Supporting Information

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Antioxidant and Hepatoprotective Activities of Flavonoids from *Bauhinia hookeri*

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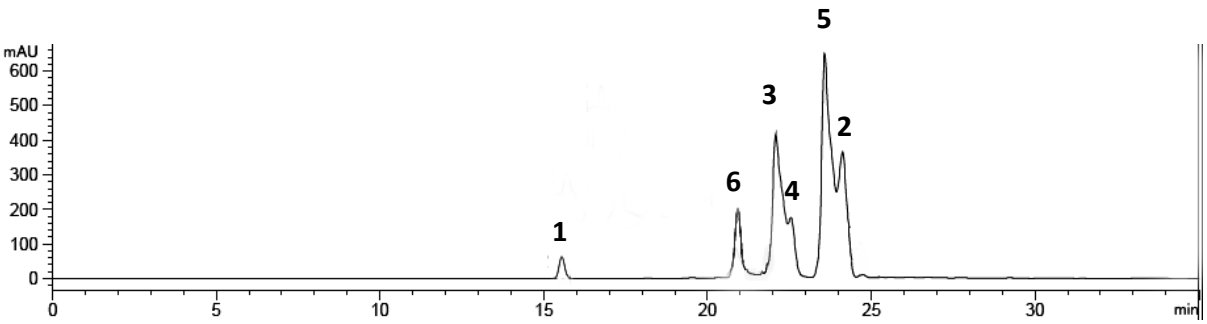

S1: HPLC chromatogram of *B. hookeri* flavonoid fraction

S2. Structure Elucidation of the Isolated Compounds

Fractionation of the flavonoid-rich fraction obtained from the 80% ethanol extract of *B. hookeri* yielded compounds **1-6** (Figure 1). Notably, this is the first report of the isolation of these compounds from *B. hookeri*. All the compounds were identified based on their UV, HRESI-MS/MS, 1D and 2D NMR data, as well as by comparing these data with those previously reported in the literature. The compounds were identified as (+)-catechin 3-*O*-*α*-L-rhamnoside (**1**), kaempferol 3-*O*-*β*-D-glucoside (**2**), quercetin 3-*O*-*β*-D-glucoside (**3**), kaempferol 3-*O*-(6″-*O*-galloyl)-*β*-D-galactoside (**4**), kaempferol 3-*O*-(6″-*O*-galloyl)-*β*-D-glucoside (**5**) and quercetin 3-*O*-(6″-*O*-galloyl)-*β*-D-glucoside (**6**).

