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Kaempferol acetylated glycosides from the seed cake of *Camellia oleifera*

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ABSTRACT

The seed cake is a big by-product after crushing cooking oil from the seeds of *Camellia oleifera* Abel. Chemical investigation on the seed cake of *C. oleifera* led to the isolation of two new kaempferol acetylated glycosides (**1** and **2**). In addition, five kaempferol glycosides (**3**–**7**) and their aglycone, kaempferol (**8**), were also obtained, in addition to gallic acid (**9**). Their structures were determined by the detailed spectroscopic analysis and acidic hydrolysis. The new compounds were characterised as kaempferol-3-*O*-[4‴-*O*-acetyl-α-L-rhamnopyranosyl-(1→6)]-[β-D-glucopyranosyl-(1→2)]-β-D-glucopyranoside (**1**) and kaempferol-3-*O*-[4‴-*O*-acetyl-α-L-rhamnopyranosyl-(1→6)]-[β-D-xylopyranosyl-(1→2)]-β-D-glucopyranoside (**2**), respectively. The DPPH radical scavenging activity of all the isolated compounds was described.

1. Introduction

Camellia oleifera Abel., a theaceous evergreen tree, is distributed and cultivated widely in the central and south China. The seeds are important oil material in China used extensively for producing a kind of cooking oil (tea oil), whose beneficial unsaturated fatty acids are comparable to those of olive oil (Long & Wang, 2008). To obtain one ton of tea oil, four times of residue composing of the remaining grounded fruit and shell will be produced, which is called "seed cake" of *C. oleifera*. As a big amount of by-product, the seed cake of *C. oleifera* is normally used as detergent, animal feeds, or organic fertiliser, due to the containing of rich polyphenols, saponins, protein, polysaccharide, etc. (Wang & Wei, 1990). Up to now, several flavonoids (Chen, Liau, Jong, & Chang, 2009; Du, Wu, & Chen, 2008; Luo, Li, & Xie, 2003; Wang, Jia, Zhu, Yang, & Zhou, 1986), lignans (Lee & Yen, 2006), tannins (Yoshida et al., 1994), and saponins (Sugimoto et al., 2009) were reported from the seeds, leaves, flower buds, seed cake, and seed oil. Our detailed chemical investigation on the seed cake of *C. oleifera* led to the isolation of two new kaempferol acetylated glycosides, together with six kaempferol derivatives and a simple phenolic compound. This paper describes the structural elucidation of the new compounds (**1** and **2**) on the basis of spectroscopic method and acidic hydrolysis. The antioxidant activity of the isolates on DPPH radical scavenging assay was also described.

2. Materials and methods

2.1. General procedures

Optical rotations were measured with a HORIBA SEPA-300 high-sensitive polarimeter. IR spectra were recorded on a Bio-Rad FTS-135 spectrometer with KBr pellets. UV spectra were recorded on a UV 210A Shimadzu spectrometer. 1D- and 2D-NMR spectra were recorded in DMSO-d_6 with a Bruker DRX-500 instrument operating at 500 MHz for ^{1}H, 125 MHz for ^{13}C, respectively. Coupling constants were expressed in Hertz and chemical shifts were given on a ppm scale with tetramethylsilane as internal standard. FABMS were recorded on a VG Auto Spec-300 spectrometer with glycerol as the matrix. HRESIMS was recorded on an API QSTAR Pular-1 mass spectrometer. DPPH radical scavenging assay was performed on an Emax precision microplate reader.

2.2. Chemicals and reagents

1,1-Diphenyl-2-picrylhydrazyl (DPPH) was purchased from Sigma–Aldrich Chemicals (Steinheim, Germany), ascorbic acid was obtained from Xinxing Chemical Industrial Reagent Institute (Shanghai, China). Column chromatography (CC) was performed

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on Sephadex LH-20 (25–100 μm, Pharmacia Fine Chemical Co., Ltd.) and MCI-gel CHP 20P (75–100 μm, Mitsubishi Chemical Co., Ltd.). TLC was carried on silica gel H-precoated plates (0.2–0.25 mm thick, Qingdao Haiyang Chemical Co.) with benzene/ethyl formate/formic acid [3:6:1 or 2:7:1, v/v/v], and spots were detected by spraying with 2% ethanolic $FeCl_3$ or anisaldehyde-H_2SO_4 reagent followed by heating.

