Supporting Information

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Xanthine Oxidase-Inhibitory Activity and Antioxidant Properties of the Methanol Extract and Flavonoids of *Artemisia Asiatica*

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S.1. Materials and Methods

S.1.1. General methods

NMR spectra were recorded on a Bruker Avance DRX 500 spectrometer at 500 MHz (^{1}H) or 125 MHz (^{13}C); the signals of the deuterated solvents were taken as reference. Two-dimensional experiments (^{1}H,^{1}H-COSY, HSQC, HMBC and NOESY) were set up, performed and processed with the standard Bruker protocol. Silica gel plates were applied for analytical and preparative TLC (Merck 5717 and 5715). For vacuum liquid chromatography (VLC), SiO_2 (Silica gel 60 G, 15 µm, Merck 11677) was applied. Centrifugal planar chromatography (CPC) was performed with a Chromatotron instrument (Model 8924, Harrison Research) on manually prepared SiO_2 (Silica gel 60 GF_{254}, Merck 7730) plates of 2 and 4 mm thickness, at a flow rate of 4 and 10 mL/min, respectively. Preparative layer chromatography (PLC) was carried out on SiO_2 plates (20×20 cm Silica gel 60 F_{254}, Merck 5715). Separation was monitored in UV light at 254 nm. Gel chromatography was performed on Sephadex LH-20 (25-100 µm, Pharmacia Fine Chemicals), with MeOH as eluent.

S.1.2. Plant material

A. asiatica was collected in September 2008 in the experimental field of the Institute of Ecology and Botany of the Centre for Ecological Research, Hungarian Academy of Sciences at Vácrátót, Hungary. A voucher specimen has been deposited at the herbarium of the Institute of Pharmacognosy, University of Szeged, voucher no. 703.

S.1.3. Extraction and isolation

Dried and ground aerial parts (2223 g) were percolated with MeOH (45 L) at room temperature. The extract (465 g) was concentrated in vacuum and fractionated by solvent−solvent partition with *n*-hexane (8 L) and $CHCl_3$ (6 L). The $CHCl_3$ phase (100.6 g) was subjected to column chromatography (CC) on polyamide (ICN), using MeOH-H_2O 2:8, 4:6, 6:4, and 8:2 mixtures as eluents. The flavonoid-containing fractions eluted with 60 and 80% MeOH were combined on the basis of their similar chromatographic compositions, and the combined fraction was separated by VLC on Si gel, using gradient mixtures of $CHCl_3$−MeOH with increasing polarity, to obtain 11 fractions. The fraction obtained with $CHCl_3$−MeOH 98:2 (F1) was subjected to gel chromatography on Sephadex LH 20 using MeOH as eluent to afford three subfractions (S1, S2 and S3). The preparative TLC of S1 and S2 on silica gel with *n*-hexane−EtOAc as 98:2 mobile phase yielded chrysoplenetin (**7**), and cirsilineol (**4**), respectively. Crystallization of S3 afforded by eupatilin (**1**), and preperative TLC of the mother liquor on silica with *n*-hexane−EtOAc 98:2 gave jaceosidin (**3**), and 6-methoxytricin (**6**). Fraction F2 of the VLC separation, eluted with $CHCl_3$−MeOH 6:4 was separated by CPC on silica gel, eluted with *n*-hexane−EtOAc mixtures of increasing polarity, affording compound **2**. When F3 was subjected to CPC on silica, eluted with *n*-hexane−EtOAc mixtures of increasing polarity, followed by separation on Sephadex LH 20, with elution with MeOH, pure compound **5** was obtained.

S.1.4. Characterization of the pure compounds

Eupatilin (**1**), yellow crystals; mp. 236-238 ºC; the ^{1}H- and ^{13}C-NMR data were identical with published data [1,2]; ESIMS (positive mode) *m/z* 345 $[M+H]^+$, 330 $[(M+H)–CH_3]^+$, 168, 162.

Hispidulin (**2**)**,** a yellow powder; the ^{1}H- and ^{13}C-NMR data were identical with published data [3], ESIMS (positive mode) *m/z* 301 $[M+H]^+$, 286 $[(M+H)–CH_3]^+$, 258 $[(M+H)–CH_3–CO]^+$, 168, 119.

Jaceosidin (**3**), a yellow amorphous powder; ^{1}H-NMR (500 MHz, DMSO-d_6 δ ppm): 13.07 (1H, s, 5-OH), 7.53 (1H, d, *J* = 8.9 Hz, H-6'), 7.53 (1H, s, H-2'), 6.92 (1H, d, *J* = 8.9 Hz, H-5'), 6.86 (1H, s, H-

3), 6.60 (1H, s, H-8), 3.89 (3H, s, 3'-OCH_3), 3.75 (3H, s, 6-OCH_3)); ^{13}C-NMR (125 MHz, DMSO-d_6, δ ppm): 182.1 (C-4), 163.7 (C-2), 157.3 (C-7), 152.7 (C-5), 152.4 (C-9), 150.7 (C-3'), 148.0 (C-4'), 131.3 (C-6), 121.5 (C-1'), 120.3 (C-6'), 115.7 (C-5'), 110.1 (C-2'), 104.0 (C-10), 102.7 (C-3), 94.3 (C-8), 59.9 (6-OCH_3), 55.9 (3'-OCH_3); NOESY correlations: H-2'/H-3, H-2'/3'-OCH_3. ESIMS (positive mode) *m/z* 331 $[M+H]^+$, 316 $[(M+H)-CH_3]^+$, 301 $[(M+H)-2\times CH_3]^+$, 273 $[(M+H)-2\times CH_3-CO]^+$, 168, 140.

Cirsilineol (**4**), a yellow powder from MeOH; the ^{1}H- and ^{13}C-NMR data were identical with those published in the literature [4,5]. ESIMS (positive mode) *m/z* 345 $[M+H]^+$, 330 $[(M+H)-CH_3]^+$, 312 $[(M+H)-CH_3-H_2O]^+$, 284 $[(M+H)-CH_3-H_2O-CO]^+$, 148, 136.

5,7,4',5'-Tetrahydroxy-6,3'-dimethoxyflavone (**5**) a yellow powder; the ^{1}H- and ^{13}C-NMR data were identical with published data [6]. A NOESY correlation was detected between the signals of H-2' (δ_H 7.15, s) and 3'-OCH_3 (δ_H 3.87, s) in DMSO-d_6. ESIMS (positive mode) *m/z* 347 $[M+H]^+$, 332 $[(M+H)-CH_3]^+$, 317 $[(M+H)-2\times CH_3]^+$, 289 $[(M+H)-2\times CH_3-CO]^+$, 168, 164.

6-Methoxytricin (**6**), a pale-yellow powder; the ^{1}H- and ^{13}C-NMR data measured in DMSO-d_6 were identical with those published in the literature [7]. In the NOESY spectrum, *Overhauser* effects were detected between H-2',6' (δ_H 7.22, s) and 3',5'-OMe [δ_H 3.85, s (6H)]. ESIMS (positive mode) *m/z* 361 $[M+H]^+$, 346 $[(M+H)-CH_3]^+$, 343 $[(M+H)-H_2O]^+$, 178, 167.

