Structural Determination and DPPH Radical-Scavenging Activity of Two Acylated Flavonoid Tetraglycosides in Oolong Tea (*Camellia sinensis*)

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Two major acylated flavonoid tetraglycosides were isolated from the methanol extract of oolong tea. Their structures were elucidated by spectroscopic methods as quercetin 3-*O*-[2^G-(*E*)-coumaroyl-3^G-*O*-β-D-glucosyl-3^R-*O*-β-D-glucosylrutinoside] (1) and kaempferol 3-*O*-[2^G-(*E*)-coumaroyl-3^G-*O*-β-D-glucosyl-3^R-*O*-β-D-glucosylrutinoside] (2). Compounds 1 and 2 exhibited scavenging activity against DPPH radical with EC_{50} values of 30.5 and 487.2 μM, respectively.

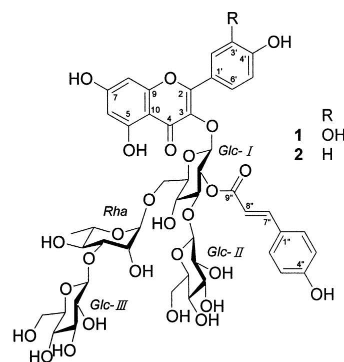
Key words *Camellia sinensis*; oolong tea; quercetin; kaempferol; 1,1-diphenyl-2-picrylhydrazyl

Tea is a beverage widely consumed in the world for over several thousand years. Various teas as well as their active ingredients, such as catechins and polyphenols, have been demonstrated to possess antioxidant and biological activities.[1] They are mainly classified into green tea (unfermented), oolong tea (partially fermented), and black tea (fully fermented) according to the degree of fermentation during their preparations, where the term 'fermentation' refers to natural browning reactions induced by oxidative enzymes in the cells of tea leaves.[2] Oolong tea possessing a taste and color somewhere between green and black teas is the most popular tea in Taiwan, and different process conditions have been practiced to generate variable products of this tea by local manufacturers.

In the production of oolong tea, young green shoots are freshly harvested and allowed to wither under sunlight for a few hours prior to undergoing the semi-fermentation process, in which tea leaves are oxidized, pan fired at *ca.* 200 °C, rolled to form ball shape, and then dried in a specialized oven at various desired temperatures. The reaction time for the contact between phenolic compounds and oxidative enzymes is empirically controlled by experts during this semi-fermentation process, and the final fermentation degree of oolong tea ranges at 20—80% depending on the demand of customers. Experientially, fermentation (oxidation) and drying in the specialized oven are two key steps for the quality control of oolong tea preparation. Therefore unique compounds are possibly generated in various preparations of oolong tea manufactured by different process conditions.[3—5]

Many kinds of compounds have been identified in various teas, such as flavan-3-ols, flavonoids, gallic acid, quinic esters of caffeic, coumaric and gallic acid, theaflavins, thearubigins, and alkaloids.[6—11] Although oolong tea is becoming increasingly more popular in the world, there is much less investigation of the infusions of different oolong tea products in comparison with the vigorous studies that have been conducted on the active ingredients found in green and black teas. Recently, six novel acylated flavonol glycosides were identified in the infusion of oolong tea, and their chemical structures were approximately predicted by electrospray ionization tandem mass spectrometry.[12] In this report, we describe the extraction, purification, and structural elucidation of the two relatively abundant acylated flavonoid tetraglycosides, **1** and **2**, from the methanol extract of oolong tea. Scavenging activities against 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical of these two compounds were evaluated.