2.3. Plant materials

The seed cake of *C. oleifera* was purchased from a tea oil factory in Fujian Province, China, where *C. oleifera* has been planted widely for producing tea oil. The fresh seeds of the title plant were obtained from the factory and identified by Mr. Yi-Fei Wang from Jinan University, China.

2.4. Extraction and isolation

The seed cake of *C. oleifera* (3.0 kg) was extracted four times with MeOH under reflux (2 h each). After concentrated to a small volume (2.5 l), the extract was partitioned with petroleum ether three times to afford a defatted fraction (420 g). Part of the defatted fraction (86 g) was subjected to a Sephadex LH-20 column, eluting with H_2O–MeOH (1:0, 4:1, 3:2, 2:3, 1:4, 0:1, v/v, each 800 ml) and finally 50% aq. acetone (800 ml) to give four fractions (Frs. 1–4). Fr. 2 (8.8 g) was applied to repeated CC over silica gel ($CHCl_3$–MeOH–H_2O, 7:3:0.5), Sephadex LH-20, and MCI-gel CHP 20P (H_2O–MeOH, 1:0–0:1), and middle pressure liquid chromatography (MPLC) on silica gel (30 × 3 cm, i.d., $CHCl_3$–MeOH–H_2O, 8:2:0.2, flowing rate: 10 ml min^{-1}) to yield compounds **1** (6 mg), **2** (7 mg), **3** (1.64 g), **4** (1.11 g) and **5** (102 mg). Fr. 3 (1.2 g) was purified by MCI-gel CHP 20P (75–100% aq. MeOH) and silica gel ($CHCl_3$–MeOH–H_2O, 8:2:0.2) CC to give **6** (30 mg) and **7** (17 mg). Fr. 4 (1.5 g) and Fr. 1 (21 g) were separately applied to MCI-gel CHP 20P CC, eluted with H_2O–MeOH (1:0–0:1) to afford compounds **8** (106 mg) and **9** (120 mg), respectively.

Kaempferol-3-*O*-[4‴′-*O*-acetyl-α-L-rhamnopyranosyl-(1→6)]-[β-D-glucopyranosyl-(1→2)]-β-D-glucopyranoside (**1**): yellow amorphous powder; $[\alpha]_D^{24}$ −26.6° (*c* 0.09, MeOH); UV (MeOH) λ_{max} (log ε) 208 (4.55), 283 (4.08) and 329 (3.94) nm; IR (KBr) ν_{max} 3441, 1652, 1507 and 1606 cm^{-1}; negative FABMS *m*/*z* 797 $[M–H]^-$; HRESIMS *m*/*z* 797.2141 $[M–H]^-$ (calcd for $C_{35}H_{41}O_{21}$, 797.2140). ^1H NMR (DMSO-d_6, 500 MHz) and ^{13}C NMR (DMSO-d_6, 125 MHz) see Table 1.

Kaempferol-3-*O*-[4‴′-*O*-acetyl-α-L-rhamnopyranosyl-(1→6)]-[β-D-xylopyranosyl-(1→2)]-β-D-glucopyranoside (**2**): yellow amorphous powder; $[\alpha]_D^{24}$ −33.3° (*c* 0.09, MeOH);UV (MeOH) λ_{max} (log ε) 202 (4.52), 269 (4.00) and 327 (3.84) nm; IR (KBr) ν_{max} 3423, 1657, 1609, 1628, 1507 and 1454 cm^{-1}; negative FABMS *m*/*z* 767 $[M–H]^-$; HRESIMS *m*/*z* 767.2049 $[M–H]^-$ (calcd for $C_{34}H_{39}O_{20}$, 767.2034); ^1H NMR (DMSO-d_6, 500 MHz) and ^{13}C NMR (DMSO-d_6, 125 MHz) see Table 1.