Chrysosplenetin (**7**), a yellow amorphous powder; the ^{1}H- and ^{13}C-NMR data were in good agreement with published data [8,9,10]. In the NOESY experiment, NOEs were observed between H-8 and 7-OMe (δ_H 3.92, s), H-2' and 3-OMe (δ_H 3.87, s), H-6' and 3-OMe, and H-2' and 3'-OMe (δ_H 3.81, s). ESIMS (positive mode) *m/z* 375 $[M+H]^+$, 370 $[(M+H)-CH_3]^+$, 345 $[(M+H)-2\times CH_3]^+$, 342 $[(M+H)-CH_3-H_2O]^+$, 317 $[(M+H)-2\times CH_3-CO]^+$, 299 $[(M+H)-2\times CH_3-CO-H_2O]^+$, 271, 151.

S.1.5. Xanthine oxidase assay

XO isolated from bovine milk (lyophilized powder) and xanthine powder was purchased from Sigma-Aldrich. The protocol used was adapted from that recommended by Sigma as follows: the production of uric acid by XO in a total volume of 0.300 mL was measured at 290 nm for 3 minutes in 96-well plate, using the plate reader FluoSTAR OPTIMA Fluorescence (BMG LABTECH, Germany).

Stock solutions were prepared as recommended: 50 mM potassium buffer, pH 7.5, 0.15 mM xanthine solution, pH 7.5 and XO 0.04 units/mL. The stock solutions of *A. asiatica* extracts (12 g/mL) and purified compounds **1-7** (600 mg/L) were prepared in DMSO solution. 0.140 mL of buffer solution and 0.100 mL of xanthine solution were added to the wells at a final concentration of 33 mM and 0.05 mM, respectively. Compounds and extracts were added in appropriate volumes so that the final concentration of DMSO in the assay did not exceed 3.3%. The reaction was initiated by the automatic addition of 0.050 mL of XO solution to a final concentration of 0.006 units/mL. Each extract/compound was tested in triplicate. Allopurinol was used as positive control. The IC_{50} values were calculated by analysing the inhibitory percentage of each concentration, using GraphPad Prism 5.04 software (GraphPad Software Inc., US) with nonlinear regression.

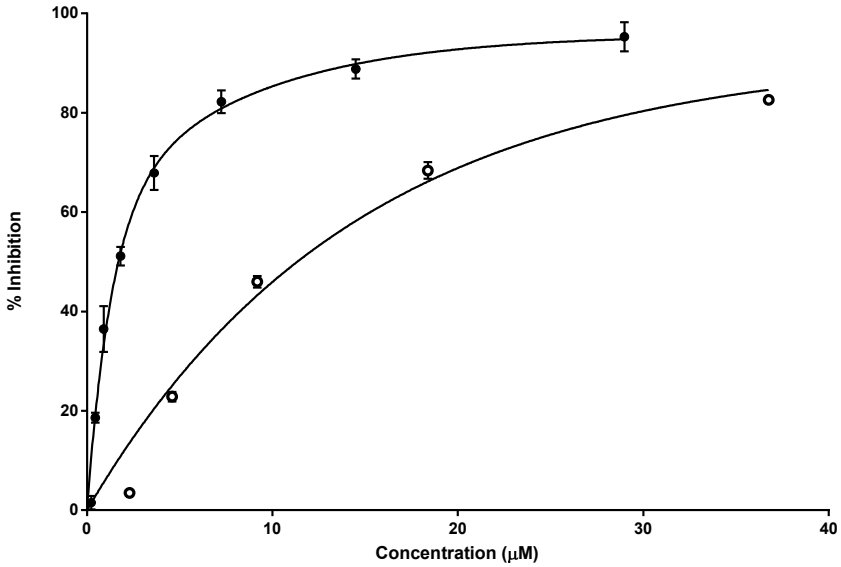

Figure 2. Dose-dependent inhibition of XO by compound **1** (●) and allopurinol (○).
Each value is the mean ± SD of three different determinations

S.1.6. DPPH assay

The capacity of the pure compounds to scavenge for radicals was tested by using the DPPH method. Briefly, reaction of the test compound with the stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) and the reduction of DPPH in a total volume of 300 µl was followed by monitoring the decrease in its absorbance at 550 nm using the FluoSTAR OPTIMA (BMG LABTECH, Germany) Fluorescence plate reader. Three stock solutions of DPPH and test samples were prepared in HPLC grade MeOH at 1 mM and 1 mg/mL, respectively. Samples were added to the wells according to the desired concentration and serial-diluted to the following wells. The reaction was started by adding 100 µL of DPPH solution to each well. The decrease in absorbance was determined after 30 and 60 min, in duplicate. During the reaction period, the mixture was kept in the dark. Quercetin was used as positive control [11].