Compound **1** was isolated as a light yellow amorphous solid. The molecular formula of **1** was established as $C_{48}H_{56}O_{28}$ by the positive HR-FAB-MS showing a quasimolecular ion peak at *m/z* 1081.3032 $[M+H]^+$, ^{13}C-NMR spectrum, and DEPT spectrum indicating 21 degrees of unsaturation. The IR spectrum of **1** displayed absorptions for hydroxyl (3383 cm^{-1}), conjugated ester (1703 cm^{-1}), conjugated ketone (1659 cm^{-1}), and benzene ring (1606, 1514 cm^{-1}) functionalities. The UV absorption maxima at 260, 271, and 354 nm, as well as the ^{1}H-NMR signals for a 5,7-dihydroxylated pattern of ring A [(δ_H 6.13 (1H, br d, J=1.6 Hz, H-6); 6.32 (1H, br d, J=1.6 Hz, H-8)], and a set of ABX coupling pattern of ring C [(δ_H 7.59 (1H, d, J=1.6 Hz, H-2′); 6.88 (1H, d, J=8.4 Hz, H-5′); 7.53 (1H, dd, J=1.6, 8.4 Hz, H-6′)] suggested that the aglycone of **1** was quercetin.[13] The ^{1}H-NMR spectrum of **1** (Table 1) also showed the resonances for a (*E*)-coumaroyl group [δ_H 7.45 (2H, d, J=8.8 Hz, H-2″, 6″); 6.79 (2H, d, J=8.8 Hz, H-3″, 5″); 7.67 (1H, d, J=16.0 Hz, H-7″); 6.41 (1H, d, J=16.0 Hz, H-8″)], four anomeric protons [δ_H 5.54 (1H, d, J=8.0 Hz, H-1 of Glc-I); 4.43 (1H, d, J=7.6 Hz, H-1 of Glc-II); 4.44 (1H, br d, J=7.6 Hz, H-1 of Glc-III); 4.62 (1H, br s, H-1 of Rha)], and other sugar protons [δ_H 3.22—4.01; 5.21]. The sugar



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moiety of **1** was proposed to comprise one internal glucose [δ_H 5.54 (H-1 of Glc-I); δ_C 100.9, 74.4, 84.6, 70.0, 76.5, 68.4], one internal rhamnose [4.62 (H-1 of Rha-I); δ_C 102.2, 71.2, 82.9, 72.4, 69.4, 17.9], and two terminal glucoses {[δ_H 4.43 (H-1 of Glc-II); δ_C 104.5, 74.6, 77.3, 71.2, 77.9, 62.4] and [δ_H 4.44 (H-1 of Glc-III); δ_C 105.4, 75.3, 77.3, 70.7, 77.5, 62.0]} by the HOHAHA, HMQC experiments, and direct comparisons of the ^{1}H- and ^{13}C-NMR data with those of the literature.[13—15] The monosaccharides obtained by acid hydrolysis of the saponin mixture were identified as D-glucose and L-rhamnose by TLC and by measurement of their specific rotation after purification.[16] The β-configuration for the three D-glucoses was determined from their large $^3J_{H1,H2}$ coupling constants (J=7.6—8.0 Hz), while the α-configuration for the L-rhamnose was judged from the broad singlet signal of anomeric proton.[17,18] The internal glucose was connected to C-3 of aglycone, which was deduced from the HMBC correlation between the anomeric proton of Glc-I (δ_H 5.54) and C-3 of aglycone (δ_C 134.9). In turn, the HMBC correlations between the anomeric proton of Rha (δ_H 4.62)/C-6 of Glc-I (δ_C 68.4), the anomeric proton of Glc-II (δ_H 4.43)/C-3 of Glc-I (δ_C 84.6), and the anomeric proton of Glc-III (δ_H 4.44)/C-3 of Rha (δ_C 82.9) suggested that the connection order of sugar moiety at the C-3 of aglycone was Glc-I (6→1)[(3→1) Glc-II] Rham (3→1) Glc-III. Furthermore, the coumaroyl unit was located at the C-2 position of Glc-I, which was also assured by the observation of the HMBC relationship between H-2 of Glc-I (δ_H 5.21) and C-9″ (δ_C 168.8). The assignment structure of **1** closely resembles the known acylated flavonoid tetraglycoside, quercetin 3-*O*-(2^G-*p*-coumaroyl-3^G-*O*-β-L-arabinosyl-3^R-*O*-β-D-glucosylrutinoside), which was isolated from oolong tea by Mihara *et al.*[19] By comparison of ^{1}H- and ^{13}C-NMR data of **1** with those of quercetin 3-*O*-(2^G-*p*-coumaroyl-3^G-*O*-β-L-arabinosyl-3^R-*O*-β-D-glucosylrutinoside), the only difference was that the C-3 substituent of Glc-I of rutin was an arabinosyl in the known flavonoid tetraglycoside instead of a glucosyl in **1**. Hence compound **1** was characterized as quercetin 3-*O*-[2^G-(*E*)-coumaroyl-3^G-*O*-β-D-glucosyl-3^R-*O*-β-D-glucosylrutinoside]. Complete ^{1}H- and ^{13}C-NMR chemical shifts were established by ^{1}H–^{1}H COSY, HMQC, HMBC, and NOESY spectra.

