Journal of Traditional and Complementary Medicine Vol. 1, No. 1, pp. 57-63


Journal of Traditional and Complementary Medicine
Journal homepage http://www.jtcm.org

# Antioxidative Flavonol Glucuronides and Anti-HBsAg Flavonol from *Rotala rotundifolia*

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## Abstract

Two new flavonol glucuronides, quercetin 3-*O*-*β*-D-2″-acetylglucuronide (**1**) and quercetin 3-O-*β*-D-2″-acetylglucuronide methyl ester (**2**), along with four known flavonoids (**3-6**) were isolated from the whole parts of *Rotala rotundifolia*. The structures of **1** and **2** were elucidated by application of various spectroscopic methods, including 1D and 2D NMR techniques. Biological evaluation showed that all of isolated flavonoid compounds have potent anti-oxidative activities by DPPH and superoxide anion methods, and kaempferol (**3**) and quercetin (**4**) exhibited significant anti-HBV activity assayed by anti-HBsAg production. The HPLC fingerprint with photodiode array detection (HPLC-DAD) for quality control of *R. rotundifolia* partitioned EtOAc layer was also established.

**Key words:** *Rotala rotundifolia*, Flavonol glucuronides, Anti-HBsAg, Anti-oxidative activity

## Introduction

*Rotala rotundifolia* (wallich ex Roxb.) (Lythraceae) is an erect herb, found widely throughout the marshes, streamside, and paddy fields, in the lowlands of tropical Asia to Japan including Taiwan and the south of China (Huang, 1993). The plant is reputed of antipyretic, detoxication, antiswelling and diuresis properties and also useful in treatments of cirrhosis ascetic fluids, gonorrhea, menstrual cramps and piles in the south of China (Jiangsu Modern Medicine College, 2004). The genus *Rotala*, belonging to family Lythraceae, contains 44 species plants distributed in the tropics and temperate regions, including six species in Taiwan. So far, there are no phytochemical and/or biological researches for the genus *Rotala* to be found. It was unveiled with that human hepatocellular carcinoma cell line HepA2 containing hepatitis B virus (HBV) genomes, which continually secretes the viral surface antigen (HBsAg) into the culture medium, can be served as a quick assay system for screening biologically active natural products for anti-HBV activity (Tseng et al., 2008). As part of our continuing search for pharmacological agents from Taiwanese plants, recently, we found that the EtOH extract of *R. rotundifolia* showed the promising effect in suppression of HBV surface antigen (HBsAg) production in HepA2 cells. Herein, we report the isolation and characterization of two new flavonol glucuronides (**1** and **2**), from the EtOH extract of titled plant, and the HPLC fingerprint established from the EtOAc layer. Besides anti-HBV assay, compounds **1-6** were also evaluated for anti-oxidant activity by 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical and superoxide anion generation.

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## Materials and Methods

### General experimental procedures

Column chromatography (CC): commercial silica gel 60 ($SiO_2$, 70-230, 230-400 mesh, Merck), and Sephadex LH-20 (Pharmacia). TLC: precoated silica gel plates (Merck 60 F-254). MPLC was performed on a system equipped with a Buchi pump B-688, Buchi B-684 fraction collector and Buchi columns. M.p.: Fisher-Johns melting point apparatus; uncorrected. Optical rotations were obtained on a JASCO P-1020 polarimeter. IR Spectra: Mattson Genesis II FT-IR spectrometer, in $cm^{-1}$. UV Spectra: GBC 918 spectrophotometer. $^{1}H$- and $^{13}C$-NMR and 2D NMR spectra: Bruker UltraShield 400 MHz spectrometer, $\delta$ in ppm, $J$ in Hz. Electrospray ionization mass spectrometry data were obtained on an ESI trap tandem mass spectrometer (Thermo Finnigan LCQ-Duo, CA, USA). High resolution ESI-MS spectra were determined using a Shimadzu IT-TOF HR mass spectrometer.

### Plant material

The whole part of *R. rotundifolia* was collected in the northern marshes of Taipei County, Taiwan, in May 2006. A voucher specimen has been deposited in the National Research Institute of Chinese Medicine, Taipei, Taiwan.