S.1.7. References of Supporting Information

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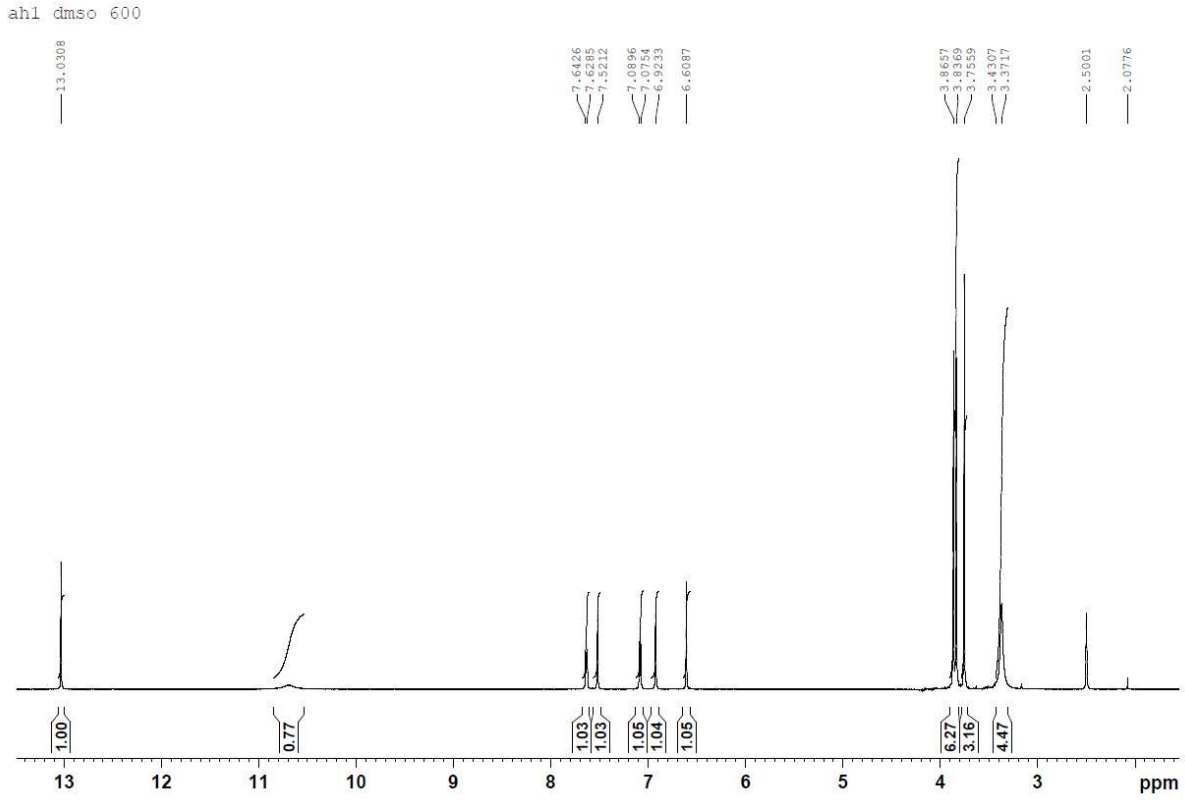
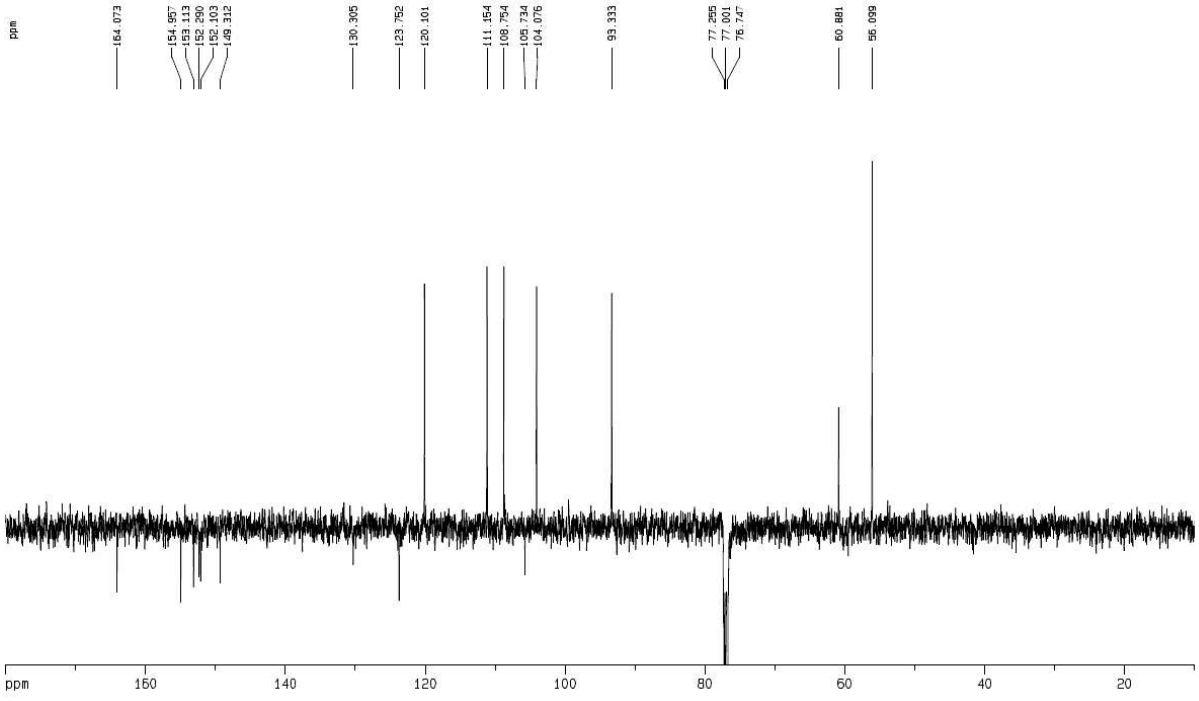
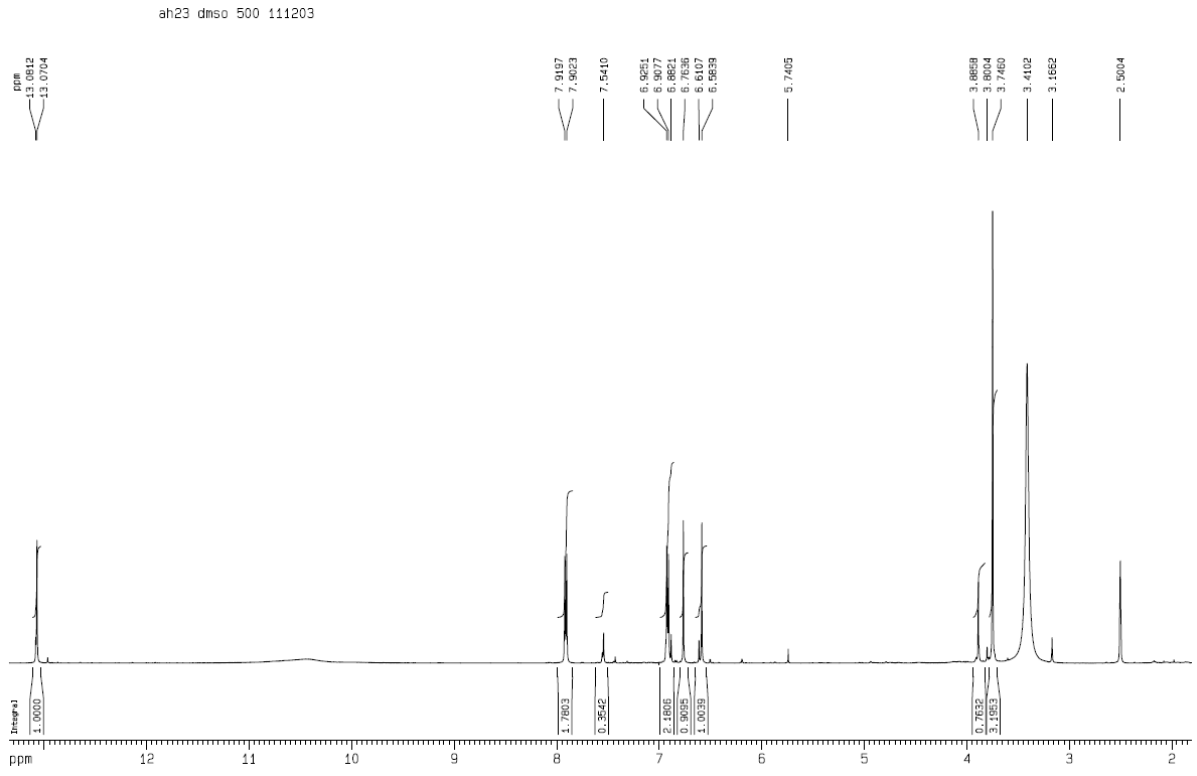


