experiment, where correlation peaks were observed between proton signals at δ 4.30, 4.23 (H-6‴) of a glucose unit and the ester carbonyl at δ 166.5 of the sinapoyl unit, proving that the attachment of the sinapoyl unit was at C-6‴. These results were consistent with a downfield shift of C-6‴(α-C) at δ 63.3 and an upfield shift of C-5‴ (β-C) at 73.9 of the kaempferol outer 3-glucosyl moiety, and further corroborated by HMBC analyses: correlation peaks were observed between the anomeric proton H-1‴ (δ 4.69) and C-5‴ (δ 73.9). From the above evidence, the structure of compound **8** was established as kaempferol-3-*O*-[(6-sinapoyl)-β-D-glucopyranosyl-(1 → 2)-*O*-β-D-glucopyranoside]-7-*O*-β-D-glucopyranoside.

Compound **9** afforded D-glucose as the sugar residues upon acid hydrolysis. It showed a quasi-molecular ion peak at *m*/*z* 1001.2532 $[M+Na]^+$, consistent with a molecular formula of $C_{44}H_{50}O_{25}Na$ based on HR ESI–MS. The ^1H NMR spectrum revealed the presence of three anomeric protons at 5.48 (*d*, 7.5), 5.06 (*d*, 7.2) and 5.14 (*d*, 7.5), indicating three glucose units. A characteristic sinapoyl moiety was also observed in the ^1H NMR, which gave two methoxy groups at δ 3.75 (each, 3H, s), two phenyl protons at δ 6.99 (each, 1H, s) and *trans* olefinic protons at δ 6.54 (*d*, 15.8) and 7.55 (*d*, 15.8). Comparative analysis of the ^{13}C NMR data of the aglycone carbons in compound **9** and kaempferol-3,7,4′-tri-O-β-D-glucoside (**5**) proved that the glycosylation sites of compound **9** were at the 3-, 7- and 4′-positions. This was further supported by HMBC correlation peaks between the anomeric protons at δ 5.48, 5.06 and 5.14 with the corresponding aglycone carbons at δ 134.0 (C-3), 159.1 (C-4′) and 162.9 (C-7), respectively. Furthermore, a comparative analysis of the ^{13}C NMR chemical shifts of the sugar units with those of compound **5** showed a significant δ 2.2 ppm downfield shift for C-6 of a glucose unit, suggesting the sinapoyl moiety was attached at this position. This was confirmed from a HMBC experiment where the signals at δ 3.35, 3.58 (H-6‴) in the ^1H NMR were correlated with the ester carbonyl (δ 166.6) of the sinapoyl unit. In addition, long-range correlations were observed between the signals at δ 3.35, 3.58 (H-6‴) and C-5‴ (δ 77.7). Meanwhile, the anomeric proton (δ 5.06) assigned to the 4′-*O*-glucose moiety was also correlated with C-5‴ (δ 77.7), which confirmed that the linkage of the sinapoyl unit was at the 6-position of the 4′-*O*-glucose moiety. From these results, the structure of compound **9** was concluded to be kaempferol-4′-*O*-(6-sinapoyl)-β-D-glucopyranoside-3,7- di-*O*-β-D-glucopyranoside.

Compound **10** was obtained as a yellow powder, and the HR ESI–MS spectrum showed an $[M+Na]^+$ ion at *m*/*z* 1001.2551, which was attributable to a molecular formula of $C_{44}H_{50}O_{25}$. Acid hydrolysis gave kaempferol and D-glucose with comparison to authentic samples. The ^1H NMR of compound **10** showed three glucose moieties from three anomeric proton signals at δ 5.75 (*d*, 7.3), 4.81 (*d*, 7.8) and 5.08 (*d*, 7.4), with the corresponding anomeric carbon signals at δ 97.8 (C-1″), 103.6 (C-1‴) and 99.7 (C-1⁗), respectively. The ^1H NMR showed that all glucosyl units were

β-linked to other glucose units or to the aglycone because the coupling constants between the anomeric protons and H-2 were always approximately 7–8 Hz (Agrawal, 1992). In the ^{13}C NMR spectrum, chemical shifts in the aglycone at C-3 and C-7 due to glycosylation relative to kaempferol (**1**) indicated that the sugar units were attached at these positions. The downfield-shifted carbon signal at δ 81.9, attributed to the C-2″ of the inner glucose unit, suggested a glucosyl (1 → 2)-β-D-glucopyranoside moiety, which was similar to that of compound **7**. These results were consistent with the HMBC correlations shown in Fig. 2, where the signals of the glucosyl anomeric protons at δ 5.75, 5.08 and 4.81 were correlated with those of the kaempferol C-3 (δ 133.0) and C-7 (δ 162.7), and the inner glucose C-2″ (δ 81.9).