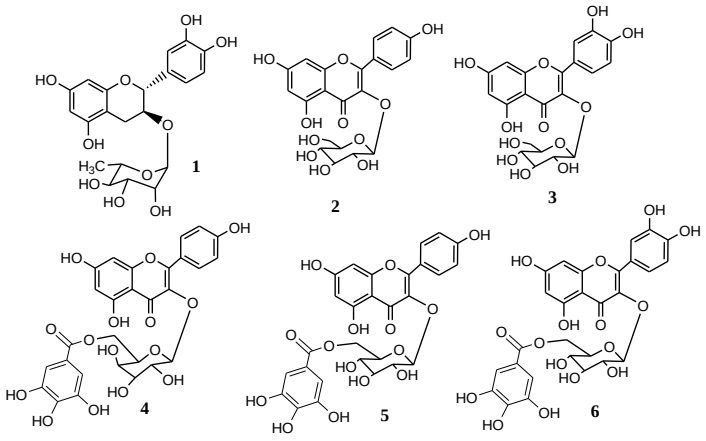

Figure 1. Structures of the isolated flavonoids from *B. hookeri*

*(+)-Catechin 3-O-α-L-rhamnoside (**1**)*

Yellowish white amorphous powder. UV λ_{max} (MeOH): 220, 235 sh, 280 nm. HR-ESIMS (negative mode) *m/z*: 435.1266 $(M–H)^-$. ^{1}H NMR (500 MHz, CD_3OD): δ_H 5.85 (1H, d, *J* = 2.3 Hz, H-6), 5.94 (1H, d, *J* = 2.3 Hz, H-8), 6.84 (1H, d, *J* = 1.8 Hz, H-2′), 6.76 (1H, d, *J* = 8.0 Hz, H-5′), 6.72 (1H, dd, *J* = 8.1, 1.8 Hz, H-6′), 4.62 (1H, d, *J* = 7.9 Hz, H-2), 3.92 (1H, m, H-3), 2.88 (1H, dd, *J* = 16.2, 5.7 Hz, H-4a), 2.64 (1H, dd, *J* = 16.2, 8.6 Hz, H-4b), rhamnosyl: 4.29 (1H, d, *J* = 1.3 Hz, H-1″), 3.74-3.30 (4H, unresolved H-2″, H-3″, H-4″, H-5″), 1.25 (3H, d, *J* = 6.2 Hz, H-6″). ^{13}C NMR (125 MHz, CD_3OD): δ_C 79.7 (C-2), 74.5 (C-3), 26.6 (C-4), 155.5 (C-5), 94.1 (C-6), 156.6 (C-7), 95.0 (C-8), 156.2 (C-9), 99.3 (C-10), 130.6 (C-1′), 113.7 (C-2′), 145.0 (C-3′), 144.9 (C-4′), 114.7 (C-5′), 118.5 (C-6′), rhamnosyl: 100.7 (C-1″), 70.6 (C-2″), 70.9 (C-3″), 72.6 (C-4″), 68.9 (C-5″), 16.5 (C-6″).

*Kaempferol 3-O-β-D-glucoside (**2**)*

Yellowish amorphous powder. UV λ_{max} (MeOH): 260,300 sh, 340 nm. HR-ESIMS (negative mode) *m/z*: 447.0911 $(M–H)^-$. ^{1}H NMR (500 MHz, CD_3OD): δ_H 6.19 (1H, d, *J* = 2.0 Hz, H-6), 6.38 (1H, d, *J* = 2.0 Hz, H-8), 6.88 (2H, d, *J* = 8.5 Hz, H-3′, H-5′), 8.05 (2H, d, *J* = 8.5 Hz, H-2′, H-6′), glucosyl: 5.24 (1H, d, *J* = 7.5 Hz, H-1″), 3.47-3.40 (2H,unresolved H-2″, H-3″), 3.31 (1H, unresolved with the solvent peak, H-4″), 3.21 (1H, m, H-5), 3.69 (1H, dd, *J* = 2.0, 11.5 Hz, H-6″a), 3.52 (1H, dd, *J* = 5.0, 11.5 Hz, H-6″b). ^{13}C NMR (125 MHz, CD_3OD): δ_C 157.6 (C-2), 134.5 (C-3), 178.5 (C-4), 162.1 (C-5), 98.9 (C-6), 165.0 (C-7), 93.8 (C-8), 158.2 (C-9), 104.8 (C-10), 121.8 (C-1′), 131.4 (C-2′,C-6′), 115.2 (C-3′,C-5′), 160.7 (C-4′), glucosyl: 103.2 (C-1″), 74.8 (C-2″), 77.1 (C-3″), 70.4 (C-4″), 77.5 (C-5″), 61.7 (C-6″).

*Quercetin 3-O-β-D-glucoside (**3**)*

Yellowish amorphous powder. UV λ_{max} (MeOH): 255,300 sh, 352 nm. HR-ESIMS (negative mode) *m/z*: 463.0897 $(M–H)^-$. ^{1}H NMR (500 MHz, CD_3OD): δ_H 6.18 (1H, d, *J* = 2.0 Hz, H-6), 6.36 (1H, d, *J* = 2.0 Hz, H-8), 6.86 (1H, d, *J* = 8.5 Hz, H-5′), 7.58 (1H, dd, *J* = 8.5, 2.1 Hz, H-6′), 7.71 (1H, d, *J* = 2.1 Hz, H-2′), glucosyl: 5.24 (1H, d, *J* = 7.6 Hz, H-1″), 3.49 (1H, dd, *J* = 9.1, 7.6 Hz, H-2″), 3.44 (1H, t, *J* = 9.1 Hz, H-3″), 3.36 (1H, t, *J* = 9.1 Hz, H-4″), 3.24 (1H, m, H-5″), 3.72 (1H, dd, *J* = 2.3, 11.8 Hz, H-6″a), 3.58 (1H, dd, *J* = 5.2, 11.8 Hz, H-6″b). ^{13}C NMR (125 MHz, CD_3OD): δ_C 158.5 (C-2), 135.1 (C-3), 178.9 (C-4), 162.5 (C-5), 99.4 (C-6), 165.5 (C-7), 94.2 (C-8), 157.9 (C-9), 105.1 (C-10), 122.7 (C-1′), 117.1 (C-2′), 149.3 (C-3′), 145.3 (C-4′), 115.5 (C-5′), 122.5 (C-6′), glucosyl: 103.9 (C-1″), 75.2 (C-2″), 77.6 (C-3″), 70.7 (C-4″), 77.8 (C-5″), 62.0 (C-6″).