2.5. Acid hydrolysis of compounds *1–4*

The method was applied as the previously reported one (Shang et al., 2006) with some minor modifications. Compounds **1–4** (5–8 mg each) were hydrolysed with 2 M HCl/dioxane (1:1, 4 ml) under reflux for 8 h, respectively. The reaction mixture was partitioned between H_2O and $CHCl_3$ (2 ml × 3). The aqueous layer was neutralised with 2 M NaOH and then dried to give a monosaccharide mixture. A solution of the sugar mixture in pyridine (2 ml) was added to L-cysteine methyl ester hydrochloride (about 1.5 mg) and kept at 60 °C for 1 h. Then trimethylsilylimidazole (1.5 ml) was added to the reaction mixture and kept at 60 °C for 30 min. Finally, the mixture was immediately subjected to GC analysis, run on a Shimadzu GC-14C gas chromatograph equipped with an H_2 flame ionisation detector. The column was a 30 m × 0.32 mm i.d. 30QC2/AC-5 quartz capillary column with the following conditions: column temperature, 180–280 °C; programmed increase, 3 °C min^{-1}; carrier gas, N_2 (1 ml min^{-1}); injector and detector temperature, 250 °C; injection volume, 4 μl; and split ratio, 1/50. In comparison with the retention time of the authentic sugars in the form of derivatives under the same condition, the sugar moieties of compounds **1–4** were determined to be D-glucose (19.15 min), L-rhamnose (15.62 min), and D-xylose (13.65 min).

2.6. DPPH radical scavenging activity

The DPPH assay was operated as previously reported with some minor modifications (Gao, Zhang, Yang, Chen, & Jiang, 2008), and ascorbic acid was used as positive control. The starting concentration for the samples or positive control were prepared as 1 mg ml^{-1}, then two fold serial dilutions was prepared to make a gradient with ten concentrations. 100 μl of samples or positive control ethanol solution was added to 100 μl of DPPH ethanol solutions (0.2 μM ml^{-1}) in a 96 well microplate and incubated at room temperature for 30 min. After incubation, the DPPH remaining was determined by the absorbance at 517 nm and the scavenging activity was determined by the following equation: % scavenging activity = 100 × $(A_{control} - A_{sample})/A_{control}$. The SC_{50} value was obtained through extrapolation from linear regression analysis and denoted the concentration of sample required to scavenge 50% of DPPH radicals. The data presented are means ± SD of three determinations.

3. Results and discussion

3.1. Identification of compounds *1–9*

The defatted MeOH extract of seed cake of *C. oleifera* was subjected to repeated CC over Sephadex LH-20, MCI-gel CHP 20P and silica gel, and MPLC on silica gel to give two new compounds **1** and **2**, along with seven known compounds (**3–9**), which are shown in Fig. 1. The known ones were identified as kaempferol-3-*O*-[α-L-rhamnopyranosyl-(1→6)]-[β-D-xylopyranosyl-(1→2)]-β-D-glucopyranoside (camelliaside B, **3**) (Du et al., 2008; Park et al., 2006; Sekine et al., 1991), kaempferol-3-*O*-[α-L-rhamnopyranosyl-(1→6)]-[β-D-glucopyranosyl-(1→2)]-β-D-glucopyranoside (**4**) (Budzianowski, 1990; Du et al., 2008; Wang et al., 1986), kaempferol-3-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside (**5**) (Wang et al., 1986; Zhou & Yang, 2000), kaempferol-3-*O*-β-D-xylopyranosyl-(1→2)-β-D-glucopyranoside (leucoside, **6**) (Wei et al., 2004), kaempferol-3-*O*-β-D-glucopyranosyl-(1→2)-β-D-glucopyranoside (**7**) (Song, 1990), kaempferol (**8**) (Luo et al., 2003; Zhou & Yang, 2000), and gallic acid (**9**) (Zhou & Yang, 2000), respectively, by detailed spectroscopic analysis and comparing with the literature data. Compounds **6**, **7** and **9** were isolated from *C. oleifera* for the first time.

Compound **1** was isolated as a yellow amorphous powder. Its molecular formula was deduced to be $C_{35}H_{42}O_{21}$ on the basis of the negative HRESIMS ($[M–H]^-$, *m*/*z* 797.2141) and the comprehensive analysis of NMR data. The IR spectrum showed absorption bands characteristic for hydroxyl (3441 cm^{-1}), conjugated carbonyl (1652 cm^{-1}) and aromatic ring (1507 and 1606 cm^{-1}) functional groups. The characteristic UV absorption maxima at 208, 283 and 329 nm due to flavonols chromophore closely resembled that of kaempferol (**8**) (Zhou & Yang, 2000). In the ^1H NMR spectrum of **1**, the presence of two *meta*-coupling signals at δ 6.41 and 6.18 (each 1H, d, *J* = 2.0 Hz, H-6, H-8), and an A_2B_2 coupling system [δ 8.03 and 6.90 (each 2H, d, *J* = 8.5 Hz, H-2′,6′, H-3′,5′)] (Table 1) were the resonances characteristic for a kaempferol skeleton. In addi-