### Extraction and Isolation

The dried whole parts of *R. rotundifolia* (3.1 kg) were extracted with 95% EtOH for 3 times (15 L) at 55 ºC. The crude extract was concentrated under reduced pressure. The residue was suspended in $H_2O$, and then successively partitioned with *n*-hexane, EtOAc, and BuOH at room temperature The EtOAc extract (100 g) was fractionated by silica gel CC eluting with $CHCl_3$-MeOH (100:0, 100:1, 50:1, 10:1, 5:1, 2:1, 0:100), to yield 9 fractions (F1-F9). F4 ($CHCl_3$-MeOH 50:1) was purified by Sephadex LH-20 CC ($CHCl_3$-MeOH 1:1) to afford kaempferol (**3**, 39.1 mg). F5 ($CHCl_3$-MeOH 20:1) was then submitted to Sephadex LH-20 CC eluting with $CHCl_3$-MeOH (1:2), to afford five subfractions (F51-F55). F53 was subjected on a Sephadex LH-20 column (100% MeOH) and then followed by semi-preparative TLC ($CHCl_3$-MeOH 6:1) (runs 2 times) to afford **2** (quercetin 3-*O*-*β*-D-glucuronide-2″-acetate methyl ester, 12.7 mg). Compounds **4** (Quercetin, 44.8 mg) and **5** (quercetin 3-*O*-*β*-D-glucuronide methyl ester, 21.6 mg) were obtained from F55 and F7 ($CHCl_3$-MeOH 10:1), respectively, by Sephadex LH-20 CC eluting with pure MeOH. F8 ($CHCl_3$-MeOH 2:1) was further separated over silica gel by MPLC (EtOAc-EtOH 9:1), to afford five subfractions (F81-F85). F82 was chromatographed on a LH-20 column using MeOH-$H_2O$ 3:1 and then purified by recrystallization to yield **1** (quercetin 3-*O*-*β*-D-glucuronide-2″-acetate, 467.9 mg) and 6 (quercetin 3-*O*-*β*-D-glucuronide, 202 mg).

### Scavenging Activity of 1, 1-Diphenyl-2-picrylhydrazyl (DPPH) Radical Assay

The radical scavenging activity of the isolates on DPPH free radical was measured using the method of Rangkadilok et al. (2007) and Chung et al. (2002) with minor modifications. The aliquot of each sample (120 μL, 5~30 μg/mL), or (±)-*α*-tocopherol (10~40 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 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.

### Scavenging Activity of Superoxide Anion Generation Assay

The superoxide anion scavenging activity assay was measured using the method of Péréz-Perez et al. (2006) and Barthomeuf et al. (2001) with minor modifications. The reaction mixture containing the pure compounds (30~200 μg/mL) prepared with 0.1 M sodium phosphate buffer solution (PBS) (pH 7.4), or an equivalent volume of PBS for the negative control, was mixed with 120 μM phenazine metho sulfate (PMS), 936 μM *β*-nicotinamide adenine dinucleotide (*β*-NADH) and 300 μM nitro blue tetrazolium (NBT) each 180μL. The reaction mixture was mixed well and incubated at room temperature in the dark for 5 minutes. NBT reduction was followed by recording the absorbance changes at 560 nm with the spectrophotometer. After the subtraction of the respective background of test samples, the results were expressed as $ED_{50}$, the concentration of test sample required to inhibited 50% superoxide anion generated in the assay system. (±)-Catechin hydrate (200~1000 μg/mL) was used as positive control in scavenging activity of superoxide anion generation assay.

**Anti-HBsAg Activity Assay**

Cell culture: The human hepatocellular carcinoma (HCC) HepA2 cell line was derived from HepG2 cells by transfecting a tandem repeated full-length HBV DNA and continually secretes HBsAg and HBeAg into the culture medium (Chang, et al., 1987; Yeh, et al., 1993). Stock cultures of HepA2 cell line was maintained in DMEM supplemented with 10% fetal calf serum and antibiotics (100 IU/mL each of penicillin and streptomycin) in a humidified atmosphere containing 5% $CO_2$ and 95% air at 37 °C. The cultures were passaged by trypsinization every 4 days. For the bioassays, cells were plated either in 24-well plates at a density of $8\times10^4$ cells/well in DMEM medium containing 10% fetal calf serum. Preparations of tested compounds: For the bioassays, the compound was dissolved in dimethyl sulfoxide (DMSO) and filtered through a 0.25 μfm fluoropore filter (Millipore, Mass, USA). Quantification of HBsAg: Cells were seeded in 24-well plates at a density of $8 \times 10^4$ cells/well in DMEM containing 10% fetal calf serum. After 24 h of incubation, the cells were washed twice with PBS, pH 7.0, and treated with various concentrations of drugs in serum-free DMEM for the time indicated. The HBsAg in the culture medium were measured by enzyme immunoassay (EIA) kit (Bio-Rad, CA, USA). The viability of cells was determined by a WST-1 cell proliferation assay. For the WST-1 assay (Levitz, 1985), WST-1 (Roche Diagnostics, Mannheim, Germany) was added to each well and incubated for 0.5 h. The amount of formazan dye formed can be correlated to the number of metabolically active cells, which is quantitatively determined using a scanning multi-well spectrophotometer (ELISA reader) at the absorbance 450 nm.