*Kaempferol 3-O-(6″-O-galloyl)-β-D-galactoside (**4**)*

Yellowish amorphous powder. UV λ_{max} (MeOH): 265, 300 sh, 345 nm. HR-ESIMS (negative mode) *m/z*: 599.1014 $(M–H)^-$. ^{1}H NMR (500 MHz, CD_3OD): δ_H 6.18 (1H, d, *J* = 2.1 Hz, H-6), 6.88 (2H galloyl, s, H-2‴and H-6‴), 6.36 (1H, d, *J* = 2.1 Hz, H-8), 6.82 (2H, d, *J* = 8.9 Hz, H-3′, H-5′), 8.02 (2H, d, *J* = 8.9 Hz, H-2′, H-6′), galactosyl: 5.04 (1H, d, *J* = 7.8 Hz, H-1″), 3.80 (1H, dd, *J* = 9.7, 7.8 Hz, H-2″), 3.57 (1H, dd, *J* = 9.7, 3.5 Hz, H-3″), 3.83 (1H, brd, *J* = 3.7 Hz, H-4″), 3.77 (1H, brt, *J* = 6.7 Hz, H-5″), 4.33 (1H, dd, *J* = 7.4, 11.2 Hz, H-6″a), 4.15 (1H, dd, *J* = 5.6, 11.2 Hz, H-6″b). ^{13}C NMR (125 MHz, CD_3OD): δ_C 159.3 (C-2), 135.6 (C-3), 179.6 (C-4), 162.9 (C-5), 100.0 (C-6), 166.2 (C-7), 94.9 (C-8), 158.4 (C-9), 105.4 (C-10), 122.6 (C-1′), 132.4 (C-2′,C-6′), 116.1 (C-3′,C-5′), 161.5 (C-4′), galactosyl: 105.4 (C-1″), 72.9 (C-2″), 75.0 (C-3″), 70.0 (C-4″), 74.6 (C-5″), 64.0 (C-6″). Galloyl: 121.1 (C-1‴), 110.1 (C-2‴, C-6‴), 146.3 (C-3‴, C-5‴), 139.8 (C-4‴), 168.0 (C-7‴).

*Kaempferol 3-O-(6″-O-galloyl)-β-D-glucoside (**5**)*

Yellowish amorphous powder. UV λ_{max} (MeOH): 260,300 sh, 348 nm. HR-ESIMS (negative mode) *m/z*: 599.1029 $(M–H)^-$. ^{1}H NMR (500 MHz, CD_3OD): δ_H 6.19 (1H, d, *J* = 2.1 Hz, H-6), 6.96 (2H galloyl, s, H-2‴and H-6‴), 6.35 (1H, d, *J* = 2.1 Hz, H-8), 6.74 (2H, d, *J* = 8.9 Hz, H-3′, H-5′), 7.94 (2H, d, *J* = 8.9 Hz, H-2′, H-6′), glucosyl: 5.22 (1H, d, *J* = 7.4 Hz, H-1″), 4.33 (1H, dd, *J* = 4.6, 11.8 Hz, H-6″a), 4.28 (1H, dd, *J* = 2.1, 11.8 Hz, H-6″b), 3.51 -3.48 (4H, unresolved glucose protons H-2″- H-5″). ^{13}C NMR (125 MHz, CD_3OD): δ_C 159.0 (C-2), 134.7 (C-3), 178.8 (C-4), 162.4 (C-5), 99.4 (C-6), 165.4 (C-7), 94.4 (C-8), 157.9 (C-9), 105.1 (C-10), 122.1 (C-1′), 131.7 (C-2′,C-6′), 115.5 (C-3′,C-5′), 160.9 (C-4′), glucosyl: 103.9 (C-1″), 75.2 (C-2″), 77.5 (C-3″), 70.9 (C-4″), 75.3 (C-5″), 63.8 (C-6″). Galloyl: 120.8 (C-1‴), 109.6 (C-2‴, C-6‴), 145.8 (C-3‴, C-5‴), 139.2 (C-4‴), 167.6 (C-7‴).