Table 1
^{1}H NMR and ^{13}C NMR (δ in ppm, J in Hz, in DMSO-d_6) data for compounds **1–2.**

Position	**1**		**2**	
	δ_C	δ_H	δ_C	δ_H
2	156.2 (s)		155.8 (s)	
3	132.7 (s)		132.8 (s)	
4	177.5 (s)		177.5 (s)	
5	161.3 (s)		161.3 (s)	
6	98.7 (d)	6.41 (d, 2.0)	98.8 (d)	6.40 (d, 2.0)
7	164.1 (s)		164.1 (s)	
8	93.7 (d)	6.18 (d, 2.0)	93.7 (d)	6.17 (d, 2.0)
9	156.4 (s)		156.5 (s)	
10	103.8 (s)		103.9 (s)	
1′	120.8 (s)		120.9 (s)	
2′,6′	130.9 (d)	8.03 (d, 8.5)	131.0 (d)	8.01 (d, 8.5)
3′,5′	115.3 (d)	6.90 (d, 8.5)	115.3 (d)	6.87 (d, 8.5)
4′	159.9 (s)		160.0 (s)	
β-D-Glc-1″	98.3 (d)	5.57 (d, 7.2)	98.2 (d)	5.54 (d, 7.2)
2″	82.5 (d)	3.54 (d, 9.7)	81.7 (d)	3.56 (d, 9.6)
3″	76.5 (d)	3.29–3.33 (m)	76.8 (d)	3.31–3.37 (m)
4″	69.6 (d)	3.29–3.33 (m)	69.5 (d)	3.31–3.37 (m)
5″	75.2 (d)	3.29–3.33 (m)	75.3 (d)	3.31–3.37 (m)
6″	65.7 (t)	3.66–3.67 (m) 3.31 (d, 12.0)	65.8 (t)	3.69 (m), 3.24 (m)
	β-D-Glc		β-D-Xyl	
1‴	104.1 (d)	4.59 (d, 7.0)	104.5 (d)	4.59 (d, 7.2)
2‴	74.4 (d)	3.20–3.23 (m)	73.9 (d)	3.17–3.28 (m)
3‴	77.1 (d)	3.08–3.14 (m)	76.2 (d)	3.17–3.28 (m)
4‴	70.3 (d)	3.08–3.14 (m)	69.1 (d)	3.17–3.28 (m)
5‴	76.5 (d)	3.08–3.14 (m)	65.8 (t)	2.99–3.02 (m), 3.03–3.07 (m)
6‴	60.8 (t)	3.16–3.19 (m) 3.20–3.23 (m)		
α-L-Rha-1⁗	100.1 (d)	4.39 (br s)	100.2 (d)	4.32 (br s)
2⁗	68.1 (d)	3.22–3.24 (m)	70.4 (d)	3.33–3.35 (m)
3⁗	69.0 (d)	3.15–3.22 (m)	68.2 (d)	3.12–3.16 (m)
4⁗	73.6 (d)	4.52–4.56 (m)	73.6 (d)	4.52–4.56 (m)
5⁗	68.1 (d)	3.15–3.22 (m)	65.8 (d)	3.12–3.16 (m)
6⁗	17.0 (q)	0.67 (d, 6.1)	17.0 (q)	0.66 (d, 6.1)
CH_3CO	20.9 (q)	1.95 (s)	20.9 (q)	1.93 (s)
CH_3CO	170.1 (s)		170.2 (s)	

