Supporting Information

***Rec. Nat. Prod*. 10:6 (2016) 766-770**

Antiproliferative Activities of Chemical Constituents Isolated from *Thymus praecox* subsp. *grossheimii* (Ronniger) Jalas

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1. Antiproliferative assays

Antiproliferative and cytotoxic activities of isolated compounds and extract were investigated in vitro on C6 (rat brain tumor), HeLa (human cervix carcinoma), and HT29 (human colon carcinoma) and Vero (African green monkey kidney epithelium) cells lines by using BrdU cell proliferation ELISA and lactate dehydrogenase (LDH) assays.

2. Preparation of cell culture

All cell lines were maintained in Dulbecco's modified eagle's medium (DMEM, Sigma) supplemented with 10% (v/v) fetal bovine serum (Sigma, Germany) and PenStrep solution (10000 U / 10 mg) (Sigma, Germany) (ATTC, American Type Culture Collection). Old medium was aspirated out of the plate while cells had reached a confluence of 80%. Next, cells were detached from the flasks using 4 mL of trypsin-EDTA (Sigma, Germany) and centrifuged. Following, the cell pellet was resuspended with 4 mL of supplemented DMEM and was counted to gain a final concentration of 5×10^4 cells/mL, and inoculated into wells (100 µL cells/well).

3. Cell proliferation assay (CPA)

A cell suspension containing 5×10^3 cells in 100 µL was pipeted into the wells of 96-well cell culture plates (COSTAR, Corning, USA). The test compounds and a positive control compound (5 fluorouracil, 5FU) were dissolved in sterile DMSO. The amount of DMSO was adjusted to 0.5% maximum. The cells were treated with test compounds and 5FU at final concentrations of 5, 10, 20, 30, 40, 50, 75, and 100 µg/mL. The final volume of the wells was adjusted to 200 µL with supplemented DMEM. The cells were then incubated at 37 °C with 5% CO_2 overnight. The antiproliferative activity of the compounds was determined using a BrdU Cell proliferation ELISA kit according to manufacturer's protocol (Roche, USA). Briefly, cells were exposed to BrdU labeling reagent for 4 h, followed by fixation in FixDenat solution for 30 min at room temperature. Then, cells were cultured with a 1:100 dilution of anti-BrdU-POD for 1 h and 30 minutes at room temperature. Substrate solution was added to each well, and BrdU incorporation was measured at 450 - 650 nm using a microplate reader (Rayto, China). Each experiment was repeated at least three times for each cell line.

4. Calculation of IC50 and % inhibition

IC50 represents the concentration of an agent that is required for 50% inhibition in vitro. The half maximal inhibitory concentration (IC50) of the test and control compounds was calculated using XLfit5 software (IDBS) and expressed in µg/mL at 95 % confidence intervals.