*Quercetin 3-O-(6″-O-galloyl)-β-D-glucoside (**6**)*

Yellowish amorphous powder. UV λ_{max} (MeOH): 260,300 sh, 355 nm. HR-ESIMS (negative mode) *m/z*: 615.0962 $(M–H)^-$. ^{1}H NMR (500 MHz, CD_3OD): δ_H 6.17 (1H, d, *J* = 2.0 Hz, H-6), 6.94 (2H galloyl, s, H-2‴and H-6‴), 6.34 (1H, d, *J* = 2.0 Hz, H-8), 6.71 (1H, d, *J* = 8.3 Hz, H-5′), 7.55 (1H, dd, *J* = 8.4, 2.1 Hz, H-6′), 7.53 (1H, d, *J* = 2.1 Hz, H-2′), glucosyl: 5.20 (1H, d, *J* = 7.6 Hz, H-1″), 4.34 (1H, dd, *J* = 4.9, 11.8 Hz, H-6″a), 4.27 (1H, dd, *J* = 1.9, 11.8 Hz, H-6″b), 3.53 -3.43 (4H, unresolved glucose protons H-2″- H-5″). ^{13}C NMR (125 MHz, CD_3OD): δ_C 158.8 (C-2), 134.8 (C-3), 178.8 (C-4), 162.3 (C-5), 99.4 (C-6), 165.3 (C-7), 94.3 (C-8), 157.8 (C-9), 105.0 (C-10), 122.5 (C-1′), 116.7 (C-2′), 149.1 (C-3′), 145.2 (C-4′), 115.4 (C-5′), 123.0 (C-6′), 103.7 (C-1″), 75.1 (C-2″), 77.5 (C-3″), 70.9 (C-4″), 75.3 (C-5″), 63.8 (C-6″). Galloyl: 120.7 (C-1‴), 109.6 (C-2‴, C-6‴), 145.7 (C-3‴, C-5‴), 139.1 (C-4‴), 167.6 (C-7‴).

S3. Materials and Methods

3.1. General Experimental Procedures

The NMR spectra were measured by a Bruker Avance 500 spectrometer (Bruker BioSpin Inc., Fällanden, Switzerland). CD_3OD was used as a solvent. DQF-COSY, HSQC and HMBC were all measured by the pulse programs originally installed by Bruker. HRESIMS was performed on a Bruker micrOTOF-$_Q$ quadrupole time-of-flight mass spectrometer (Bremen, Germany). The ionization technique was an electrospray. The mass spectrometer was operated in the negative mode with capillary voltage, 4000 V; end plate offset, –500 V. Heated drying gas (N_2) flow rate was 12 L/min; the drying gas temperature was 200 ^oC. For MS/MS measurements, argon was used as a collision gas and the voltage over the collision cell varied from 20 to 70 eV. The data were analyzed using Compass Data Analysis Software (version 4.0 SP5; Bruker Daltonics). Analytical HPLC was performed using an Agilent HPLC-DAD system consisted of quaternary pump G1311A connected to a diode array and multiple wavelength detector G1315D, interface module D-7000, autosampler G-1329A. Chromatographic separation was performed on a ZORBAX Eclipse XDB-C18 column (4.6 × 150 mm; 5 μm) column (Agilent Technologies). The mobile phase consisted of acetonitrile (A) and 0.4% phosphoric acid (B). The elution profile was 0–3 min, 100% B (isocratic); 3–30 min, 0–30% A in B; 30–35 min, 30–70% A in B; 35–37 min, 70% A in B (isocratic) with constant flow rate of 1 mL /min. Silica gel 60 (200–300 mesh) was obtained from Merck, Darmstadt, Germany. Sephadex LH-20 was purchased from Amersham Biosciences, Sweden. RP-C18 was obtained from Sigma-Aldrich GmbH, Germany and precoated silica gel TLC GF254 was purchased from Riedel-De Häen-AG, Seelze Germany.