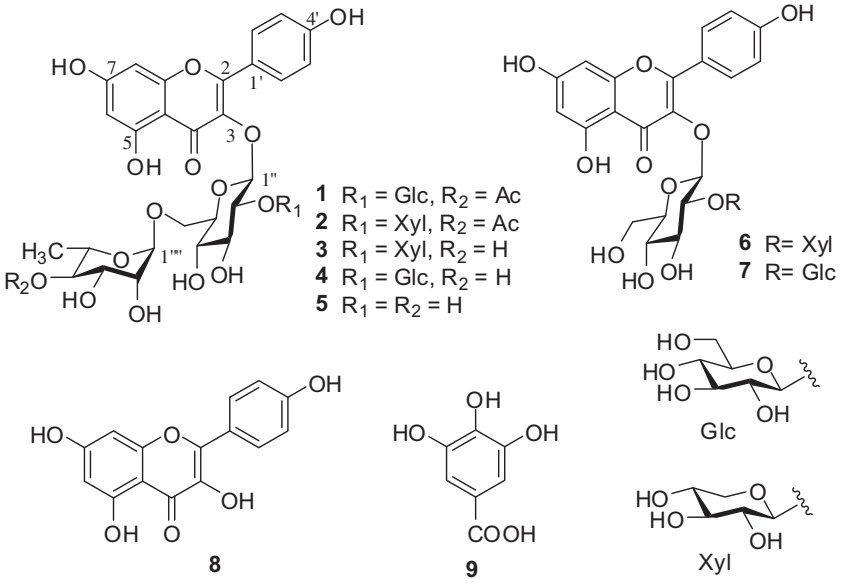

Fig. 1. Structures of compounds **1–9** from the seed cake of *C. oleifera.*

tion, three anomeric signals [δ 5.57 (d, J = 7.2 Hz, H-1″), 4.59 (d, J = 7.0 Hz, H-1‴) and 4.39 (br s, H-1⁗)], an acetyl methyl signal at δ 1.95 (s), and a methyl signal at δ 0.67 (d, J = 6.1 Hz) arising from a rhamnosyl C-6 position were also observed, together with signals between δ 3.08 and 3.66 attributed to the remaining protons of three sugar residues. These spectral features indicated that

1 was a kaempferol acetylated triglycoside, which was confirmed by the analysis of its ^{13}C NMR spectral data (Table 1). Acid hydrolysis of **1** yielded the D-glucose and L-rhamnose as sugar residues, which were determined by GC analysis of its corresponding trimethylsilylated L-cysteine adduct. The anomeric proton coupling constants indicated the presence of two β-glucosyl units and one α-rhamnosyl unit (Allgeier, 1968). The NMR spectral data of **1** was closely related to those of **4** (Budzianowski, 1990), except for the appearance of an acetyl group in **1**, indicated that **1** is an acetylated derivative of **4** (Table 1). The sugar sequence and location were deduced from the HMBC experiment, in which correlations of the inner glucosyl anomeric proton at δ 5.57 (H-1″) with the aglycone C-3 at δ 132.7, the rhamnosyl anomeric proton at δ 4.39 (H-1‴′) with the inner glucosyl C-6″ at δ 65.7, and the terminal glucosyl anomeric proton at δ 4.59 (H-1‴) with the inner glucosyl C-2″ at δ 82.5 were observed distinctively (Fig. 2). In addition, correlation of the rhamnosyl H-4‴′ (δ 4.55) correlated to the acetyl carbonyl carbon (δ 170.1) demonstrated the acetyl group located on C-4‴′ of the rhamnosyl unit. Therefore, the structure of compound **1** was established as kaempferol-3-*O*-[4‴′-*O*-acetyl-α-L-rhamnopyranosyl-(1→6)]-[β-D-glucopyranosyl-(1→2)]-β-D-glucopyranoside.

Compound **2** was obtained as a yellow amorphous powder with a molecular formula $C_{34}H_{40}O_{20}$, as deduced by the negative HRESIMS at *m/z* 767.2049 $[M-H]^-$. The UV, IR and NMR spectra of **2** were similar to those of **3**, which indicated the presence of kaempferol unit as aglycone, and a glucosyl, a xylosyl and a rhamnosyl unit as sugar moieties. Acidic hydrolysis of **2** gave D-glucose, D-xylose and L-rhamnose as the sugar residue, which were determined to have β configuration for glucosyl and xylosyl units and α-configuration for rhamnosyl unit, on the basis of the coupling constants of the anomeric protons [δ 5.54 (d, J = 7.2 Hz), 4.59 (d, J = 7.2 Hz), and 4.32 (br s)]. In addition, a set of additional signals due to an acetyl group [δ_H 1.93 (s); δ_C 20.9 (q) and 170.2 (s)] was observed in the NMR spectra of **2**, indicating that **2** was an acetyl cognate of **3**. The sequence of sugars and the linkage site to the aglycone of **2** were confirmed by 2D NMR experiments, in which respective HMBC correlations of the rhamnosyl (δ 4.32, H-1‴′), xylosyl (δ 4.59, H-1‴) and glucosyl (δ 5.54, H-1″) anomeric protons with C-6″ (δ 65.8) and C-2″ (δ 81.7) of the glucosyl unit, and the C-3 (δ 132.8) position of aglycone were observed (Fig. 2). The acetyl group was determined to be located on C-4‴′ of the rhamnosyl unit, deduced from the HMBC correlation of H-4‴′ of the rhamnosyl unit at δ 4.56 with the carbonyl carbon (δ 170.2) of the acetyl group. Accordingly, compound **2** was characterised as kaempferol-3-*O*-[4‴′-*O*-acetyl-α-L-rhamnopyranosyl-(1→6)]-[β-D-xylopyranosyl-(1→2)]-β-D-glucopyranoside.