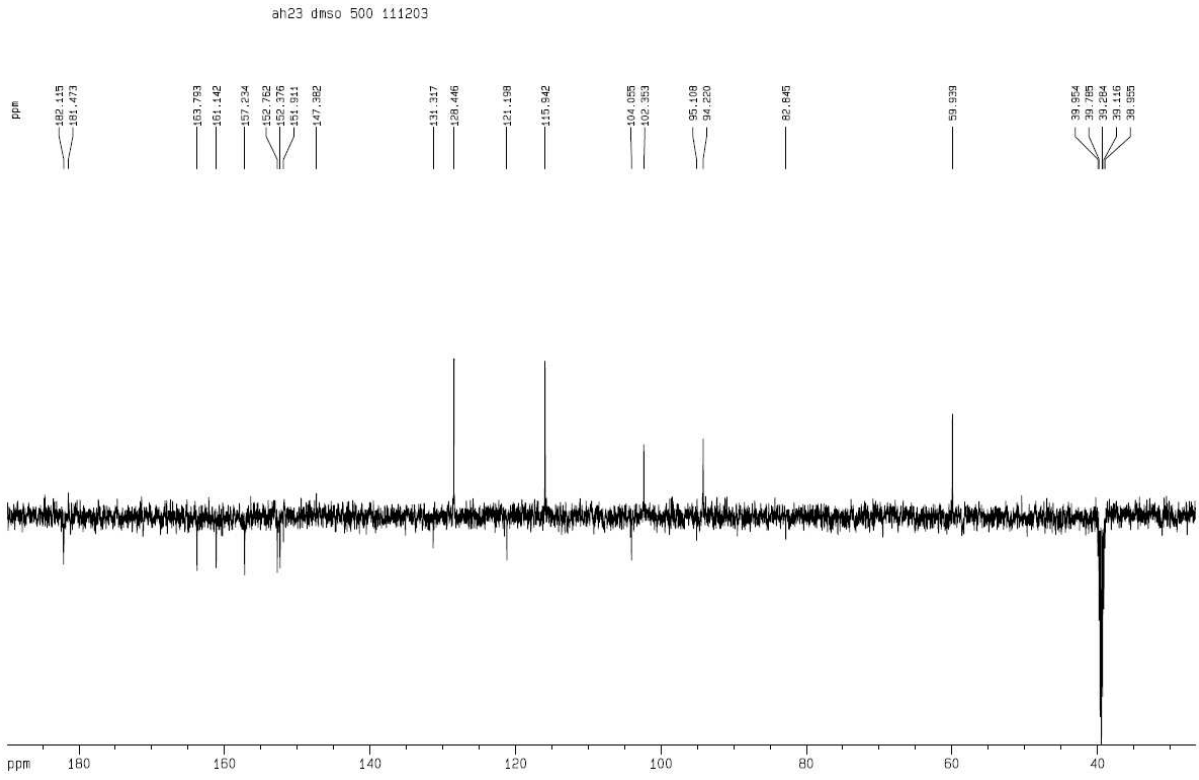
S2: ^{1}H-NMR Spectrum (600 MHz, DMSO-d_6) of Compound **1** eupatilin



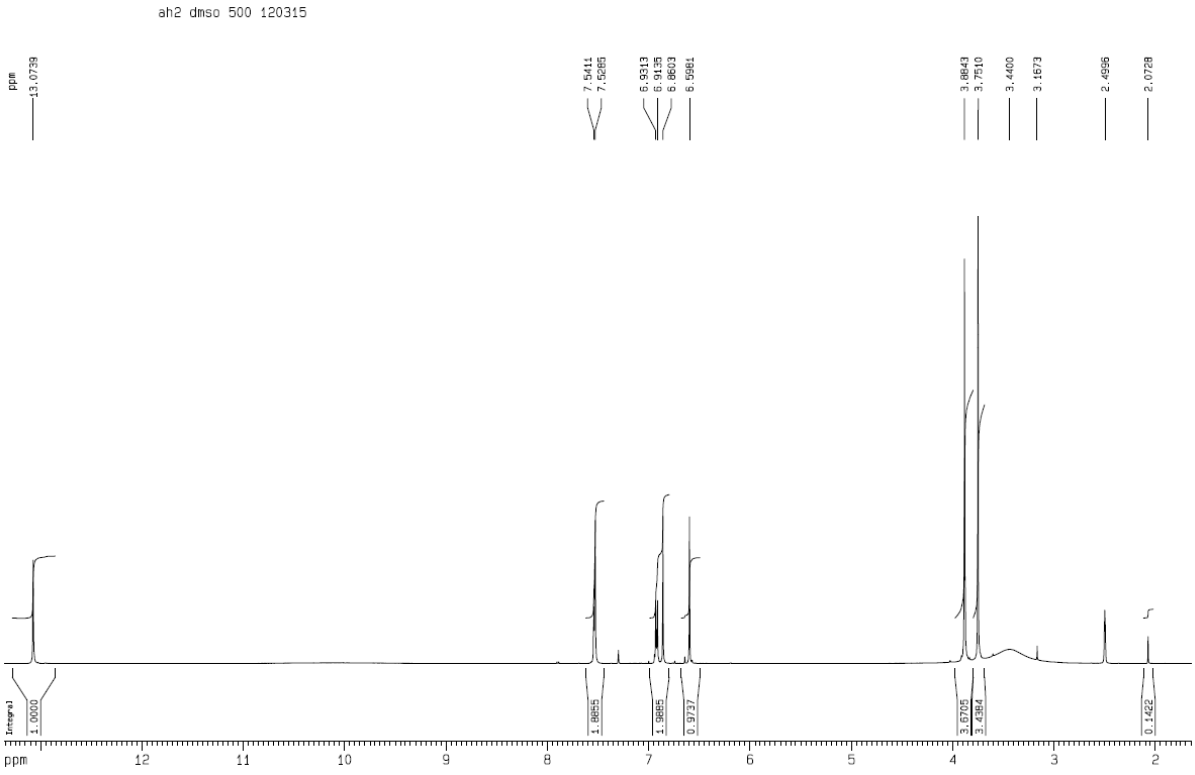
S3: JMOD Spectrum (500 MHz, $CDCl_3$) of Compound **1** eupatilin



