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Antiinflammatory and Antioxidant Flavonoids and Phenols from *Cardiospermum halicacabum* (倒地鈴 Dào Dì Líng)

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ABSTRACT

Seventeen compounds, quercetin-3-*O*-α-L-rhamnoside (**1**), kaempferol-3-*O*-α-L-rhamnoside (**2**), apigenin-7-*O*-β-D-glucuronide (**3**), apigenin 7-*O*-β-D-glucuronide methyl ester (**4**), apigenin 7-*O*-β-D-glucuronide ethyl ester (**5**), chrysoeriol (**6**), apigenin (**7**), kaempferol (**8**), luteolin (**9**), quercetin (**10**), methyl 3,4-dihydroxybenzoate (**11**), *p*-coumaric acid (**12**), 4-hydroxybenzoic acid (**13**), hydroquinone (**14**), protocathehuic acid (**15**), gallic acid (**16**), and indole-3-carboxylic acid (**17**), were isolated from the ethanol extract of Taiwanese *Cardiospermum halicabum*. All chemical structures were determined by physical and extensive spectroscopic analyses such as ^{1}H Nuclear Magnetic Resonance spectroscopy (NMR), ^{13}C NMR, ^{1}H-^{1}H Correlation spectroscopy (^{1}H-^{1}H COSY), Heteronuclear Multiple Quantum Coherence spectroscopy (HMQC), Heteronuclear Multiple-bond Correlation spectroscopy (HMBC), and Nuclear Overhauser Effect spectroscopy (NOESY), as well as comparison with literature values. Furthermore, the High-Performance Liquid Chromatography- Photodiode Array Detector (HPLC-DAD) fingerprint profile was established for the determination of major constituents in the EtOAc extract and retention times of the isolated compounds. All isolated compounds were also evaluated for antiinflammatory and antioxidant activities.

Key words: Anti-oxidative activity, Antiinflammatory activity, *Cardiospermum halicabum*, Flavanoids, HPLC fingerprint assay

INTRODUCTION

Cardiospermum halicacabum (倒地鈴 Dào Dì Líng) is a creeping perennial plant belonging to the Sapindaceae, widely distributed in tropical and subtropical region, especially Taiwan, India, and Africa.[1] *C. halicacabum* is a folk medicine for treatment of diuretic, gastroenteritis, rheumatism, lumbago, fever, and nervous diseases. Previous pharmacological studies in animal model show that the extracts of *C. halicacabum* exhibited antiulcer,[2] analgesic, and vasodepressant activities.[3] Phytochemistry studies showed that the title plant contains phenolic acid derivatives, flavonol and its glycosides, many of which have antiinflammatory and antioxidant activities.[4-6]

In our continuing search for bioactive secondary metabolites

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from Taiwanese plants, we found that an EtOH extract of *C. halicacabum* showed the inhibition against lipopolysaccharide (LPS)-induced nitric oxide (NO) production and antioxidant activity by 1,1-Diphenyl-2-picrylhydrazyl (DPPH) testing. Our subsequent phytochemical analysis of the EtOH extract has led to the isolation of 10 flavonoids, quercetin-3-*O*-α-L-rhamnoside (**1**),[7] kaempferol-3-*O*-α-L-rhamnoside (**2**),[8] apigenin-7-*O*-β-D-glucuronide (**3**),[9] apigenin 7-*O*-β-D-glucuronide methyl ester (**4**),[8] apigenin 7-*O*-β-D-glucuronide ethyl ester (**5**),[8] chrysoeriol (**6**),[10] apigenin (**7**),[11] kaempferol (**8**),[10] luteolin (**9**),[8] quercetin (**10**)[10] and phenolic acid derivatives, methyl 3,4-dihydroxybenzoate (**11**),[12] *p*-coumaric acid (**12**),[11] 4-hydroxybenzoic acid (**13**),[13] hydroquinone (**14**),[14] protocathehuic acid (**15**),[13] gallic acid (**16**),[13] and indole-3-carboxylic acid (**17**).[15] The structures of all isolated compounds were identified by extensive spectroscopic methods including MS and 2D-NMR experiments and comparison with the reported data. To establish the HPLC-DAD fingerprinting profile of the EtOAc extract were described. All isolates were also evaluated for antiinflammatory and antioxidant activities.

MATERIALS AND METHODS

General experimental procedures

Optical rotations were measured with JASCO P-2000 polarimeter (Japan spectroscopic Co. LTD, Japan). Infrared (IR) and ultraviolet (UV) spectra were measured on a Nicolet AVATAR 320 FT-IR spectrophotometer (Nicolet instrument corporation, USA) and a U-3310 spectrophotometer (Hitachi high-technologies corporation, Japan), respectively. ESIMS data were performed on the Waters Quattro Ultima mass spectrometer (Waters , USA). 1D and 2D NMR spectra were performed on a Bruker NMR spectrometer (Unity Plus 400 MHz, Bruker corporation, Germany) using $CDCl_3$, CD_3OD, C_5D_5N and DMSO-d_6 as solvent for measurement. Sephadex LH-20 (GE healthcare bio-sciences ABSE-75184 Uppsala, Sweden) and silica gel (70–230 mesh and 230–400 mesh, Merck LTD., Germany) were used for column chromatography, and precoated silica gel 60 F-254 plates (Merck LTD., Germany) were used for thin-layer chromatography (TLC). The spots on TLC were detected by spraying with 10% H_2SO_4 and then heating on a hot plate. HPLC separations were performed on a Shimadzu LC-20A series apparatus (Pump: LC-20AT; UV detector: SPD-20A, Shimadzu corporation, Japan), equipped with a 250 × 20 mm preparative Cosmosil $5C_{18}$-ARII column (Nacalai Tesque, INC. Japan). HPLC fingerprint plot was done on a Waters 600S separations module (Pump: Waters 600; UV detector: Waters 996 Photodiode Array Detector; Controller: Waters 600S; Autosampler: Waters 717plus; Degasser: Waters In-Line Degasser), with a Cosmosil $5C_{18}$-ARII column (5 μm, 4.6 × 250 mm, Nacalai Tesque, INC. Japan).