3.2. Chemicals

Bovine serum albumin (BSA), RPMI-1640 medium, fetal bovine serum (FBS), penicillin/streptomycin solution and other cell culture reagents were purchased from Lonza (Basel, Switzerland). Reduced glutathione (GSH), 5,5′-dithiobis-2-nitrobenzoic acid (DTNB, Ellman's reagent), thiobarbituric acid (TBA), and trichloroacetic acid (TCA) were purchased from Sigma–Aldrich Chemical Company (St. Louis, MO, USA). Silymarin 99% (Indena S.P.A, Milano, Italy) was kindly supplied by (Medical Union Pharmaceuticals Co (MUP), Cairo, Egypt). All other chemicals were of analytical grade and of the highest purity.

3.3. Plant Material

The leaves of *B. hookeri* were collected in July 2011 from the botanical garden of the Faculty of Agriculture, Cairo University, Cairo, Egypt. The plant was botanically identified by Eng. Therese Labib, the taxonomy specialist at the herbarium of El-Orman Botanical Garden, Giza, Egypt. A voucher specimen of *B. hookeri* was deposited at the herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Ain Shams University, Cairo, Egypt (ASU BHF2011).

3.4. Extraction and Isolation

Air-dried powdered leaves of *B. hookeri* (1.7 kg) were extracted three times with 80% EtOH. The total extract was concentrated then freeze dried. The dried powder was re-dissolved in absolute EtOH and filtered to remove the sugars. The EtOH soluble part was concentrated and freeze dried to obtain 140 g of the extract. Column fractionation was performed with a portion of the extract (100 g) using a Sephadex LH-20 column (5 × 100 cm). Elution was performed with H_2O followed by H_2O-MeOH mixtures of decreasing polarities, and fractions were combined based on their analytical HPLC and TLC profiles to afford 8 major fractions (I-VI). Elution with 20% MeOH afforded fraction II (6.5 g). A portion of fraction II (4 g) was re-chromatographed over a Sephadex LH-20 column (5 × 40 cm) and H_2O-MeOH mixtures were used for elution to obtain 3 major sub-fractions. Major Sub-fraction 1 (900 g) was fractionated by Sephadex LH-20 column chromatography (5 × 40 cm) and elution was performed using EtOAc: EtOH mixtures of increasing polarities to yield 3 sub-fractions. Sub-fraction 2 (140 mg) was re-chromatographed over a Sephadex LH-20 column (2 × 20 cm) and eluted with

H_2O-MeOH mixtures followed by purification over RP-18 column (2 × 20 cm), eluted with 10% MeOH, to obtain compound **1** (24 mg). Major Sub-fraction 2 (1.2 g) was re-chromatographed over a Sephadex LH-20 column (5 × 40 cm) and elution was performed using EtOAc: EtOH mixtures of increasing polarities to yield 2 sub-fractions. Sub-fraction 1 (560 mg) was subjected to CC over Sephadex LH-20 (2 × 20 cm) using H_2O-MeOH mixtures to obtain compound **2** (60 mg). Sub-fraction 2 (340 mg) was re-chromatographed over a Sephadex LH-20 column (2 × 20 cm) and eluted with H_2O-MeOH mixtures followed by the purification of each sub-fraction over RP-18 (2 × 20 cm), to obtain compounds **3** (52 mg) and **4** (27 mg). Major Sub-fraction 3 (2 g) was fractionated over a Sephadex LH-20 column (5 × 40 cm) and was eluted with EtOAc: EtOH mixtures of increasing polarities to yield 5 sub-fractions. Sub-fraction 2 (420 mg) was re-chromatographed over Sephadex LH-20 (2 ×20 cm) eluted with H_2O–MeOH mixtures to obtain compound **5** (60 mg). Sub-fraction 5 (250 mg) was re-chromatographed over Sephadex LH-20 (2 x 20 cm) eluted with H_2O-MeOH mixtures to obtain compound **6** (38 mg).