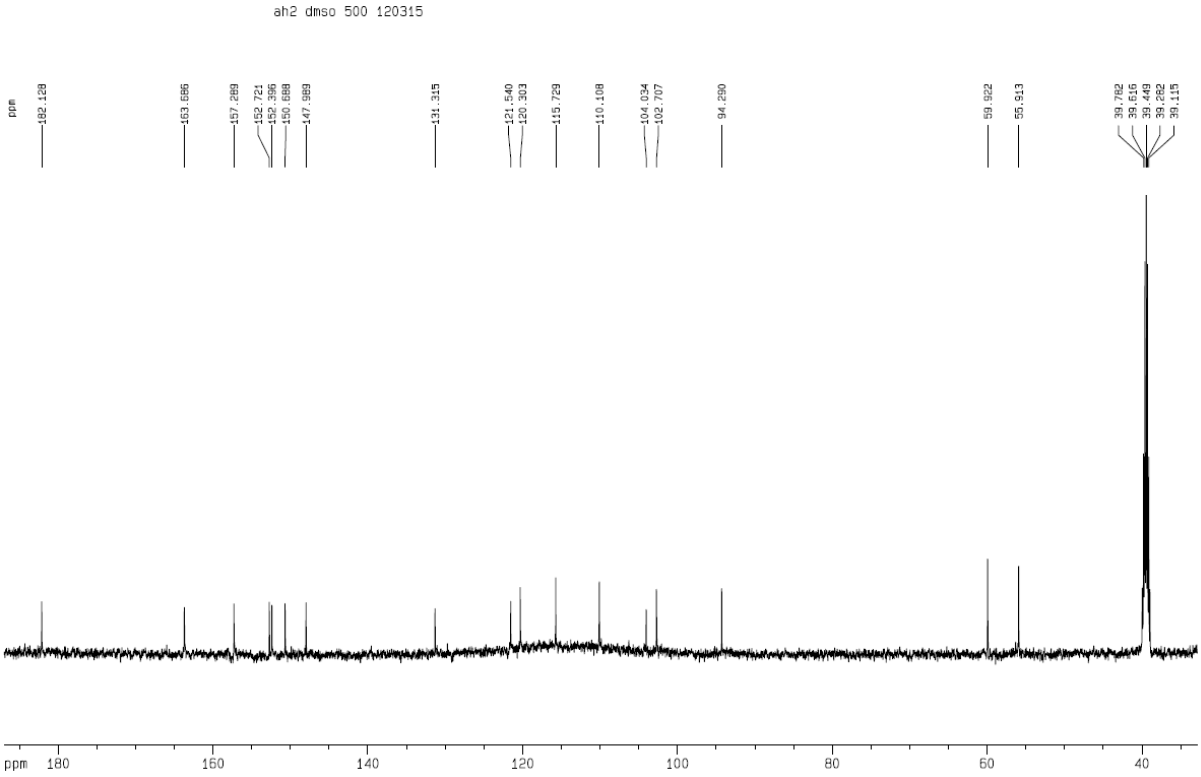
S4: ^{1}H-NMR (500 MHz, DMSO-d_6) Spectrum of Compound **2** hispidulin



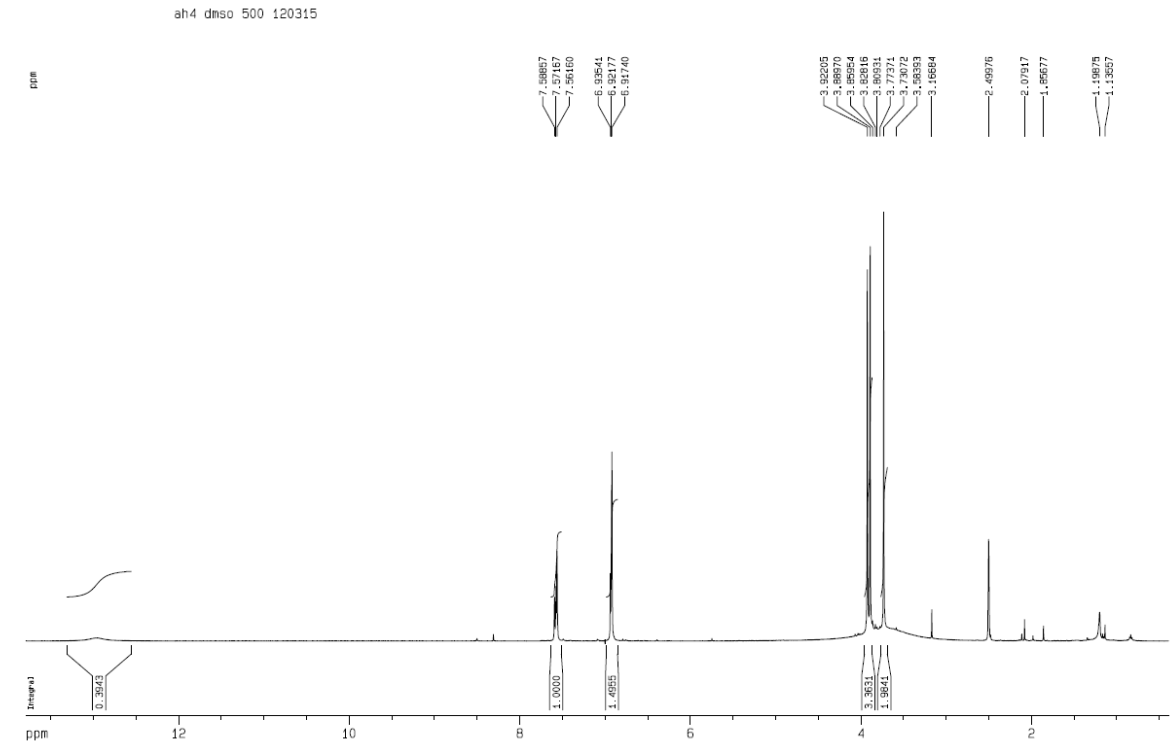
S5: JMOD (500 MHz, DMSO-d_6) Spectrum of Compound **2** hispidulin