Plant material

The dried whole part of *Cardiospermum halicabum* was collected in Taitung, County, Taiwan, in September 2009. A voucher specimen (NRICM 20090815A) was identified by Dr. Syh-Yuan Hwang and deposited in the National Research Institute of Chinese Medicine, Taipei, Taiwan.

Extraction and isolation

The dried whole parts of *C. halicabum* (12 kg) were extracted with 95% EtOH (120 L) twice at 50°C, and then extracted with 70% EtOH (120 L) twice at 50°C. The dark crude extracts were combined and concentrated under reduced pressure. The residue was suspended in H_2O, and then sequentially partitioned with *n*-hexane, EtOAc, and BuOH, obtained four layers. After concentrated under reduced pressure, the EtOAc extract (KCHE, 570 g) was fractionated by silica gel column (7.5 × 100 cm) eluting with $CHCl_3$/MeOH (100:0, 100:1, 50:1, 10:1, 5:1, 2:1, 0:100), to yield 12 fractions (Fr. A–L). Fr. C (ca 30 g) was subjected by Sephadex LH-20 column (7 × 100 cm) with CH_2Cl_2/MeOH (1:1) to yield five subfractions (Fr. C1–C8). Fr. C1 (1.7 g) was recrystallized from CH_2Cl_2 and MeOH to give yellow powder, compound **6** (Chrysoeriol, 613 mg). Fr. C3 (3.3 g) was repeatedly separated by Sephadex LH-20 (100% MeOH, 4.5 × 100 cm) and chromatographed on silica gel using a gradient of CH_2Cl_2/MeOH to obtain compounds **7** (Apigenin, 527 mg), **14** (Methyl 3,4-dihydroxybenzate, 4 mg), and **15** (*p*-Coumaric acid, 80 mg). Fr. C4 (0.7 g) was submitted to Sephadex LH-20 column (3 × 135 cm) eluting with MeOH and then followed by Reverse Phase-HPLC (RP-HPLC) (MeOH/H_2O = 65:35) to afford compounds **11** (Hydroquinone, 4 mg), **12** (4-Hydroxybenzoic acid, 5 mg), and **17** (Indole-3-carboxylic acid, 6 mg). The MeOH/CH_2Cl_2-insoluble portion of Fr. C6 (104 mg) furnished compound **8** (Kaempferol, 30 mg). Fr. E (ca 17 g) was subjected on Sephadex LH-20 column (7 × 100 cm) with CH_2Cl_2/MeOH (1:1) to yield seven subfractions (Fr. E1–E7). Fr. E3 (2.1 g) was repeatedly chromatographed on Sephadex LH-20 column (3 × 135 cm) to yield compounds **9** (Luteolin, 201 mg), **10** (Quercetin, 128 mg), and **13** (Protocathehuic acid, 895 mg). Fr. G (ca 27 g) was subjected by Sephadex LH-20 column (7 × 100 cm) with CH_2Cl_2/MeOH (1:1) to yield nine subfractions (Fr. G1–G9). Fr. G5 (1.6 g) was repeatedly chromatographed on Sephadex LH-20 column (4 × 72 cm) to yield compound **2** (Kaempferol-3-*O*-α-L-rhamnoside, 860 mg). Fr. G6 (1.7 g) was subjected by Sephadex LH-20 column (4 × 120 cm) with CH_2Cl_2/MeOH (1:1) and purified by RP-HPLC (MeCN/H_2O, solvent rate:15-22-26-30-40, flowing time: 5-13-23-28-30) to afford compounds **4** (apigenin-7-*O*-β-D-glucuronide methyl ester, 105 mg, Rt: 25.4 min) and **5** (apigenin-7-*O*-β-D-glucuronide ethyl ester, 126 mg, Rt: 29.4 min). Using the same methods as that of Fr. G6 on Sephadex LH-20 (3 × 135 cm), compound **16** (35 mg) was obtained from Fr. G7 (0.4 g). Fr. I (ca 30 g) was separated by Sephadex LH-20 column (7 × 100 cm) with CH_2Cl_2/MeOH to give ten subfractions (Fr. I1–I10). Crystallization of Fr. I3 (5.6 g) and I5 (4.3 g), compounds **1** (Quercetin-3-*O*-α-L-rhamnoside, 4.3 g) and **3** (apigenin 7-*O*-β-D-glucuronide, 3.4 g), were afforded, respectively.

Quercetin-3-*O*-*α*-L-rhamnoside (**1**): Yellow, amorphous powder. M.p. 190°C disappeared. $[\alpha]_D^{25}$ −141 (*c* 0.4, MeOH). UV (ACN) λ_{max} nm: 222, 254, 348. IR ν_{max} (KBr)/cm^{-1}: 3324,