**HPLC Fingerprint Assay**

Materials and Reagents: EtOAc layer partitioned from the *R. rotundifolia*, six reference compounds (**1-6**) isolated from the EtOAc extract. Acetonitrile and methanol (LC grade) were purchased from Merck, HOAc was analytical grade. 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 Hitachi L system equipped with one four channels pump (Hitachi, L-2130, Japan), a diode array detector (Hitachi, L-2450, Japan), a vacuum degasser (Biotech, model 2003, Sweden), and an EZChrom Elite workstation. The Cosmosil 5C18-AR-II (5 μm, 4.6 × 250 mm, Nacalai Tesque, INC. Japan) eluted at a rate of 1.0 ml/min. The mobile phase consisted of 0.5% HOAc in water (A) and acetonitrile (B) using a gradient program of 5-10% (B) in 0-15 min, 10-18% (B) in 15-60 min, 18-36% (B) in 60-105 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 **1** to **6** was accurately weighed and dissolved in MeOH, the terminate concentration was ca. 500 μg/ml. Preparation of sample solution, the EtOAc layer of *R. rotundifolia* was dried under vacuum, then accurately weighed 100 mg and dissolved in methanol in a 10 ml volumetric flask, this sample solution was filtered through a 0.45 μm filter (Millipore) before use.

**Quercetin 3-*O*-*β*-D-2″-acetylglucuronide (1):**

Yellow, amorphous powder. Mp. 137~140 ºC. $[\alpha]_D^{25}$ -21.2 (*c* 1.04, DMSO). UV (MeOH) $\lambda_{max}$ nm: 213, 256, 356. IR $\nu_{max}$ (KBr) $cm^{-1}$: 3419, 1734, 1653, 1608, 1508, 1305, 1203, 1085. For $^1H$ (400 MHz, DMSO-$d_6$) and $^{13}C$ (100 MHz) NMR spectroscopic data, see Table 1. HR-ESI-MS: *m/z* 543.0716 $[M + Na]^+$ (calcd for $C_{23}H_{20}O_{14}Na$ 543.0751).

**Quercetin 3-*O*-*β*-D-2″-acetylglucuronide methyl ester (2):**

Yellow, amorphous powder. Mp. 158~161 ºC, [α]25 D -57.5 (*c* 0.80, DMSO); UV (MeOH) $\lambda_{max}$ nm: 206, 257, 356; IR $\nu_{max}$ (KBr) $cm^{-1}$: 3423, 1739, 1655, 1608, 1508, 1305, 1203, 1088. For $^1H$ (400 MHz, DMSO-$d_6$) and $^{13}C$ (100 MHz) NMR spectroscopic data, see Table 1. HR-ESI-MS: *m/z* 557.0871 $[M + Na]^+$ (calcd for $C_{24}H_{22}O_{14}Na$ 557.0907).