Compound **2** was isolated as a light yellow amorphous solid. Its HR-FAB-MS spectrum showed a quasi-molecular ion peak at m/z 1065.3080 $[M+H]^+$, which corresponded to the molecular formula $C_{48}H_{56}O_{27}$, indicating 21 degrees of unsaturation. The IR spectrum of **2** indicated signals for hydroxyl (3383 cm^{-1}), conjugated ester (1698 cm^{-1}), conjugated ketone (1654 cm^{-1}), and phenyl group (1601, 1518 cm^{-1}) functionalities. The UV spectrum showed absorption bands at 267 and 355 nm characteristic of kaempferol.[15] The ^{1}H-NMR spectrum of **2** (Table 1) also showed the resonances for a kempferol moiety [δ_H 6.15 (1H, br d, J=2.0 Hz, H-6); 6.34 (1H, br d, J=2.0 Hz, H-8); 7.96 (2H, d, J=8.0 Hz, H-2′, 6′); 6.90 (2H, d, J=8.8 Hz, H-3′, 5′)], a (*E*)-coumaroyl group [δ_H 7.45 (2H, d, J=8.8 Hz, H-2″, 6″); 6.80 (2H, d, J=8.8 Hz, H-3″, 5″); 7.67 (1H, d, J=15.6 Hz, H-7″); 6.41 (1H, d, J=15.6 Hz, H-8″)], and four anomeric protons [δ_H 5.54 (1H, d, J=8.0 Hz, H-1 of Glc-I); 4.43 (1H, d, J=8.0 Hz, H-1 of Glc-II); 4.41 (1H, br d, J=7.2 Hz, H-1 of Glc-III); 4.60 (1H, br s, H-1 of Rha-I)].

Table 1. ^{1}H- and ^{13}C-NMR Data for **1** and **2** (400, 100 MHz in CD_3OD)

	1		**2**	
	δ_C	δ_H	δ_C	δ_H
Aglycone				
2	158.8		159.0	
3	134.9		134.8	
4	178.9		179.0	
5	162.8		162.8	
6	99.8	6.13 br d (1.6)	99.9	6.15 br d (2.0)
7	165.5		165.6	
8	94.9	6.32 br d (1.6)	95.0	6.34 br d (2.0)
9	158.2		158.3	
10	105.8		105.8	
1′	123.2		122.8	
2′	117.5	7.59 d (1.6)	132.3	7.96 d (8.8)
3′	145.9		116.3	6.90 d (8.8)
4′	149.6		161.2	
5′	116.2	6.88 d (8.4)	116.3	6.90 d (8.8)
6′	123.5	7.53 dd (1.6, 8.4)	132.3	7.96 d (8.8)
Glc-I				
1	100.9	5.54 d (8.0)	100.8	5.54 d (8.0)
2	74.4	5.21 t (9.8)	74.4	5.20 t (9.2)
3	84.6	3.94 t (8.8)	84.5	3.92 t (8.4)
4	70.0	3.56 m	70.1	3.50 m
5	76.5	3.57 m	76.6	3.54 m
6	68.4	3.56 m, 3.89 m	68.4	3.51 m, 3.88 m
Glc-II				
1	104.5	4.43 d (7.6)	104.7	4.43 d (8.0)
2	74.6	3.22 t (8.4)	74.7	3.20 t (8.0)
3	77.3	3.33 m	77.4	3.30 m
4	71.2	3.30 m	71.2	3.30 m
5	77.9	3.33 m	78.0	3.32 m
6	62.4	3.62 m, 3.86 m	62.4	3.60 m, 3.86 m
Glc-III				
1	105.4	4.44 br d (7.6)	105.5	4.41 br d (7.2)
2	75.3	3.29 m	75.3	3.28 m
3	77.3	3.46 m	77.4	3.42 m
4	70.7	3.41 m	70.7	3.40 m
5	77.5	3.27 m	77.5	3.28 m
6	62.0	3.71 m, 3.76 m	62.0	3.72 m, 3.75 m
Rha				
1	102.2	4.62 br s	102.2	4.60 br s
2	71.2	4.01 br s	71.3	4.00 br s
3	82.9	3.64 m	83.0	3.60 m
4	72.4	3.46 m	72.4	3.48 m
5	69.4	3.52 m	69.4	3.49 m
6	17.9	1.11 d (6.4)	18.0	1.11 d (6.4)
Coumaroyl				
1″	127.3		127.3	
2″, 6″	131.4	7.45 d (8.8)	131.4	7.45 d (8.8)
3″, 5″	116.7	6.79 d (8.8)	116.8	6.80 d (8.8)
4″	161.0		161.1	
7″	147.4	7.67 d (16.0)	147.3	7.67 d (15.6)
8″	115.1	6.41 d (16.0)	115.1	6.41 d (15.6)
9″	168.8		168.7	