Table 1. ^{1}H- and ^{13}C-NMR spectroscopic data of compounds **1** and **2**

No.	Compound **1** (Pyridine-d_6)		Compound **2** (MeOH-d_4)	
	δ_H (mult, J in Hz)[b]	δ_C (mult)[c]	δ_H (mult, J in Hz)[b]	δ_C (mult)[c]
2		158.1 (s)		159.1 (s)
3		135.9 (s)		136.1 (s)
4		179.0 (s)		179.5 (s)
5		162.8 (s)		163.3 (s)
6	6.67 (d, 2.0)	99.6 (d)	6.16 (d, 2.0)	100.1 (d)
7		165.7 (s)		166.7 (s)
8	6.62 (d, 2.0)	94.4 (d)	6.33 (d, 2.0)	94.9 (d)
9		157.6 (s)		158.5 (s)
10		105.3 (s)		105.6 (s)
1'		122.2 (s)		122.6 (s)
2'	8.00 (d, 2.4)	117.0 (d)	7.74 (d, 8.8)	131.9 (s)
3'		147.2 (s)	6.91 (d, 8.8)	116.5 (d)
4'		150.4 (s)		161.5 (s)
5'	7.67 (dd, 8.4, 2.0)	116.4 (d)	6.91 (d, 8.8)	116.5 (d)
6'	7.28 (d, 8.4)	122.2 (d)	7.74 (d, 8.8)	131.9 (d)
Sugar moiety				
1"	6.24 (*br* s)	103.9 (d)	5.36 (d, 1.2)	103.5 (d)
2"	5.06 (dd, 3.2, 1.6)	71.9 (d)	4.22 (dd, 3.2, 1.6)	71.9 (d)
3"	4.63 (dd, 8.8, 3.2)	72.4 (d)	4.70 (dd, 6.0, 3.2)	72.1 (d)
4"	4.28 (m)	73.2 (d)	3.33 (m)	73.2 (d)
5"	4.38 (m)	72.0 (d)	3.32 (m)	72.0 (d)
6"	1.45 (d, 6.0)	18.3 (q)	0.91 (d, 5.2)	17.6 (q)

2359, 1659, 1608, 1504, 1364, 1202. For ^{1}H (400 MHz, DMSO-d_6) and ^{13}C (100 MHz) NMR spectroscopic data, see Table 1. ESI-MS: m/z 449 $[M+H]^+$.

Kaempferol-3-*O*-*α*-L-rhamnoside (**2**): Yellow, amorphous powder. M.p. 180°C disappeared. $[\alpha]_D^{25}$ −157 (c 0.4, MeOH). UV (ACN) λ_{max} nm: 223, 263, 343. IR ν_{max} (KBr) cm^{-1}: 3357, 2360, 1658, 1609, 1505, 1361, 1209, 1177, 1087. For ^{1}H (400 MHz, DMSO-d_6) and ^{13}C (100 MHz) NMR spectroscopic data, see Table 1. ESI-MS: m/z 455 $[M+Na]^+$.

Apigenin-7-*O*-*β*-D-glucuronide (**3**): Yellow, amorphous powder. M.p. 230°C disappeared. $[\alpha]_D^{25}$ −40 (c 0.2, MeOH). UV (ACN) λ_{max} nm: 222, 266, 336. IR ν_{max} (KBr) cm^{-1}: 3394, 2359, 1659, 1605, 1497, 1444, 1346, 1246, 1177, 1061. For ^{1}H (400 MHz, DMSO-d_6) and ^{13}C (100 MHz) NMR spectroscopic data, see Table 1. ESI-MS: m/z 447 $[M+H]^+$.

Apigenin-7-*O*-*β*-D-glucuronide methyl ester (**4**): White, amorphous powder. M.p. 245°C disappeared. $[\alpha]_D^{25}$ −55 (c 0.4, Acetone). UV (ACN) λ_{max} nm: 222, 266, 336. IR ν_{max} (KBr) cm^{-1}: 3389, 1742, 1665, 1608, 1499, 1444, 1351, 1301, 1247, 1179, 1094, 1059. For ^{1}H (400 MHz, DMSO-d_6) and ^{13}C (100 MHz) NMR spectroscopic data, see Table 1. ESI-MS: m/z 461 $[M+H]^+$.

Apigenin-7-*O*-*β*-D-glucuronide ethyl ester (**5**): White, amorphous powder. M.p. 232°C disappeared. $[\alpha]_D^{25}$ −98 (c 0.4, MeOH). UV (ACN) λ_{max} nm: 222, 266, 337. IR ν_{max} (KBr) cm^{-1}: 3433, 2073, 1737, 1611, 1502, 1354, 1304, 1247, 1217, 1187, 1096, 1057, 1016. For ^{1}H (400 MHz, DMSO-d_6) and ^{13}C (100 MHz) NMR spectroscopic data, see Table 1. ESI-MS: m/z 475 $[M+H]^+$.

Chrysoeriol (**6**): Yellow, amorphous powder. M.p. >300°C disappeared. $[\alpha]_D^{25}$ +243 (c 0.2, 50% Acetone). UV (ACN) λ_{max} nm: 222, 267, 348. IR ν_{max} (KBr) cm^{-1}: 2348, 2283, 1649, 1624, 1561, 1502, 1433, 1349, 1270, 1207, 1167, 1028. ESI-MS: m/z 301 $[M+H]^+$.

Apigenin (**7**): White, amorphous powder. M.p. >300°C disappeared. $[\alpha]_D^{25}$ +207 (c 0.2, 50% Acetone). UV (ACN) λ_{max} nm: 222, 266, 337. IR ν_{max} (KBr) cm^{-1}: 3444, 2077, 1629, 1502, 1358, 1246, 1182. ESI-MS: m/z 271 $[M+H]^+$.

Kaempferol (**8**): Brown, amorphous powder. M.p. 270°C disappeared. $[\alpha]_D^{25}$ −27 (c 0.4, MeOH). UV (ACN) λ_{max} nm: 223, 265, 362. IR ν_{max} (KBr) cm^{-1}: 3354, 2283, 1660, 1611, 1510, 1379, 1258, 1176. ESI-MS: m/z 328 $[M+ACN+H]^+$.

Luteolin (**9**): Yellow, amorphous powder. M.p. >300°C disappeared. $[\alpha]_D^{25}$ +49 (c 0.2, MeOH). UV (ACN) λ_{max} nm: 223, 252, 348. IR ν_{max} (KBr) cm^{-1}: 3422, 2263, 1702, 1656, 1612, 1504, 1446, 1368, 1266, 1192, 1167, 1121, 1097, 1032. ESI-MS: m/z 287 $[M+H]^+$.

Quercetin (**10**): Yellow, amorphous powder. M.p. >300°C disappeared. $[\alpha]_D^{25}$ +9 (c 0.4, MeOH). UV (ACN) λ_{max} nm: 221, 254, 365. IR ν_{max} (KBr) cm^{-1}: 3451, 1658, 1519, 1321, 1170, 1112, 1093, 1011. ESI-MS: m/z 344 $[M+H]^+$.