**Table 1.** $^1H$- and $^{13}C$-NMR data of compounds **1** and **2** (DMSO-$d_6$, 400 MHz and 100 MHz).

| No | 1 | | | 2 | | |
|---|---|---|---|---|---|---|
| | $\delta_H$ (*J* in Hz) | $\delta_C$ (mult) | HMBC | $\delta_H$ (*J* in Hz) | $\delta_C$ (mult) | HMBC |
| 2 | | 157.1 (s) | | | 156.2 (s) | |
| 3 | | 133.7 (s) | | | 132.8 (s) | |
| 4 | | 177.6 (s) | | | 176.4 (s) | |
| 5 | 12.47 (br s) | 156.6 (d) | 5,6,10 | 12.54 (br s) | 157.1 (d) | 5,6,10 |
| 6 | 6.08 (br s) | 99.2 (d) | 5,7,8,10 | 6.18 (br s) | 100.6 (d) | 5,7,8,10 |
| 7 | | 165.5 (s) | | | 169.1 (s) | |
| 8 | 6.26 (br s) | 94.1 (d) | 6,7,9,10,11 | 6.37 (br s) | 94.8 (d) | 6,7,9,10,11 |
| 9 | | 161.0 (s) | | | 161.4 (s) | |
| 10 | | 103.7 (s) | | | 102.4 (s) | |
| 1′ | | 120.9 (s) | | | 120.6 (s) | |
| 2′ | 7.56 (br s) | 116.7 (d) | 1′, 3′, 4′, 6′, 2 | 7.43 (br s) | 115.8 (d) | 1′, 3′, 4′, 6′, 2 |
| 3′ | | 145.2 (s) | | | 145.7 (s) | |
| 4′ | | 148.9 (s) | | | 150.0 (s) | |
| 5′ | 6.83 (d, 8.0) | 115.7 (d) | 1′, 3′ | 6.81 (d, 8.4) | 115.6 (d) | 1′, 3′ |
| 6′ | 7.38 (br d, 8.0) | 121.8 (d) | 1′, 2′, 4′, 5′, 2 | 7.51 (br d, 8.4) | 122.1 (d) | 1′, 2′, 4′, 5′, 2 |
| 1″ | 5.37 (d, 8.0) | 99.7 (d) | 3 | 5.49 (d, 8.0) | 99.6 (d) | 3 |
| 2″ | 4.80 (t, 8.0) | 74.1 (d) | 1″,3″, 1‴ | 4.80 (t, 8.4) | 74.2 (d) | 1″,3″, 1‴ |
| 3″ | 3.47 (t, 9.2) | 74.3 (d) | 4″ | 3.46 (t, 8.0) | 74.2 (d) | 2″,4″ |
| 4″ | 3.41 (t, 9.2) | 72.1 (d) | 3″,5″ | 3.50 (m) | 71.9 (d) | 5″ |
| 5″ | 3.44 (m) | 75.0 (d) | 1″,6″ | 3.81 (d, 8.4) | 76.1 (d) | 1″,4″,6″ |
| 6″ | | 173.0 (s) | | | 169.4 (s) | |
| 1‴ | | 169.9 (s) | | | 169.9 (s) | |
| 2‴ | 1.94 (s) | 21.1 (q) | 1‴ | 2.03 (s) | 21.4 (q) | 1‴ |
| 3‴ | | | | 3.58 (s) | 52.4 (q) | 6″ |

[a]Assignments aided with $^1H$-$^1H$ COSY, HMQC and HMBC. [b]Recorded at 400 MHz. [c]Recorded at 100 MHz.

## Results and Discussion

The EtOH extract of the whole parts of *R. rotundifolia* was suspended in $H_2O$ and further partitioned with *n*-hexane, EtOAc, and BuOH, successively. Of these solvents partitioned extracts, the EtOAc-soluble extract exhibited more potent anti-HBsAg ($EC_{50}$ = 6.00 μg/mL) and scavenging activity against free radicals (DPPH) (see Table 2). Repeated column chromatography of the EtOAc extract yielded two new compounds, flavonol glucuronides **1** and **2**, together with the known flavonols and its glycosides (**3**-**6**), kaempferol (**3**) (Nawwar et al., 1984), quercetin (**4**) (Chu et al., 2004), quercetin 3-*O*-*β*-D-glucuronide methyl ester (**5**) (Nawwar et al., 1984), and quercetin 3-*O*-*β*-D-glucuronide (**6**) (Nawwar et al., 1984) were also isolated and identified by comparing their physical and spectroscopic data with those of authentic samples and references. Their structures are shown in Figure 1.