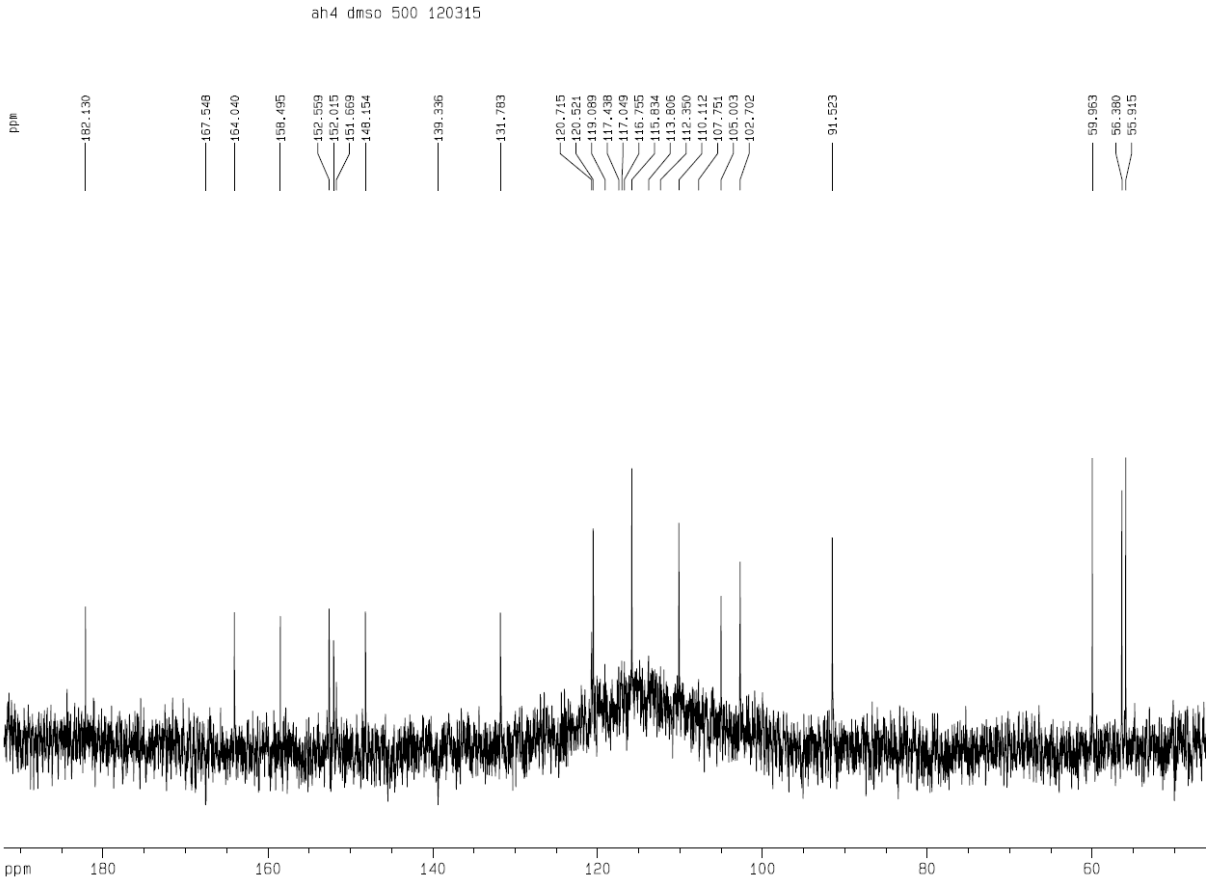
S6: ^{1}H-NMR (500 MHz, DMSO-d_6) Spectrum of Compound **3** jaceosidin



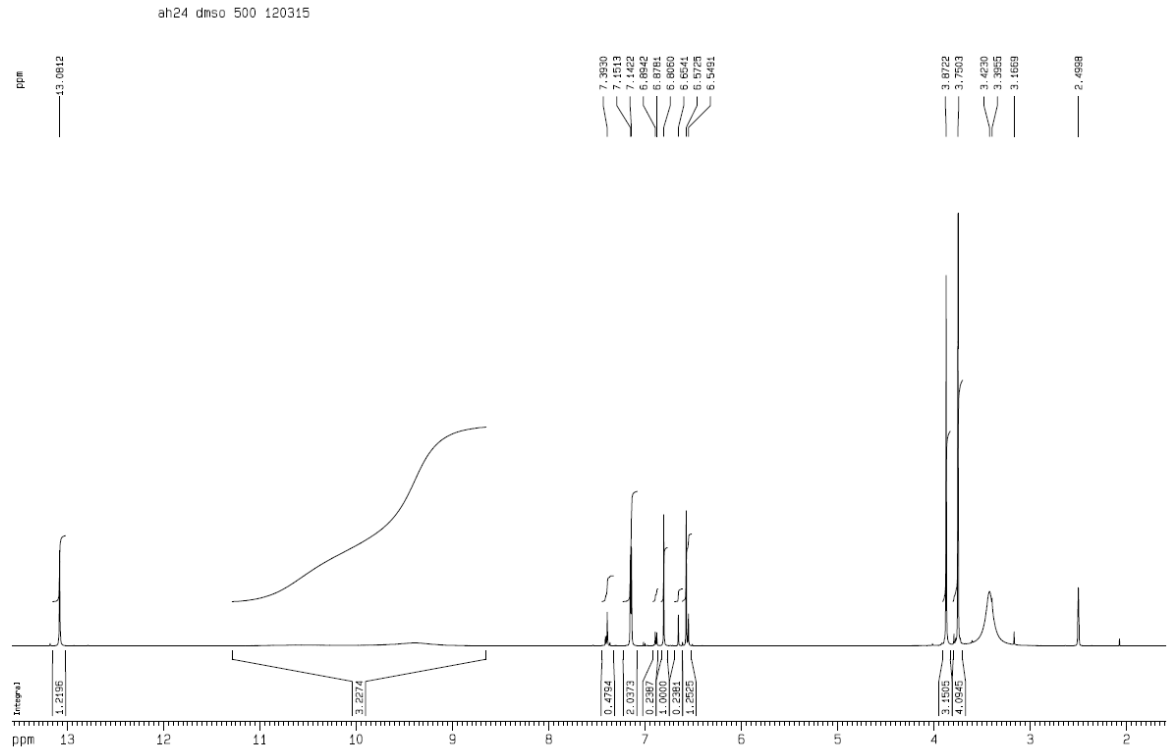
S7: ^{13}C-NMR (500 MHz, DMSO-d_6) Spectrum of Compound **3** jaceosidin



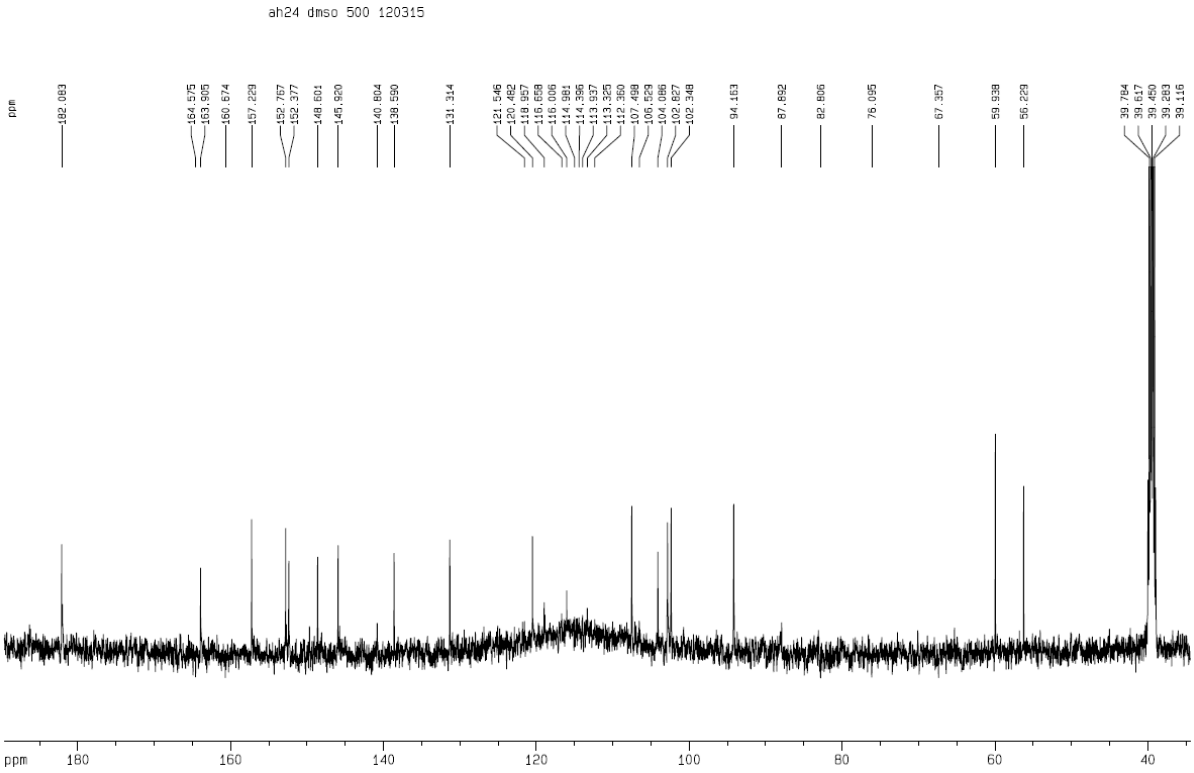
S8: ^{1}H-NMR (500 MHz, DMSO-d_6) Spectrum of Compound **4** cirsilineol