Hydroquinone (**11**): White, amorphous powder. M.p. 174°C disappeared. UV (ACN) λ_{max} nm: 219, 254. IR ν_{max} (KBr) cm^{-1}: 3753, 3678, 3652, 3403, 2348, 2282, 1665, 1594, 1509, 1421, 1315, 1246, 1166. ESI-MS: m/z 193 $[M+2ACN+H]^+$.

4-hydroxybenzoic acid (**12**): White, amorphous powder. M.p. 150°C disappeared. $[\alpha]_D^{25}$ 7 (c 0.4, MeOH). UV (ACN) λ_{max} nm: 219, 254. IR ν_{max} (KBr) cm^{-1}: 3753, 3678, 3652, 3403, 2348, 2282, 1665, 1594, 1509, 1421, 1315, 1246, 1166. ESI-

MS: *m*/*z* 137 [M−H]⁻.

Protocathehuic acid (**13**): Brown, amorphous powder. M.p. 185°C disappeared. $[\alpha]_D^{25}$ +3 (*c* 0.4, MeOH). UV (ACN) λ_{max} nm: 221, 259, 293. IR ν_{max} (KBr) cm^{-1}: 3856, 3692, 3678, 3651, 3631, 3589, 3569, 3358, 2348, 2283, 2078, 1891, 1686, 1655, 1618, 1535, 1460, 1439, 1025. ESI-MS: *m*/*z* 153 [M−H]⁻.

Methyl 3,4-dihydroxybenzoate (**14**): Brown, amorphous powder. M.p. 140°C disappeared. $[\alpha]_D^{25}$ +143 (*c* 0.2, 50% Acetone). UV (ACN) λ_{max} nm: 221, 260, 292. IR ν_{max} (KBr) cm^{-1}: 3485, 2282, 1677, 1597, 1523, 1434, 1280, 1239, 1204, 1028. ESI-MS: *m*/*z* 199 [M+MeOH−H]⁻.

Gallic acid (**15**): White, amorphous powder. M.p. 250°C disappeared. $[\alpha]_D^{25}$ +3 (*c* 0.4, MeOH). UV (ACN) λ_{max} nm: 221, 270. IR ν_{max} (KBr) cm^{-1}: 3856, 3753, 3738, 3475, 2095, 1630, 1524, 1449, 1341, 1310, 1261, 1200, 1107, 1035. ESI-MS: *m*/*z* 169 [M−H]⁻.

p-Coumaric acid (**16**): White, amorphous powder. M.p. 280°C disappeared. $[\alpha]_D^{25}$ −7 (*c* 0.4, MeOH). UV (ACN) λ_{max} nm: 226, 360. IR ν_{max} (KBr) cm^{-1}: 3652, 3398, 2282, 1676, 1604, 1512, 1450, 1312, 1251, 1173. ESI-MS: *m*/*z* 163 [M − H]⁻.

Indole-3-carboxylic acid (**17**): Yellow, amorphous powder. M.p. 210°C disappeared. $[\alpha]_D^{25}$ +60 (*c* 0.2, MeOH). UV (ACN) λ_{max} nm: 226, 280. IR ν_{max} (KBr) cm^{-1}: 3651, 2934, 2348, 2247, 1691, 1421, 1308, 1200. ESI-MS: *m*/*z* 162 [M+H]⁺.

HPLC fingerprint assay

Materials and Reagents: Seventeen reference compounds (**1**–**17**) isolated from the EtOAc extract (KCHE) of *C. halicacabum*. Acetonitrile (LC grade) were purchased from E. Merck. Milli-Q ultra-pure water (Millipore, Q-gard 1/Quantum EX) was used throughout the study. Apparatus and conditions: The HPLC was performed on a Waters system equipped with a Waters 600S controller, a Waters 600 pump, a photodiode array detector (Waters 996), a Waters In-line degasser, and Waters 717plus autosampler. The Cosmosil $5C_{18}$-AR-II (5 μm, 4.6 × 250 mm) eluted at a rate of 0.8 mL/min. The mobile phase consisted of water (A) and acetonitrile (B), together with 1% tri-fluoroacetic acid TFA , using a gradient program of 5%-25%-25%-30% (B) in 0-10-30-60 min. DAD detector was set at 254 nm for acquiring chromatograms, UV spectra and 3D-plots were recorded between 200 and 400 nm. Preparation of standard solutions, each compound was accurately weighed and dissolved in MeOH, the terminate concentration was ca. 100 μg/mL. Preparation of sample solution, the KCHE extract from *C. halicacabum* was dried under vacuum, then 10 mg was weighed accurately and dissolved in MeOH (1 mL). All the tested solutions were filtered through a 0.45 μm filter (Millipore) before use.

Scavenging activity of DPPH radical assay

The radical scavenging activity of the isolates on DPPH free radical was measured using the method of Chung *et al.*[16] with minor modifications. The aliquot of each sample (120 μL, 30–5 μg/mL), or (±)-*α*-tocopherol (40–10 μg/mL) was mixed with 30 μL of 0.75 mM DPPH methanol solution in 96-well microplate. The mixture was shaken vigorously with orbital shaker in the dark at room temperature for 30 min and then measured the absorbance at 517 nm with enzyme-linked immunosorbent assay (ELISA) reader. The negative control was the measurement using methanol to replace the sample in the react solution. The DPPH radical scavenging activity of the isolates were compared with the negative control and positive control (±)-*α*-tocopherol. The final results were performed as the concentrations of ED_{50}, which is the concentration of sample required to cause 50% inhibition against DPPH radicals in react solution.