Compound **1** was isolated as a yellow amorphous solid, and its molecular formula was determined to be $C_{23}H_{20}O_{14}$ from the analysis of its HR-ESI-MS ([*m/z*, 543.0716, $[M + Na]^+$) and NMR spectral data (Table 1). The IR spectrum exhibited absorption broad bands at 3419 $cm^{-1}$ (OH), 1734 $cm^{-1}$ (OC=$OCH_3$), 1653 $cm^{-1}$ (chelated C=O), and characteristic bands attributable to aromatic rings at 1608 and 1508 $cm^{-1}$. The UV spectrum showed absorptions at $\lambda_{max}$ 356 and 258 nm suggesting the presence of flavonol moiety (Xu et al., 2004). The $^{13}C$ NMR spectrum of **1** displayed signals for 23 carbons including one methyl carbon, ten methines of which five were aromatic, along with twelve quaternary carbons containing three carbonyl carbons at $\delta_C$ 177.6, 173.0 and 169.9 ppm. The $^1H$ NMR spectrum of **1** showed one singlet peak at $\delta_H$ 12.471attributed to an inter-molecular H-bonded hydroxyl group, together with a downfield signal $\delta_C$ 177.6 for a chelated phenolic ketone group in the $^{13}C$ NMR spectrum, suggesting the presence of a 5-hydroxyflavanoid moiety. The appearance of two *meta*-coupled peaks (br s) at $\delta_H$ 6.26 and 6.08, were assigned to H-8 and H-6 of A-ring in **1**, respectively. The aromatic proton signals at $\delta_H$ 7.56 (H-2′, br s), 7.38 (H-6′, d, *J* = 8.0 Hz), and 6.83 (H-5′, br d, *J* = 8.0 Hz) shown an AMX coupling pattern was deduced to be a 3′,4′-dihydroxy substitution of B-ring. These findings, together with three quaternary carbons ($\delta_C$ 133.7, 157.1, and 177.6) in C-ring, suggested that **1** contains a 3,5,3′,4′-tetrahydroxy flavonol, which is in agreement with published data of quercetin (Chu et al., 2004). The $^1H$ and $^{13}C$ NMR spectra showed a set characteristic signals for a hexose moiety ($\delta_C$ 72.1, 74.1, 74.3, 75.0, 99.7, and 173.0), and for an anomeric proton ($\delta_H$ 5.37, d, *J* = 8.0 Hz), compared with the reference data (Nawwar et al., 1984), which was assigned as *β*-D-glucuronide. The pending signals for an acetate group ($\delta_H$ 1.94, s; $\delta_C$ 21.2, 169.9) were also found. Moreover, the HMBC spectrum of 1 (Figure 2) showing the correlations between anomeric proton and C-3 ($\delta_C$ 133.7) of the flavonol moiety, and between H-2″-Glc and C-1‴ ($\delta_C$ 169.9) of the acetyl group, unambiguously confirmed the location of glucuronide moiety in **1** as shown. Together with above corroboration, the structure of **1** was characterized as quercetin 3-*O*-*β*-D-2″-

**Table 2.** Antioxidant assays of EtOH extract, EtOAc extract and compounds **1-6**.

| Sample | DPPH (Removal effect, %)[a] | DPPH ($ED_{50}$)[b] | $O^-$ ($ED_{50}$)[c] |
|---|---|---|---|
| EtOH ex. | 98.28 | 15.59 ± 0.38 | -[d] |
| EtOAc ex. | 99.82 | 4.52 ± 0.65 | - |
| **1** | 93.58 | 17.88 ± 0.73 | 1.05 ± 0.03 ×$10^{-1}$ |
| **2** | 92.75 | 34.17 ± 2.07 | 3.29 ± 0.13 ×$10^{-1}$ |
| **3** | 97.15 | 40.17 ± 2.37 | 6.64 ± 0.14 ×$10^{-1}$ |
| **4** | 96.49 | 11.19 ± 0.63 | 3.21 ± 0.16 ×$10^{-1}$ |
| **5** | 96.08 | 17.68 ± 0.42 | 2.52 ± 0.14 ×$10^{-1}$ |
| **6** | 96.55 | 19.24 ± 0.65 | 1.33 ± 0.09 ×$10^{-1}$ |
| (±)-*α*-Toçopherol[e] | - | 28.20 ± 0.87 | - |
| Catechin[f] | - | - | 1.85 ± 0.07 |

[a] Test samples were treated at 200 μg/ml. [b] ED50 values express the concentration in g/ml for solvent fractions and μM for pure compounds. Values are mean ± SD of three separated experiments. [c] $ED_{50}$ values express the concentration in mM for pure compounds. Values are mean ± SD of three separated. [d] Not tested. [e] Positive control for DPPH assay. [f] Positive control for $O^-$ assay.