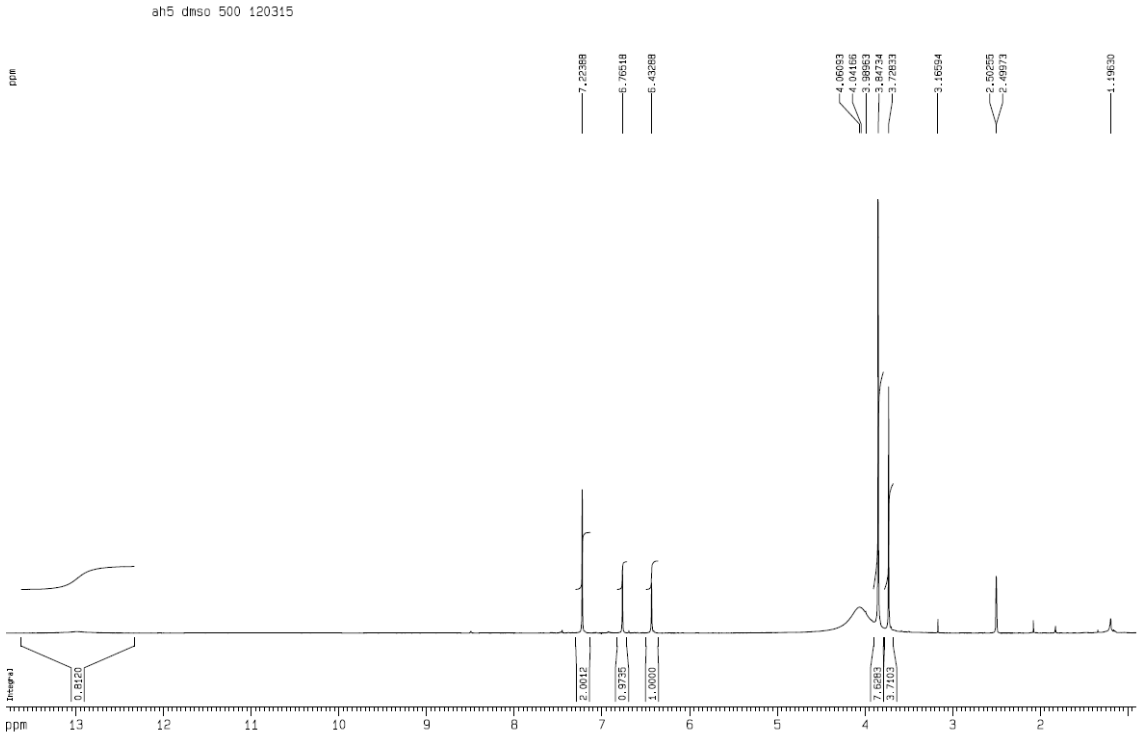
S9: ^{13}C-NMR (500 MHz, DMSO-d_6) Spectrum of Compound **4** cirsilineol



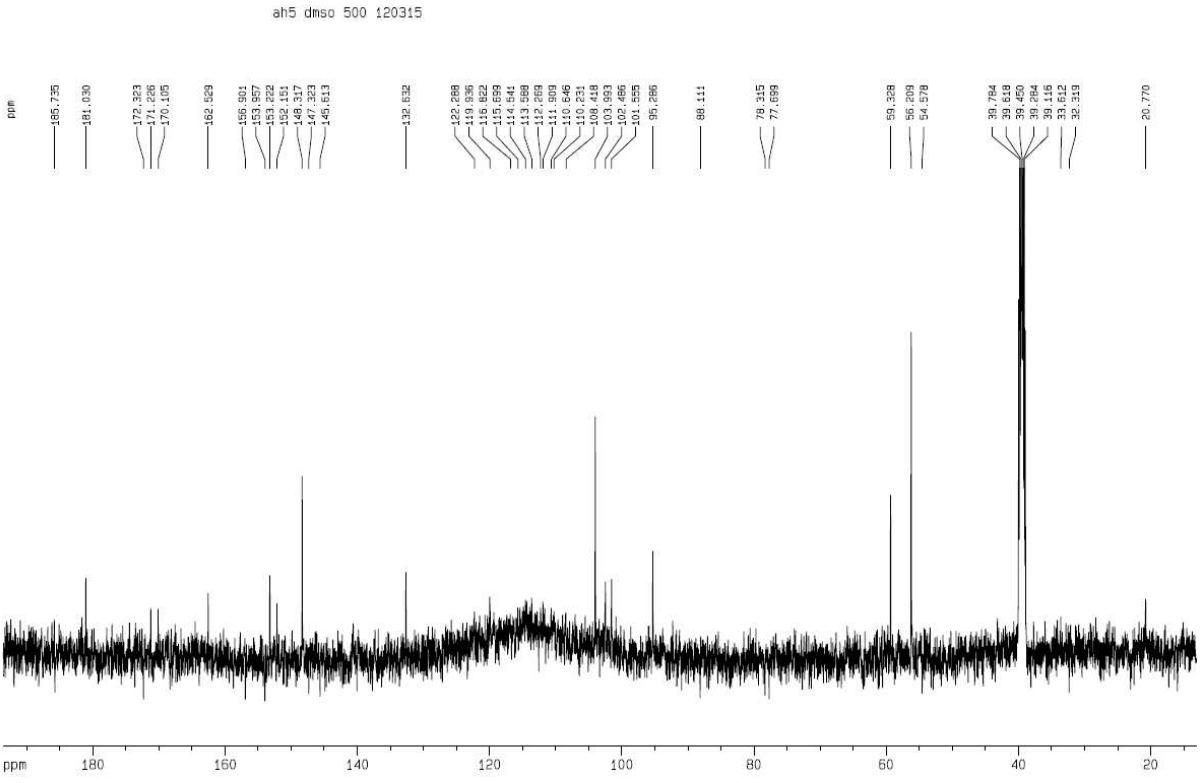
S10: ^{1}H-NMR (500 MHz, DMSO-d_6) Spectrum of Compound **5**
5,7,4',5'-tetrahydroxy-6,3'-dimethoxyflavone