5. Cytotoxic activity assay

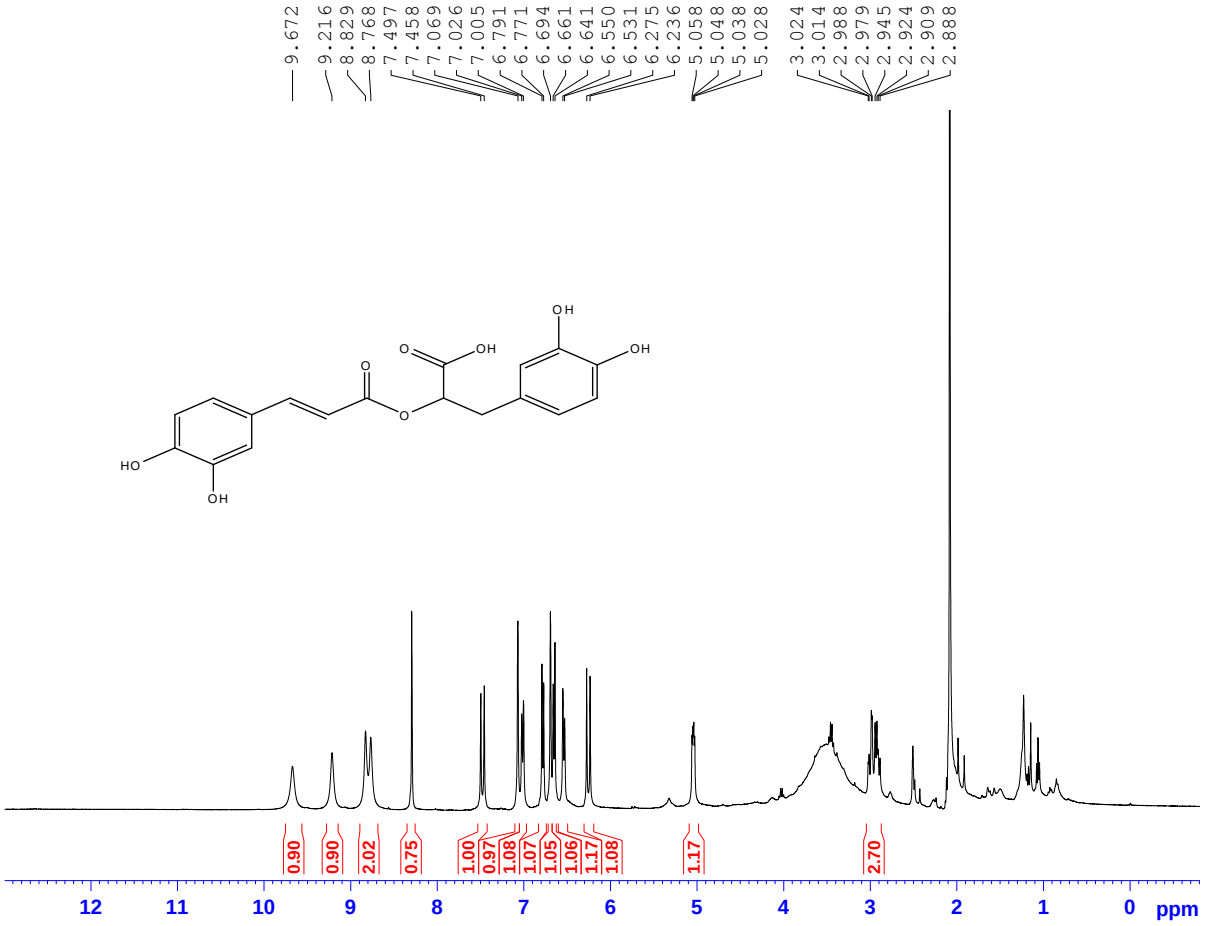
The cytotoxicity of test compounds and 5FU on cells was determined through a Lactate Dehydrogenase (LDH) Cytotoxicity Detection kit (Roche, USA). Briefly, 5×10^3 cells in 100 µL were seeded into 96-well microtiter plates as triplicates and treated with 50, 75, and 100 µg/mL concentrations of test compounds and 5FU as described above at 37 °C with 5% CO_2 overnight. LDH activity was determined by measuring absorbance at 492 - 630 nm using a microplate reader.

6. Cell imaging

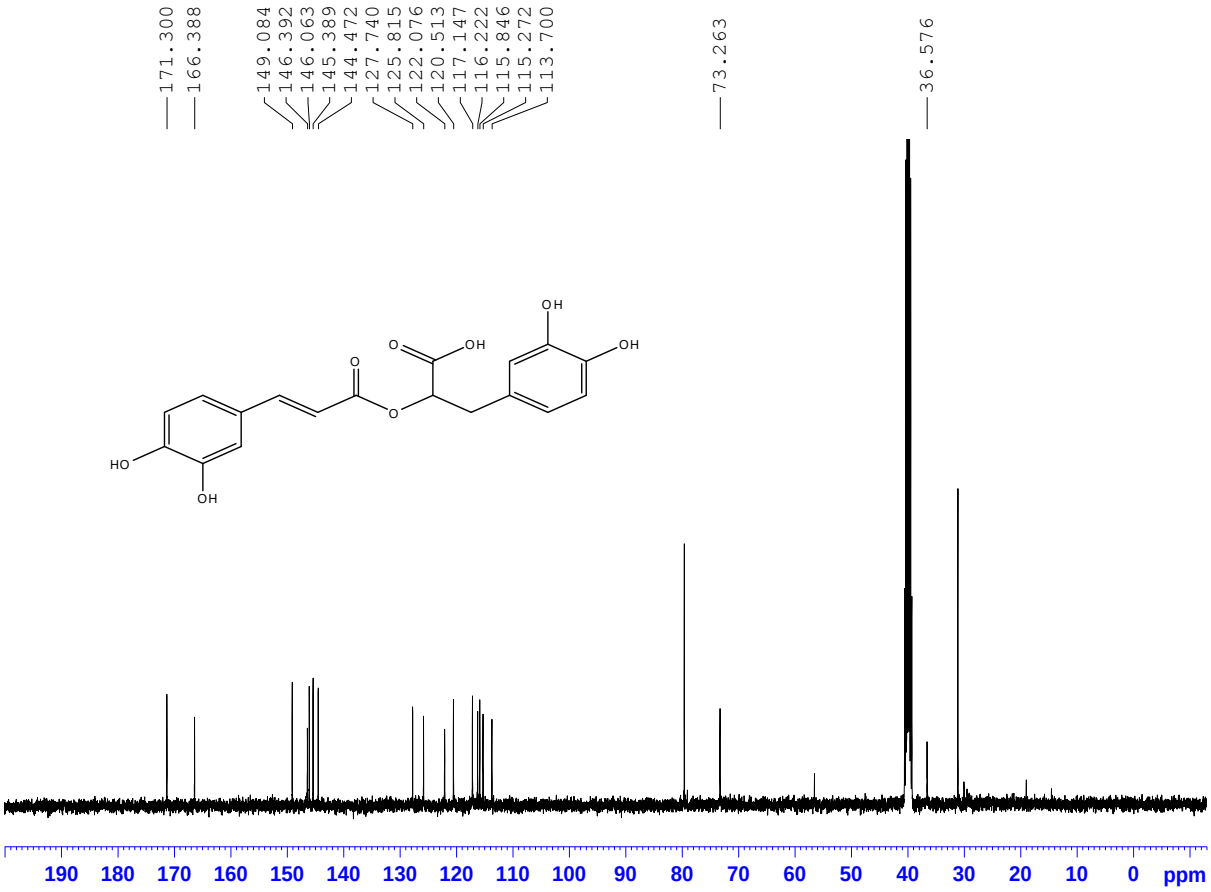
Cells were seeded in 96-well plates at a density of 5×10^3 cells per well and allowed 24 h for attachment. Using all doses of test compounds treatment was performed for 24 h, during which morphology changes were assessed by phase contrast microscopy. Images of vehicle (DMSO), test compounds treated cells were taken at the end of experimental period using a digital camera attached inverted microscope (Leica IL10, Germany).