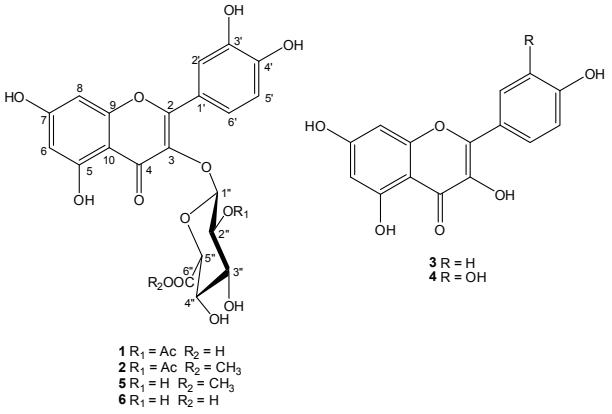


**Figure 1.** Chemical tructures of compounds **1-6**.

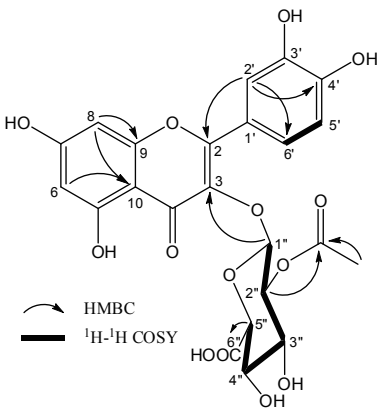


**Figure 2.** The key HMBC correlations of compound **1**.

acetylglucuronide.

Compound **2** was obtained as a yellow amorphous solid, and its molecular formula was determined to be $C_{24}H_{22}O_{14}$ from the analysis of its HR-ESI-MS and NMR spectroscopic data. Due to the similar IR, UV, and $^{1}H$ and $^{13}C$ NMR spectra as those of **1**, the major skeleton of **2** was comprised of a flavonol and a glucuronide moiety, except for the additional presence of a carboxylic methyl signal ($\delta_H$ 3.58, s, 3H, $\delta_C$ 52.4) in **2**. By means of the HMQC and HMBC spectra, the methyl ester of 2 was further assigned at the C-6″ of glucuronide group. Consequently, the structure of **2** was concluded to be quercetin 3-*O*-*β*-D-2″-acetylglucuronide methyl ester.

Compounds **1-6**, were further evaluated for antioxidant and anti-HBsAg activities. As shown in Table 2, most of isolates (**1**, **4-6**) exhibit more potent DPPH radical scavenging activity than positive control, (±)-*α*-tocopherol; especially, all of isolated flavonols and its glycosides (**1-6**) have more potent anti-superoxide anion effects compared with catechin. Notably, **3** showed less effect than that of other flavonols with two hydroxy groups by *ortho* in the B-ring, revealing that the number of hydroxyl group in flavonols would play a crucial role for the DPPH radical scavenging ability; this conclusion is consistent with Hou's reports (Hou et al., 2004a, 2004b), and implies the importance of water solubility for the improvement of antioxidant activities. Moreover, **3** ($EC_{50}$ = 1.97 μg/mL) and **4** ($EC_{50}$ = 3.39 μg/mL) had promising anti-HBsAg effects, compared with glucuronide derivatives **1**, **2**, **5** and **6**, which displayed no detectable anti-HBV activity within the concentration at 10 μg/mL. The EtOAc extract showed more potent DPPH radical scavenging activity than all of purified isolates, suggesting that these isolated flavonols or its glucuronide derivatives could perform the synergistic interactions to express the antioxidant activities.

These findings promoted us to study the HPLC fingerprint for the quality control of bioactive EtOAc layer (Figure 3). By comparing the retention times with the reference compounds (**1-6**), the six main peaks were identified. The area percent method provides the relative content of the compounds **1-6** in the EtOAc layer, showing the relative contents of 3-*O*-*β*-D-2″-acetylglucuronide (1, $t_R$ 81.1 min, 25.91%), quercetin 3-*O*-*β*-D-2″-acetylglucuronide methyl ester (2, $t_R$ 86.1 min, 12.61%), kaempferol (3, $t_R$ 100.1 min, 1.62%), quercetin (4, $t_R$ 87.2 min, 8.01%), quercetin 3-*O*-*β*-D-glucuronide methyl ester (5, $t_R$R 71.1 min, 10.09%), and quercetin 3-*O*-*β*-D-glucuronide (**6**, $t_R$ 58.6 min, 18.06%). Thus, the HPLC profile of the EtOAc layer revealed that the amounts of flavonol glucuronids **1**, **2**, **5**, and **6** are much more than that of flavonols, **3** and **4**, in the EtOAc layer.