The ^{1}H- and ^{13}C-NMR data of **1** and **2** were essentially identical in the carbohydrate region, revealing that the two compounds exhibited the same sugar compositions and linkages. The molecular weight of **2** was 16 mass units less than that of **1**, which supported the conjecture that the aglycone of **2** was kaempferol instead of quercetin in **1**. Accordingly, compound **2** was elucidated as kaempferol 3-*O*-[2^G-(*E*)-coumaroyl-3^G-*O*-β-D-glucosyl-3^R-*O*-β-D-glucosylrutinoside].

Compounds **1** and **2** were evaluated for their antioxidant activities through scavenging activity assay against DPPH radical with BHT (2,6-di-*tert*-butyl-4-methylphenol) and

quercetin as positive controls (EC_{50}=56.8, 7.2 μM).[20] Compound **1** showed moderate scavenging activity with an EC_{50} value of 30.5 μM, while compound **2** exhibited weaker scavenging activity with an EC_{50} value of 487.2 μM.

Experimental

General Experimental Procedures Optical rotations were measured by Optical Activity AA-10R automatic spectropolarimeter. UV spectra were measured in MeOH on a Shimadzu UV-1700 spectrophotometer. IR spectra were recorded on a Bruker vector 22 FT-IR spectrometer. NMR spectra were recorded in CD_3OD at room temperature on a Varian Mercury plus 400 NMR spectrometer, and the solvent resonance was used as internal shift reference (TMS as standard). The 2D NMR spectra were recorded using standard pulse sequences. FAB-MS and HR-FAB-MS spectra were recorded on a Finnigan/Thermo Quest MAT 95XL spectrometer. TLC was performed using silica gel 60 F_{254} plates (Merck). Diaion HP-20 (Mitsubishi) and Sephadex LH-20 (Pharmacia biotech) were used for column chromatography. HPLC was performed on a Hitachi L-7000 chromatograph with a Thermo Betasil C-18 (5 μm) column (250×10 mm).

Plant Material The tea plant (*Camellia sinensis* L.) was cultivated in Lugu village, Nantou County, Taiwan. Oolong tea was prepared by a local manufacturer, Mr. Jim-Fang Huang, in November, 2006. A voucher specimen has been deposited in Graduate Institute of Biotechnology, National Pingtung University of Science and Technology.

Extraction and Isolation Oolong tea (500 g) was powdered and extracted three times with methanol (4 l) at room temperature (7 d each). The methanol extract was evaporated *in vacuo* to generate a black residue, which was suspended in H_2O (500 ml) then partitioned sequentially using EtOAc and *n*-BuOH (500 ml×3). The *n*-BuOH fraction (45 g) was subjected to a Diaion HP-20 column (6×55 cm), and eluted with mixtures of water and methanol of reducing polarity as eluents. Eleven fractions were collected as follows: fr. 1 [500 ml, water], fr. 2 [500 ml, water–methanol (9 : 1)], fr. 3 [1000 ml, water–methanol (8 : 2)], fr. 4 [1000 ml, water–methanol (7 : 3)], fr. 5 [1000 ml, water–methanol (6 : 4)], fr. 6 [1000 ml, water–methanol (5 : 5)], fr. 7 [1000 ml, water–methanol (4 : 6)], fr. 8 [1000 ml, water–methanol (3 : 7)], fr. 9 [1000 ml, water–methanol (2 : 8)], fr. 10 [(1000 ml, water–methanol (1 : 9))], and fr. 11 (3000 ml, methanol). Fraction 8 (6.1 g) was further chromatographed on a Sephadex LH-20 column (3×45 cm), eluted with water–methanol (1 : 1 to 0 : 1) to afford nine fractions (each 500 ml), fr. 8A—fr. 8I. Fr. 8D (105.2 mg) was further purified by preparative HPLC using a Thermo Betasil C-18 column eluted with water–acetonitrile (9 : 1 to 1 : 1), 2 ml/min, to afford **1** (19.1 mg, t_R=19.7 min) and **2** (21.1 mg, t_R=20.9 min), respectively.