NO Measurement of antiinflammatory activity

The macrophage cell line RAW 264.7 was obtained from ATCC (Rockville, MD, U.S.A.) and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 5% heat-inactivated fetal calf serum, 100 U/mL penicillin and streptomycin, respectively, and grown at 37°C with 5% CO_2 in fully humidified air. Cells were plated at a density of 5×10^4 cells/well in 96-well culture plate and stimulated with LPS (1.0 μg/mL) in the presence or absence of different concentrations of tested compounds (0.1–40 μM) for 24 h simultaneously. All compounds were dissolved in dimethyl sulfoxide (DMSO) and further diluted with sterile phosphate buffered saline (PBS). Nitrite (NO_2^-) accumulation in the medium was used as an indicator of NO production, which was measured by adding Griess reagent (1% sulfanilamide in H_2O and 0.1% naphthylenediamine in 5% phosphoric acid). $NaNO_2$ was used to generate a standard curve, and nitrite production was determined by measuring optical density at 540 nm. All experiments were performed in triplicate. NO production by LPS stimulation was designated as 100% for each experiment. Quercetin was as a positive control.

RESULTS AND DISCUSSION

The EtOH extract of the whole parts of *C. halicacabum* was suspended in H_2O and further successively partitioned with *n*-hexane, EtOAc, and BuOH. After evaporation of the each solvent, the EtOAc extract exhibited potent antiinflammatory activity by NO production testing (IC_{50} = 32.12 μg/mL) and antioxidant activity against free radicals (DPPH) (ED_{50} = 28.97 μg/mL). The EtOAc residue was subjected to column chromatography on silica gel and Sephadex LH-20, and HPLC to yield 17 compounds, including 10 flavanoids and 7 phenolic acids derivatives. All isolated compounds were identified by detailed spectroscopic analyses, especially Mass spectroscopy (MS) and 2D NMR experiments, comparing those of authentic samples and references. Their structures are shown in Figure 1. We further established the HPLC-DAD fingerprinting profile of the EtOAc extract of which 17 main peaks were identified.

Compound **1** was obtained as yellow amorphous powder with molecular formula $C_{21}H_{21}O_{11}$, as established by positive ESIMS, in combination with NMR spectroscopic data. IR absorptions at 3324, 1659, 1608, and 1504 cm^{-1} and UV data at 254 and 348 nm implied the existence of hydroxyl, conjugated carbonyl groups, and aromatic ring, respectively. In the ^1H NMR spectrum [Table 1], five aromatic proton signals at δ_H 6.67 (H-6,

d, J = 2.0 Hz), 6.62 (H-8, d, J = 2.0 Hz), 7.28 (H-5′, d, J = 8.4 Hz), 7.67 (H-6′, dd, J = 8.4, 1.6 Hz), and 8.00 (H-2′, d, J = 1.6 Hz), and proton resonances for sugar moiety at 5.06 (H-1″, d, J = 6.8 Hz), 4.63 (H-2″, dd, J = 8.8, 3.2 Hz), 4.63 (H-3″, m), 4.28 (H-4″, m), 4.38 (H-5″, m), and 1.45 (H-6″, d, J = 6.0 Hz) were observed. The ^{13}C NMR spectra showed resonance for 21 carbons differentiated by the distortionless enhancement by polarization transfer (DEPT) experiments, including one carbonyl (C-4, δ_C 179.0), five aromatic methines (δ_C 99.6, C-6; 94.4, C-8; 117.0, C-2′; 116.4, C-5′; 122.2, C-6′), nine quaternary aromatic signals (δ_C 158.1, C-2; 135.9, C-3; 162.8, C-5; 165.7, C-7; 157.6, C-9; 105.3, C-10; 122.2, C-1′; 147.2, C-3′; 150.4, C-4′), and five oxygenated methines (δ_C 103.9, 73.2, 72.4, 72.0, 71.9) and methyl (δ_C 18.3). Those data indicated compound **1** as having a basic flavonol structure with rhamnose moiety. The planar structure and NMR assignments for **1** were established by detailed analysis of two-dimensional NMR (2D NMR), including ^{1}H-^{1}H COSY, HMQC, and HMBC correlations [Figure 2]. The ^{1}H-^{1}H COSY spectra, coupling with HMQC, allowed the establishment of two spin systems, corresponding to two structural fragments of C-5′/C-6′ and C-1″/C-2″/C-3″/C-4″/C-5″/C-6″. The HMBC correlations of H-2′/C-2, C-1′, C-3′, and C-4′, H-5′/C-2, C-1′, and H-5′/C-3′ and C-4′ indicated 3′,4′-dihydroxy substitution of aromatic ring located at C-2. Acid hydrolysis of compound **1** gave an aglycone, quercetin (**10**) and a sugar moiety, the latter was identified by HPLC analysis as L-rhamnose. Furthermore, HMBC spectrum of **1** indicated the bonding position of L-rhamnose to be C-3 according to the correlations of H-1″/C-3. The α-anomeric configuration for the rhamnose was judged by the coupling constant. On the basis of the above spectroscopic evidence, compound **1** was deduced as quercetin-3-*O*-α-L-rhamnoside.

Compound **2** was obtained as a yellow amorphous powder. ESIMS (positive) showed a quasi-molecular ion peak at *m/z* 455 $[M+Na]^+$, corresponding to the formula $C_{21}H_{20}O_{10}$, and agreed with its ^{1}H and ^{13}C NMR data. Similar to **1**, compound **2** showed IR bands at 3357, 1658, 1609, and 1505 cm^{-1} and UV absorptions at 223, 263, and 343 nm, indicating conjugated carbonyl groups, and aromatic ring, respectively. It was found that the ^{13}C-NMR spectroscopic data [Table 1] of compound **2** were closely related to those of **1**. However, detailed cross comparison of ^{1}H-NMR spectroscopic data showed that the ABX signals in compound **1** were replaced by A_2B_2 pattern system (δ_H 7.74, d, J = 8.8 Hz, 2H; 6.91, d, J = 8.8 Hz; δ_C 131.9 × 2, 116.5 × 2) in **2**. This was in accordance with the molecular formula of **1**, which contained one more oxygen atom than that of **2**. The complete structure of **2** was further supported by detailed anaylsis of ^{1}H-^{1}H COSY, HSQC, and HMBC spectra data. According to the above observation, compound **2** was determined as kaempferol-3-*O*-α-L-rhamnoside.