3.5. Biological Assays

3.5.1. Cell Culture

HepG2 cell line was maintained in RPMI-1640 complete media (L-glutamine supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ mL penicillin G, and 100 µg/mL streptomycin). Cells were grown at 37 °C in a humidified atmosphere of 5% CO_2. All experiments were performed with cells in the logarithmic growth phase. To select the appropriate concentration of the tested compounds to be used for the cytoprotective study, a cytotoxicity assay was conducted. Treatment of HepG2 cells with compounds **1**-**6** (50, 100 and 200 µM) and the total flavonoid fraction (50, 100 and 200 µg/mL) maintained more than 90% of the cell viability (data not shown), indicating that the tested compounds and the total fraction are non-cytotoxic to liver cells up to 200 µM and 200 µg/mL, respectively. Therefore, these concentrations were used for the subsequent investigations.

3.5.2. Cytorotective Activity

HepG2 monolayer culture after attachment was pretreated for 2 h with a serial dilutions 50, 100 and 200 µM of compounds **1**-**6**. The total fraction or the standard hepatoprotective agent silymarin was assayed at the concentrations (50, 100 and 200 µg/mL). At the end of the 2 h incubation, the treatments were discarded and fresh aliquot of 40 mM CCl_4 (0.05% dimethyl sulfoxide (DMSO) in phosphate buffered saline) was added to each well and incubation was continued for another 2 h. The medium and cell lysates were collected and stored at –20 °C until analysis. Positive control was a set of cells maintained in culture medium and treated only with CCl_4 (40 mM); while the negative control was a set of cells maintained in phosphate-buffered saline.

3.5.3. Assessment of AST and ALT Leakage

The activities of the marker enzymes ALT and AST were measured in the culture medium as biochemical markers of hepatocyte membrane integrity. The enzyme activities were assessed colorimetrically as reported before [1].

3.5.4. Assessment of SOD

Superoxide dismutase activity was determined in the cell lysate via the inhibition of pyrogallol autoxidation. The cell protein contents were determined with a BCA Protein Assay Kit (Pierce Biotechnology, Rockford, USA). An aliquot of 20 µL (50 µg protein) was added to a microcuvette containing 10 µL pyrogallol solution (10 mM dissolved in 10 mM HCl) and 1 mL Tris–HCl buffer (50 mM, pH 8.2) containing 1 mM diethylenetriaminopentaacetic acid. The change in absorbance per minute at 420 nm was recorded for 2 min [2].

3.5.5. Determination of GSH

GSH was estimated in the cell lysate by monitoring the reduction of Ellman's reagent. The reduced chromogen (5-thio-2-nitrobenzoic acid) is measured at 412 nm and is directly proportional to GSH concentration [3]. Briefly, proteins were precipitated by mixing equal volumes of the cell culture supernatant and 10% trichloroacetic acid, 0.005M EDTA solution followed by centrifugation at 600 × *g* for 15 min. To 0.5 mL of the resulting supernatant, 0.85 mL phosphate buffer (0.1 M, pH 8) and 0.05 mL Ellman's reagent (10 mM) were added in a microcuvette and the optical density was measured at 412 nm.

3.5.6. Assessment of MDA Levels

Lipid peroxidation was estimated spectrophotometrically using the thiobarbituric acid reactive substance (TBARS) method, as described by Uchiyama and Mihara [4] using 1,1,3,3-tetraethoxypropane as a standard. Absorbance was then read at 532 nm. Results were expressed in terms of mmol/mL malondialdehyde (MDA) formed in cell lysates.

3.5.7. Statistical Analysis

Data are presented as the mean ± SD. Comparisons were carried out using one-way analysis of variance followed by Tukey–Kramer's test for post hoc analysis. Statistical significance was acceptable to a level of $P < 0.05$, while $P < 0.01$ were considered highly significant, and $P < 0.001$ were considered extremely significant. All statistical analyses were performed using GraphPad InStat software, version 3.05 (GraphPad Software, La Jolla, CA). Graphs were plotted using GraphPad Prism software, version 5.00 (GraphPad Software, La Jolla, CA).

References

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