7. Morphological assessment of the cytotoxic activity of compounds

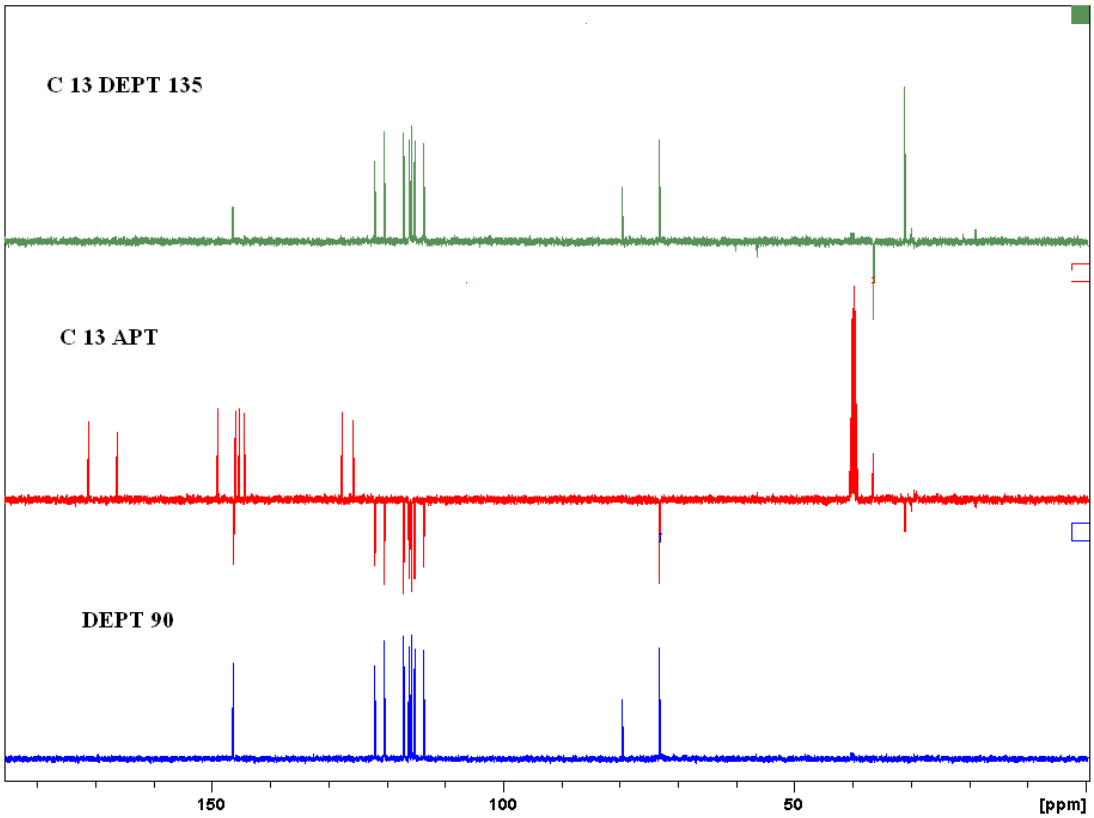
Morphological assessment of each cell line treated with compounds and extract found the compound to be antiproliferative because cells showed characteristic features of apoptotic cell death. Figure 3 shows inverted microscope images of the morphology of untreated or treated C6, HeLa, HT29, and Vero cell lines with 5, 10, 20, 30, 40, 50, 75, and 100 μg/mL of the compounds of *xperia*. The results in figure 3 show the normal structure of numerous control cells growing in the medium. Figure 3 also shows the same cells line following 24 hours of incubation with compounds and extract of *xperia*. However, according to this, the numbers of cells have importantly decreased and structurally, they look different from untreated cells. They have lost their astrocyte-like and fibroblast-like structures and they seem to be globular upon treatment. In addition the treated C6 cells seem to shrink and they developed a round shape and they have lost their neuronal-like structure. They seem to clump together which is typical of apoptosis. Especially, at high concentrations above 50 μg/mL, the numbers of cells seem to lessen and separate from one another and they look smaller.