Compound **3** was obtained as yellow amorphous powder, Mp. 230°C, with molecular formula $C_{21}H_{18}O_{11}$, as determined by ESIMS and its NMR data. The UV data, with absorption maxima at λ_{max} 222, 266, 336 nm, and IR absorption bands attributable to hydroxyl (3394 cm^{-1}), conjugated carbonyl (1659 cm^{-1}), aromatic (1605 and 1497 cm^{-1}) functionalities, suggested

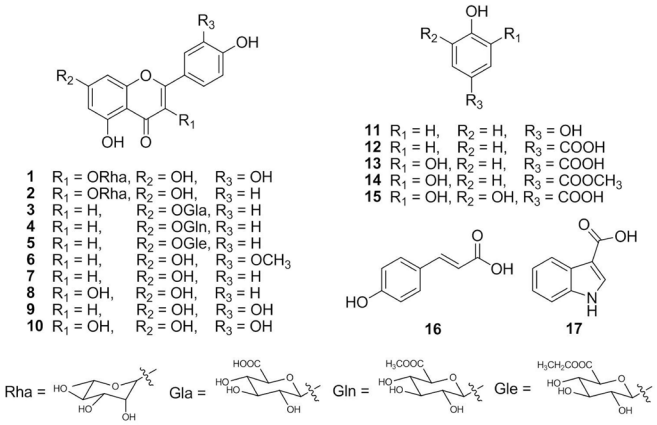

Figure 1. The structures of compounds **1**–**17** isolated from *C. halicacabum*

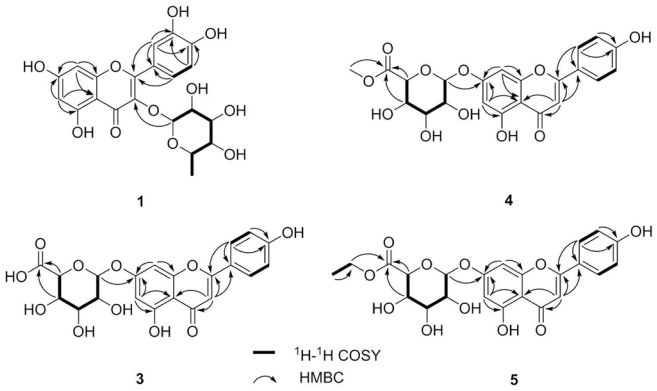

Figure 2. The key HMBC and ^{1}H-^{1}H COSY correlations of compounds **1** and **3-5**

the presence of flavanoid skeleton. The ^{1}H NMR spectrum of compound **3** [Table 2] showed a pair of doublet aromatic proton signals at δ_H 6.44 (H-6, d, J = 2.0 Hz) and 6.76 (H-8, d, J = 1.6 Hz) and A_2B_2-type aromatic proton signals at δ_H 7.80 (H-2′, H-6′, d, J = 8.4 Hz, 2H) and 6.88 (H-3′, H-5′, d, J = 8.4 Hz, 2H). The evidences was suggested that the aglycone was a 4’,5,7-trihydroxyflavone (also called apigenin). In addition, the ^{1}H and ^{13}C NMR spectra also indicated the presence of a glucuronic acid moiety from the following signals [δ_H 3.98, d, J = 8.4 Hz; δ_C 174.4], which are the characteristics for the H-5″ and C-6″ of the sugar moiety, respectively. The HMBC correlations between H-1″ (δ_H 5.12)/C-7 (δ_C 164.6) suggested that the sugar located at C-7 positions. The sugar moiety was further confirmed to be β-glucose on the basis of coupling constant (J = 6.8 Hz) of anomeric proton in the ^{1}H-NMR spectrum of **3** and the D-form was identified by acid hydrolysis with an authentic sample. From the above corroboration, compound **3** was defined as apigenin-7-*O*-β-D-glucuronide.

Compound **4**, obtained as a yellow solid, was assigned the molecular formula $C_{22}H_{20}O_{11}$, as determined form ESIMS, ^{13}C NMR, and DEPT spectroscopic data. The ^{1}H and ^{13}C NMR spectra showed resonance characteristics of flavonol skeleton with a hexose moiety, similar to those of compound **3**. However, in the ^{1}H and ^{13}C NMR spectra, an additional oxygenated methyl group at δ_H 3.62 and δ_C 52.0 was observed, which was