The HPLC fingerprint evidences support the anti-HBsAg results that only **3**, **4**, and EtOAc extract showed available activities compared with other isolates, and also imply that the flavonols, not the glycosides, in the EtOAc extract of *R. rotundifolia* are crucial components for anti-HBsAg activities.

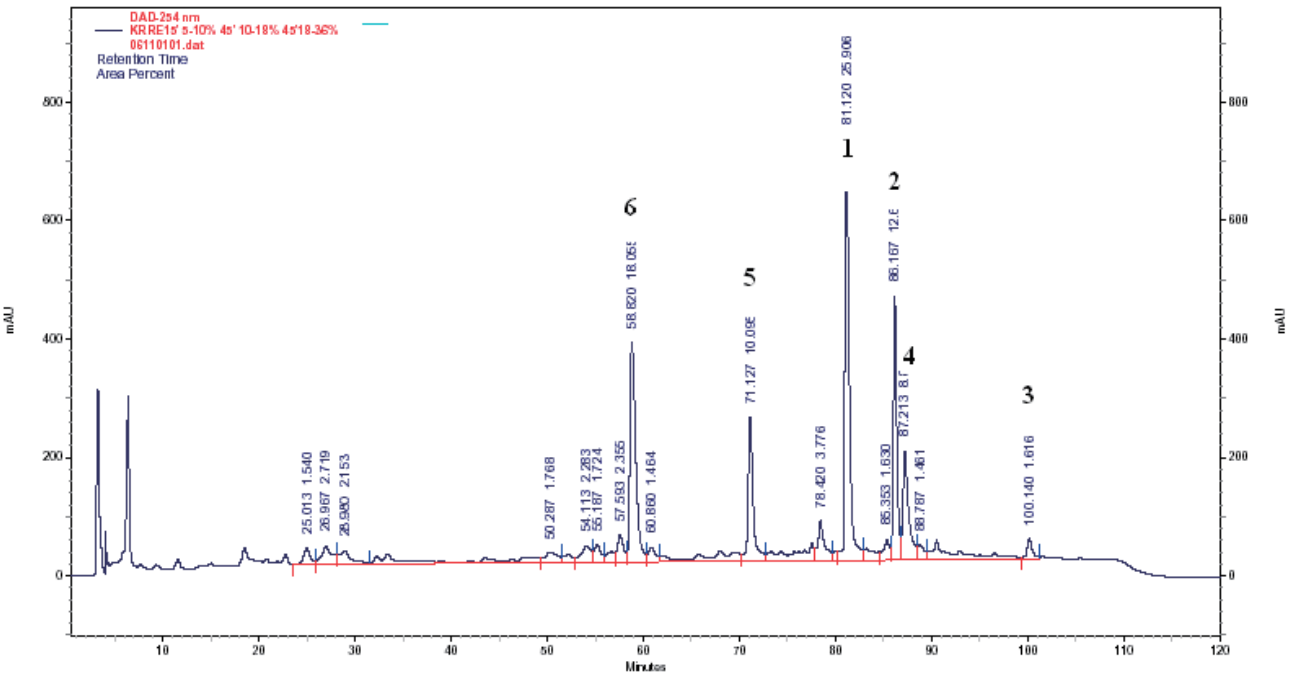


**Figure 3.** The HPLC Profile of EtOAc Layer of *R. rotundifolia*.

## Acknowledgements

The grants from National Science Council (NSC098-2320-B-077-005-MY3), Republic of China is gratefully acknowledged. We are also grateful to Dr. Chien-Chang Shen and Ms. Fei-Pei Kao, as well as Dr. Ming-Jaw Don and Mr. Tai-Hung Chen, NRICM, for the technical assistance of NMR and mass measurement, respectively. The Instrument Center of National Taiwan University, for the HR-ESI-MS measurements, and the National Center for High-Performance Computing, for checking the database, are also appreciated.