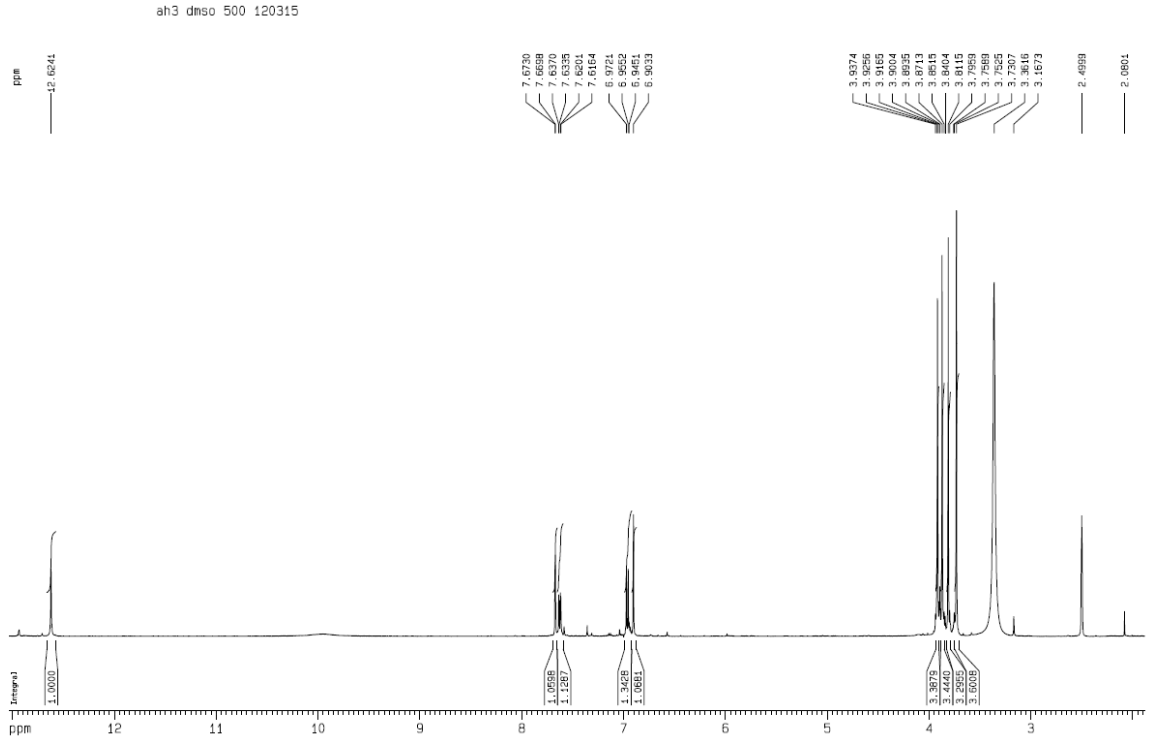
S11: ^{13}C-NMR (500 MHz, DMSO-d_6) Spectrum of Compound **5**
5,7,4',5'-tetrahydroxy-6,3'-dimethoxyflavone



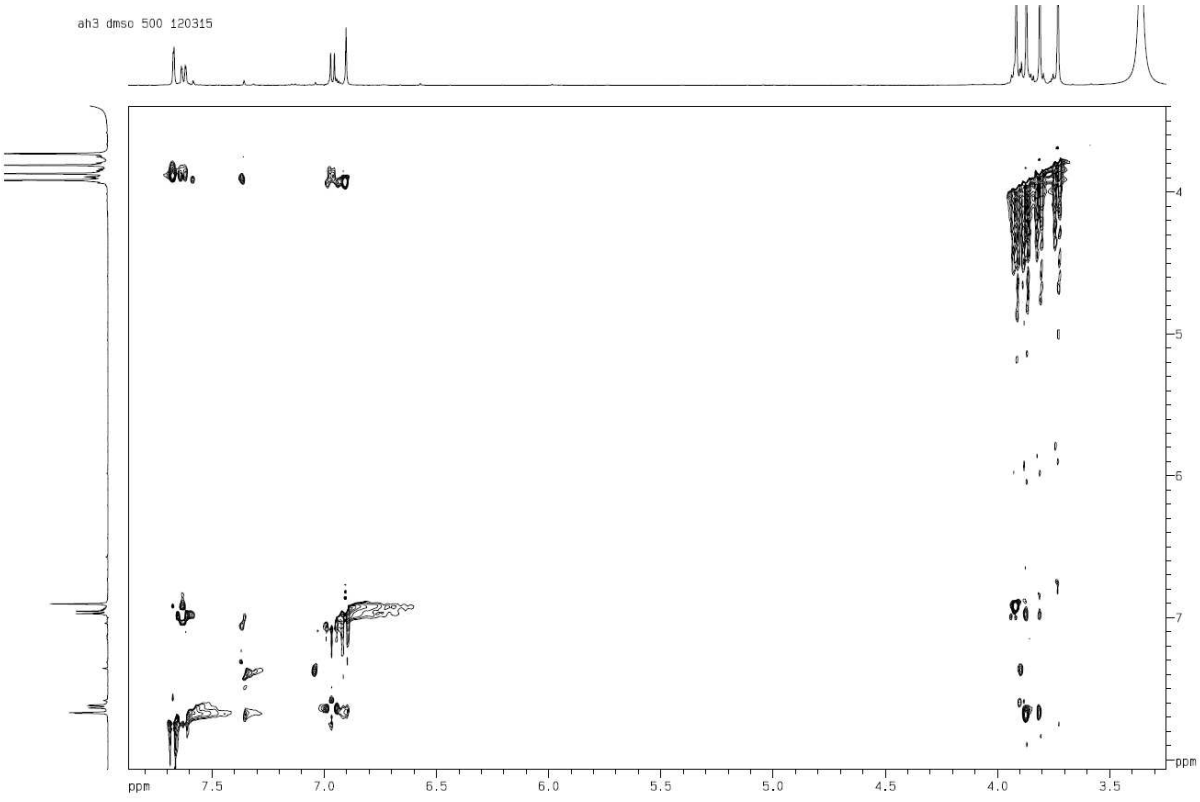
S12: ^{1}H-NMR (500 MHz, DMSO-d_6) Spectrum of Compound **6** 6-methoxytricin



S13: ^{13}C-NMR (500 MHz, DMSO-d_6) Spectrum of Compound **6** 6-methoxytricin



S14: ^{1}H-NMR (500 MHz, DMSO-d_6) Spectrum of Compound **7** chrysosplenetin



S14: ROESY (500 MHz, DMSO-d_6) Spectrum of Compound **7** chrysosplenetin