Table 2. ^{1}H- and ^{13}C-NMR spectroscopic data of compounds **3-5**

No.	Compound **3** (DMSO-d_6)		Compound **4** (Pyridine-d_5)		Compound **5** (Pyridine-d_5)	
	δ_H (mult, J in Hz)[b]	δ_C (mult)[c]	δ_H (mult, J in Hz)[b]	δ_C (mult)[c]	δ_H (mult, J in Hz)[b]	δ_C (mult)[c]
2		166.6 (s)		163.5 (s)		163.4 (s)
3	6.58 (s)	104.0 (d)	6.87 (s)	103.8 (d)	6.86 (s)	103.8 (d)
4		184.0 (s)		182.6 (s)		182.6 (s)
5		162.7 (s)		162.5 (s)		162.5 (s)
6	6.44 (d, 2.0)	101.2 (d)	6.84 (d, 2.0)	100.4 (d)	6.82 (*br* s)	100.4 (d)
7		164.6 (s)		164.8 (s)		164.8 (s)
8	6.76 (d, 1.6)	96.0 (d)	7.11 (*br* s)	95.2 (d)	7.11 (*br* s)	95.2 (d)
9		158.9 (s)		157.7 (s)		157.7 (s)
10		107.1 (s)		106.6 (s)		106.6 (s)
1'		122.9 (s)		121.8 (s)		121.7 (s)
2'	7.80 (d, 8.8)	129.6 (d)	7.80 (d, 8.4)	128.8 (d)	7.80 (d, 8.4)	128.8 (d)
3'	6.88 (d, 8.8)	117.0 (d)	7.18 (d, 8.4)	116.7 (d)	7.18 (d, 8.4)	116.7 (d)
4'		162.8 (s)		162.7 (s)		162.7 (s)
5'	6.88 d (d, 8.8)	117.0 (d)	7.18 (d, 8.4)	116.7 (d)	7.18 (d, 8.4)	116.7 (d)
6'	7.80 d (d, 8.8)	129.6 (d)	7.80 (d, 8.4)	128.8 (d)	7.80 (d, 8.4)	128.8 (d)
Sugar moiety						
1"	5.12 (d, 6.8)	101.4 (d)	6.00 (d, 7.2)	101.5 (d)	6.00 (d, 6.4)	101.5 (d)
2"	3.55 (m)	74.5 (d)	4.91 (d, 9.6)	74.3 (d)	4.87 (d, 9.6)	74.3 (d)
3"	3.55 (m)	76.5 (d)	4.58 (dd, 9.6, 8.8)	77.1 (d)	4.60 (dd, 9.2, 8.8)	77.2 (d)
4"	3.55 (m)	73.2 (d)	3.44 (m)	72.5 (d)	3.46 (m)	72.7 (d)
5"	3.98 (d, 9.2)	77.4 (d)	3.41 (m)	77.4 (d)	3.42 (m)	77.4 (d)
6"		174.4 (s)		170.1 (s)		169.6 (s)
1'''			3.62 (s)	52.0 (q)	4.15 (m)	61.3 (t)
2"					1.07 (t, 7.2)	13.9 (q)

correlated to C-6″ (δ_C 170.1) in the HMBC spectrum, indicating that methyl group located at C-6″ of the sugar moiety. Consequently, the structure of **4** was determined to be apigenin 3-*O*-*β*-D-glucuronide methyl ester.

The molecular formula of compound **5** was determined to be $C_{23}H_{22}O_{11}$ from the analysis of its ESIMS and NMR spectroscopic data. Due to the similar IR, UV, and ^{1}H and ^{13}C NMR spectra as those of compounds **3** and **4**, the basic skeleton of **5** was comprised of a flavonol and a glucuronic acid moiety, except for the additional presence of a ethyl signal (δ_H 4.15, m; 1.07, t, J = 7.2 Hz; δ_C 61.3, 13.9) in **5**. The HMBC correlations of H-1‴ and H-2‴ with C-6″ (δ_C 169.6) as well as the COSY correlation of H-1‴/H-2‴ indicated that the methyl group at C-6 in **4** was replaced by the ethyl group in compound **5**. The assignments of ^{1}H and ^{13}C NMR data of compound **5** were accomplished by ^{1}H-^{1}H COSY, HMQC, and HMBC correlations. Therefore, compound **5** was unambiguously identified as apigenin 7-*O*-*β*-D-glucuronide ethyl ester.

The UV and NMR spectroscopic features of the other flavonols, chrysoeriol (**6**), apigenin (**7**), kaempferol (**8**), luteolin (**9**), quercetin (**10**), were very similar to one another, suggesting that they were flavonoids, similar to compounds **1**–**5**. The UV spectra of flavonols **1**–**10** are illustrated in Figure 3. Compounds **6**–**10** also were identified by analyses of NMR spectroscopic data and comparison with data reported in the literature. In addition, seven phenolic acids derivatives were isolated from the EtAOc extract of *C. halicacabum*, and 3,4-dihydroxybenzoate (**11**), *p*-coumaric acid (**12**), 4-hydroxybenzoic acid (**13**), hydroquinone (**14**), protocathehuic acid (**15**), gallic acid (**16**), indole-3-carboxylic acid (**17**), determined based on the spectroscopic analysis.

We have studied the fingerprinting profile [Figure 4] of the bioactive EtOAc extract of *C. halicacabum* by using HPLC-DAD method. The mobile phase consisted of water (A) and acetonitrile (B) with 1% TFA, using a gradient program of 5%-25%-25%-30% (B) in 0-10-30-60 min. These isolated flavonoids and phenolic acids were detected at 280 nm and identified by matching the retention time (Rt) with responding compounds and their UV spectroscopic characteristics. The 17 Rts for the isolated compounds **1**–**17** were, respectively, shown as **1**: 22.79; **2**: 25.96; **3**: 24.04; **4**: 32.85; **5**: 43.51; **6**: 57.56; **7**: 53.69; **8**: 55.94; **9** & **10**: 35.87; **11**: 7.30; **12**: 16.34; **13**: 11.44; **14**: 18.03; **15**: 6.04; **16**: 21.02; **17**: 24.55 min. The major components existed in the EtOAc extract were almost identified.

Moreover, all of the isolated compounds were tested for antiinflammatory activity by the inhibition effects on the LPS-induced NO production in RAW 264.7 macrophages. As summarized in Table 3, compounds **3**–**5**, **7**, **8**, **12**, **16**, and **17** showed potent inhibitory activities against NO production and did not affect cell viability. Among them, phenolic acids derivatives **12** (IC_{50} = 2.64 μg/mL) and **16** (IC_{50} = 1.66 μg/mL) exhibited more potent than flavonoids. In addition, these isolated compounds also were tested for antioxidant activities by using the stable DPPH method. As shown in Table 4, compounds **1**, **8**, **9**, **10**, **15**, and **16** had potent antioxidant activities;