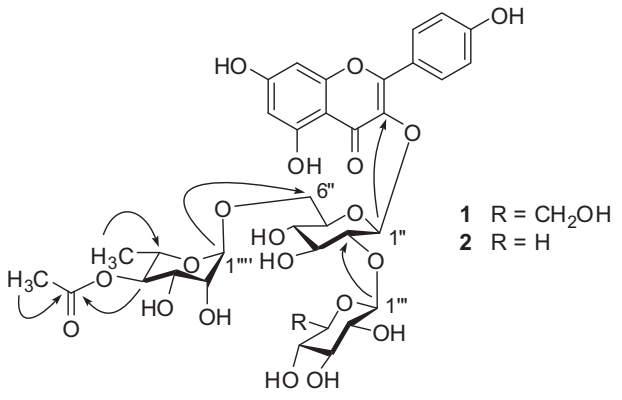

Fig. 2. Key HMBC correlations of compounds **1** and **2.**

Table 2
DPPH radical scavenging activity of compounds **1–9.**

Compounds	SC_{50} (μM)[a,b]
1	>100
2	>100
3	>100
4	>100
5	71.1 ± 0.5
6	85.4 ± 0.8
7	45.0 ± 0.7
8	35.6 ± 0.3
9	13.1 ± 0.1
Ascorbic acid	37.5 ± 0.6

[a] SC_{50}: DPPH radical scavenging activity (concentration in μM necessary for 50% reduction of DPPH radical).
[b] Values represent means ± SD (n = 3).

In this study, a certain amount of gallic acid (**9**), a potent natural antioxidant contained in various edible plants, was obtained. Since hydrolysable tannins were reported from the leaves of *C. oleifera* (Yoshida et al., 1994), compound **9** may come from the degradation of hydrolysable tannins during the crushing process of the fruit. Further study on the fresh fruit of *C. oleifera* is now in progress.

3.2. DPPH radical scavenging capacity

The antioxidant activity of compounds **1–9** was evaluated on the DPPH radical scavenging assay, and the results were shown in Table 2. Comparing to the positive control, ascorbic acid, compound **9** (gallic acid), the well-known natural antioxidant found widely in plants, exhibited higher DPPH radical scavenging activity, while kaempferol derivatives **1–7** showed lower activities. This is consistent with the previous reports and suggests that three or two *ortho*-phenolic hydroxy groups in the flavonoid B-rings are key factors for enhancing their activities (Guo, Zhao, Shen, Hou, & Xin, 1999; Okawa, Kinjo, Nohara, & Ono, 2001). The activity order of kaempferol derivatives was shown to be **8** > **7** > **5** > **6** > **1**–**4**, suggesting that the active centre of flavonol glycosides (**1–7**) lies in the aglycone part of the molecules, and the more glycosidic residues, the less active for the molecule. If a bigger substituent like *O*-glycosyl linked at C-3 position of flavonoids, the activity was greatly reduced probably for the steric hindrance (Braca et al., 2003; Cioffi et al., 2002).

Though the antioxidant activity of kaempferol derivatives on DPPH radical scavenging assay was moderate, they were reported to have inhibitory activities on lipopolysaccharide-induced nitric oxide production in BV2 microglia (Kim, Jang, Lee, Sung, & Kim, 2009), $CB_{1-6}V$ and HSV-1 virus infection (Yao & Wang, 1999), and protein kinase CK_2 *in vitro* and in HL-60 cells (Lin, Liu, Chen, & Liang, 2006). Glycosylated kaempferol derivatives were also reported from some vegetables and fruits, e.g. tronchuda cabbage (*Brassica oleracea* var. *costata*) (Ferreres et al., 2005), *Styphnolobium japonicum* (Kite et al., 2009), and apricot (*Prunus armeniaca* L.) (Ruiz, Egea, Gil, & Tomás-Barberán, 2005), etc. The high content of kaempferol derivatives suggested that the seed cake of *C. oleifera* may provide a good source of health promoting compounds, namely, flavonoids. Further evaluation on this by-product and its plant materials is now in progress.

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