The two methoxy groups at δ 3.80 (each, 3H, s), two phenyl protons at δ 7.03 (each, 1H, s), and a *trans* olefinic protons at δ 6.95 and 7.56 (each, *d*, 15.8) proved the presence of a sinapoyl moiety. In the ^{13}C NMR spectrum, a carbon signal due to the C-4‴ (β-C) of a glucose unit shifted upfield and appeared at δ 67.6 revealing that the sinapoyl moiety might be substituted at C-3‴ or C-5‴ of the glucose unit. In the HMBC analysis, long-range correlations were observed between a triplet signal at δ 4.96 (*t*, 9.4) and the ester carbonyl at δ 166.2, which proved that the sinapoyl unit was attached at this position. The observation of long-range correlations between the anomeric proton at δ 4.81 (H-1‴) and the signal at δ 3.29 by ^{1}H–^{1}H COSY analysis proved that the signal at δ 3.29 could be assigned to H-2‴. In addition, a ^{1}H-^{1}H COSY correlation between the signal at δ 3.29 (H-2‴) and the signal at δ 4.96 (*t*, 9.4) was also detected, which revealed that the triplet signal at δ 4.96 could be assigned to H-3‴. Additionally, the anomeric proton at δ 4.81 (H-1‴) was correlated with the carbon signal at δ 77.4 in the HMBC spectrum, which demonstrated that the signal at δ 77.4 should be assigned to C-3‴. Therefore, the sinapoyl group in compound **10** was connected to C-3‴. This assignment was further supported by a downfield carbon chemical shift at δ 77.4 (C-3‴) and upfield chemical shifts at C-2‴ (δ 72.3) and C-4‴ (δ 67.6). From these results, compound **10** was characterized as kaempferol-3-*O*-[(3-sinapoyl)-β-D-glucopyranosyl-(1 → 2)-*O*-β-D-glucopyranoside]-7-*O*-β-D-glucopyranoside.

Additionally, seven known compounds (**1**–**7**, Fig. 1) were elucidated from NMR and MS analyses. By comparing NMR spectroscopic data with those in the literatures, their structures were determined to be kaempferol (**1**) (Ayoub et al., 2013), kaempferol-7-*O*-β-D-glucopyranoside (**2**) (Lim et al., 2006), astragalin (**3**) (Kim et al., 2004), isorhamnetin-3-*O*-β-D-glucopyranoside (**4**) (Yeskaliyeva et al., 2006), kaempferol-3,7,4′-tir-*O*-β-D-glucopyranoside (**5**) (Kim et al., 2014), kaempferol-3-O-β-D-glucopyranosyl-(1 → 2)-β-D-glucopyranoside-7-O-β-D-glucopyranoside (**6**) (Manguro et al., 2003), kaempferol-3-O-[(2-O-sinapoyl)-β-D-glucopyranosyl-(1 → 2)-β-D-glucopyranoside]-7-O-β-D-glucopyranoside (**7**) (Jung et al., 2009).

3.2. Determination of SOD and LDH activities of the isolated compounds

All the compounds listed in Table 2 exhibited certain antioxidant activity. Two dosage groups were used in the experiment and the results indicated that the LDH and SOD activities of the isolated compounds occurred in a concentration-dependent manner. Kaempferol (**1**) exhibited the highest LDH activity among the evaluated compounds at the level of 100 μg/mL. This could be mainly ascribed to the structural polyhydroxyl groups. Owing to a methoxy substitution at C-3′, the antioxidant activity of kaempferol-3-O-β-D-glucopyranoside (**3**) was slightly better than isorhamnetin-3-O-β-D-glucopyranoside (**4**) and this result was agreed with previous studies (Yokozawa et al., 1998). The antioxidant activities of kaempferol- 3,7,4′-tri-O-β-D-glucopyranoside (**5**) at the level of 100 μg/mL was a little weaker than compound **9**, which could be explained by an additional substitution of a sinapoyl group at the 6‴-position. However, no significant difference was noted at the level of 200 μg/mL. Similarly, the substitution of a sinapoyl group at the C-2‴ (**7**) and C-3‴ (**10**) led to an increase in antioxidant activities compared to compound **6**, but the additional substitution of a sinapoyl group at the C-6‴ (**8**) showed a decrease in both LDH and SOD activities. This decrease may be due to the substitution position of the sinapoyl group. Jung et al. (2009) had also demonstrated that the sinapic acid moiety of kaempferol derivatives may play a crucial role in increasing antioxidant activity, but it was noteworthy that the compounds studied in the report were all substituted at the 2″-position. Thus, position of the substituted sinapoyl group in kaempferol derivatives could be considered as another important factor that influences their antioxidant activity.

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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.phytol.2015.06.014.

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