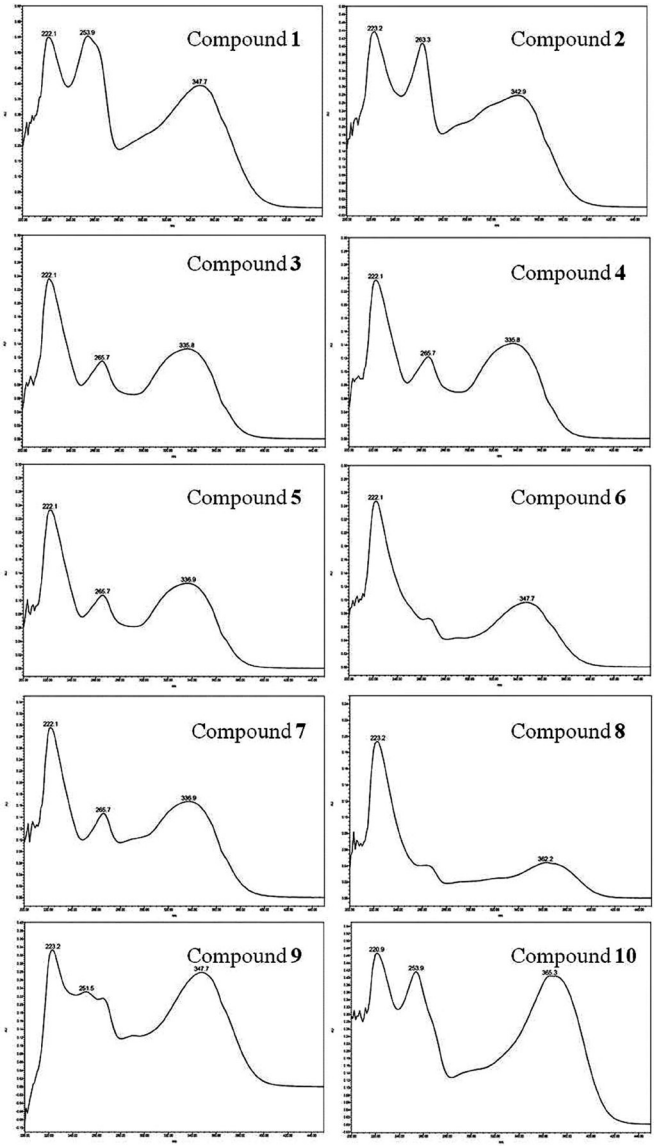

Figure 3. The UV spectra of compounds **1-10**

Table 3. Anti-NO production activity of the isolated compounds

Compound	IC_{50}[a] (μg/mL)	Cell viability (%)
EtOAc extract	32.12 ± 0.83[b]	102.34 ± 1.90
3	3.93 ± 0.23	103.65 ± 0.40
4	14.86 ± 1.86	101.63 ± 1.41
5	22.52 ± 0.47	109.21 ± 2.99
7	10.00 ± 1.24	104.34 ± 1.93
8	13.44 ± 0.36	98.24± 0.12
12	2.64 ± 0.37	95.38 ± 4.21
16	1.66 ± 0.41	94.22 ± 3.93
17	22.52 ± 0.47	107.41 ± 2.14

[a]IC_{50} : Inhibitory concentration 50%. [b]All values are presented as mean ± SD (*n* = 3). [c]Compounds **1**, **2**, **6**, **9–11**, and **13–15** are inactive (IC_{50} value > 40 μg/mL). [d]Quercetin (Sigma, 98% HPLC, IC_{50} = 10.92 ± 0.24 μg/mL) is as the positive control.

especially, compound **16** (ED_{50} = 2.45 μg/mL) showed more potent DPPH radical scavenging activity than positive control catechin (ED_{50} = 5.43 μg/mL).

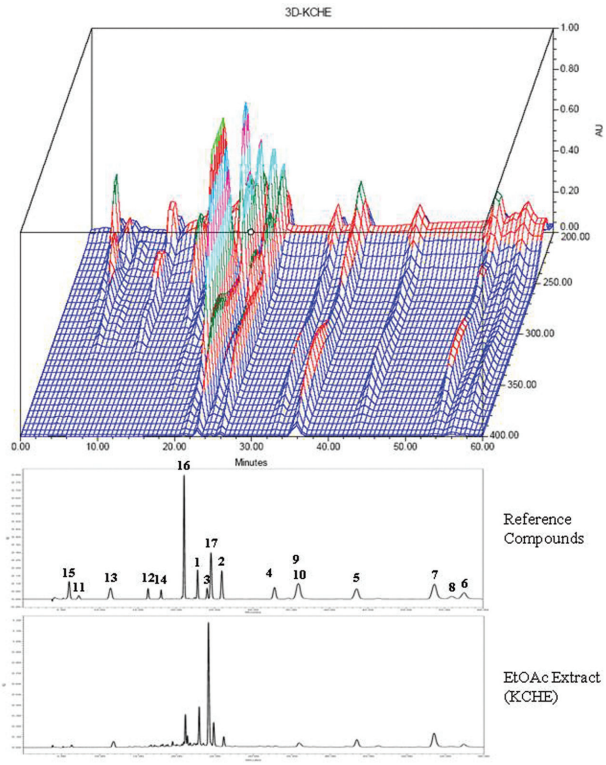

Figure 4. The HPLC-DAD profile of EtOAc extract and reference compounds of *C. halicacabum*

Table 4. Antioxidant activity of the EtOAc extract and the isolated compounds

Compound	DPPH free radical scavenger effect (%)	ED_{50} (μg/mL)
EtOAc extract	93.58 ± 1.05	28.97 ± 0.35
1	91.25 ± 3.05	19.53 ± 1.05
8	99.15 ± 1.75	15.24 ± 0.67
9	97.53 ± 0.84	6.89 ± 0.04
10	99.61 ± 0.51	7.77 ± 0.02
15	88.71 ± 3.53	21.93 ± 1.32
16	99.26 ± 0.95	2.45 ± 0.11
Catechin	99.21 ± 1.25	5.43 ± 0.14

[a]All values are presented as mean ± SD (n = 3). [b]Compounds **2–7**, **11–14**, and **17** are inactive (ED_{50} value > 40 μg/mL).

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