## Supporting information

$^{1}$H and $^{13}$C NMR spectra of compounds **1-2** are available as Supporting Information.

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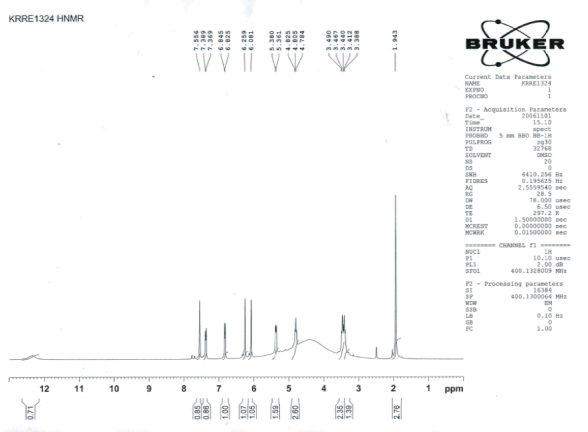


**Figure S1:** $^{1}$H NMR spectrum of compound **1** (400 MHz) in DMSO-$d_6$

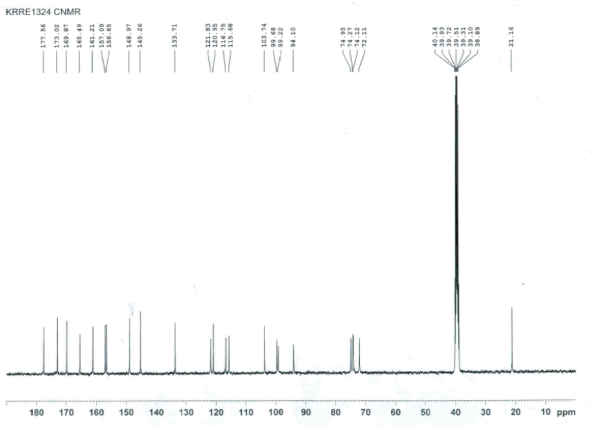


**Figure S2:** $^{13}$C NMR spectrum of compound **1** (100 MHz) in DMSO-$d_6$

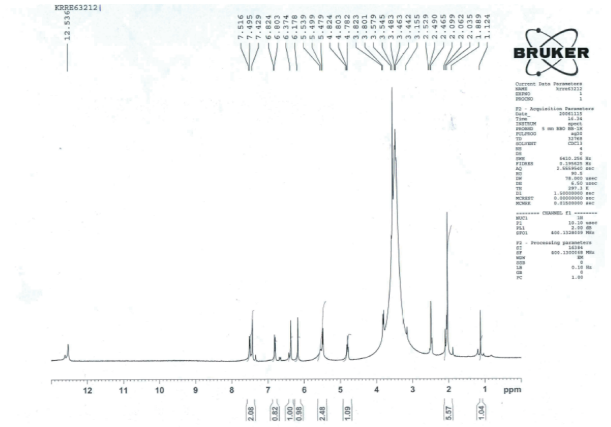


**Figure S3:** $^{1}$H NMR spectrum of compound **2** (400 MHz) in DMSO-$d_6$

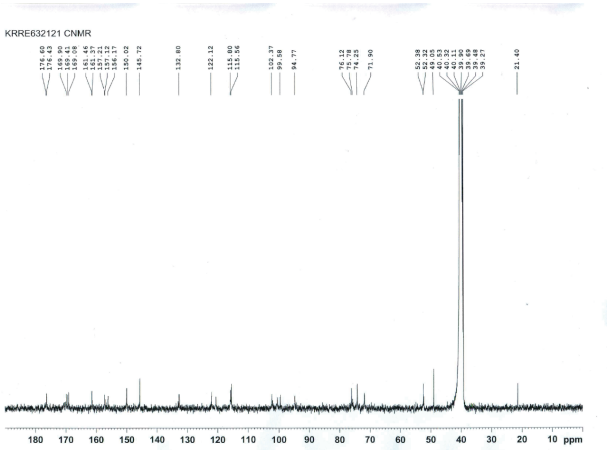


**Figure S4:** $^{13}$C NMR spectrum of compound **2** (100 MHz) in DMSO-$d_6$