Quercetin 3-*O*-[2^G-(*E*)-Coumaroyl-3^G-*O*-β-D-glucosyl-3^R-*O*-β-D-glucosylrutinoside] (**1**): Yellow amorphous solid, $[\alpha]_D^{25}$ −60.1° (*c*=0.6, MeOH); UV (MeOH) λ_{max} (log ε) nm: 260 (4.01), 271 (4.02), 320 (4.51), 354 (4.18, sh); IR (KBr) cm^{-1}: 3383, 1703, 1659, 1606, 1514, 1450, 1363, 1325, 1163, 1065, 832; ^{1}H- and ^{13}C-NMR data, see Table 1; FAB-MS *m/z* 1081 $[M+H]^+$ (5), 613 (9), 460 (41), 391 (30), 368 (6), 307 (100); HR-FAB-MS *m/z* 1081.3032 (Calcd for $C_{48}H_{57}O_{28}$, 1081.3035).

Kaempferol 3-*O*-[2^G-(*E*)-Coumaroyl-3^G-*O*-β-D-glucosyl-3^R-*O*-β-D-glucosylrutinoside] (**2**): Yellow amorphous solid, $[\alpha]_D^{25}$ −87.6° (*c*=0.5, MeOH); UV (MeOH) λ_{max} (log ε) nm: 267 (4.10), 321 (4.46), 355 (4.20, sh); IR (KBr) cm^{-1}: 3383, 1698, 1654, 1601, 1518, 1441, 1358, 1265, 1172, 1070, 832; ^{1}H- and ^{13}C-NMR data, see Table 1; FAB-MS *m/z* 1065 $[M+H]^+$ (8), 779 (9), 550 (20), 460 (41), 391 (82), 307 (100); HR-FAB-MS *m/z* 1065.3080 (Calcd for $C_{48}H_{57}O_{27}$, 1065.3086).

Acid Hydrolysis of 1, 2 and the Saponin Mixture Compound **1** (5 mg) was separately dissolved in 2 N CF_3COOH (3 ml) and refluxed for 5 h. The reaction mixture was diluted with water (3 ml) and extracted with EtOAc (6 ml×3). Through TLC comparison with authentic samples using CH_2Cl_2–MeOH (9 : 1) as a developing system, *trans*-*p*-coumaric acid (*Rf* 0.40) and quercetin (*Rf* 0.70) were detected in the EtOAc layer.[21] The aqueous layer was also identified by TLC on Si gel (developing solvent CH_2Cl_2–MeOH–H_2O, 10 : 5 : 1) as rhamnose (*Rf* 0.65) and glucose (*Rf* 0.45) in comparison with authentic samples. Acid hydrolysis of compound **2** by the same method for **1** led to decomposition of coumaric acid, kaempferol, rhamnose, and glucose. A 100-mg aliquot of the crude saponin mixture was dissolved in 2 N CF_3COOH (10 ml) and refluxed for 5 h. After extraction with EtOAc, the aqueous layer was repeatedly evaporated with MeOH under vacuum to remove the solvent completely. Purification of the sugar mixture was achieved by preparative silica TLC (developing solvent CH_2Cl_2–MeOH–H_2O, 15 : 5 : 1) to yield L-rhamnose (5.1 mg, $[\alpha]_D^{25}$ 9.5°, *c*=0.5, MeOH) and D-glucose (11.3 mg, $[\alpha]_D^{25}$ 38.1°; *c*=0.6, MeOH), respectively.[16]

Determination of Scavenging Activity against DPPH Radical Scavenging activity against DPPH radical was determined by a modified method according to Shimada *et al.*[20] DPPH radical was prepared in methanol as a 400 μM solution. DPPH solution (150 μl) was added to 50 μl of different tested samples in methanol (final concentration was 10—1000 μM) in each well of a 96-well flat-bottom EIA microtitration plate. The mixture was shaken vigorously and kept in the dark for 90 min. The absorbance was measured on a microplate spectrophotometer (SpectraMax Plus384, Molecular Devices, CA, U.S.A.) at 517 nm against methanol without DPPH as the blank reference. The percent DPPH scavenging effect was calculated using the following equation:

$$\text{scavenging effect (\%)} = [1 - (\text{absorbance of sample/absorbance of control})] \times 100$$

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