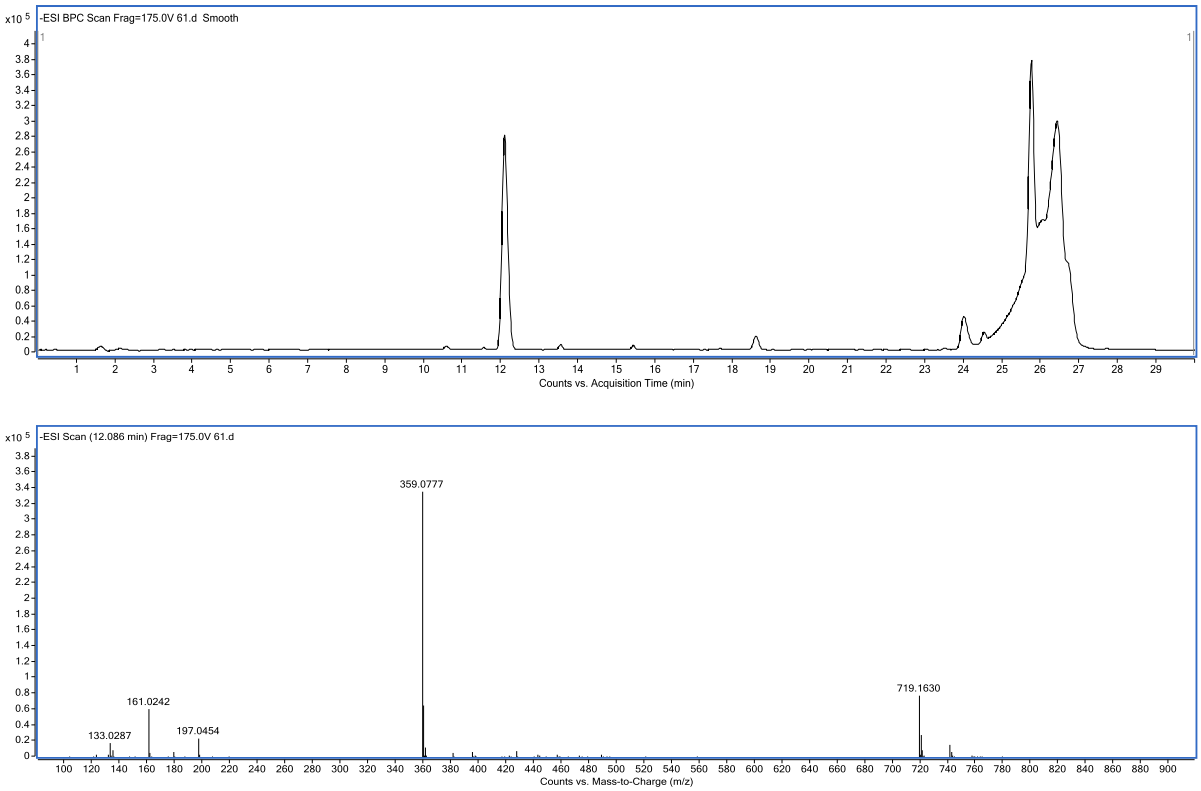
S1: ^{1}H-NMR (400 MHz, DMSO-*d6*) Spectrum of Compound **1** (rosmarinic acid)



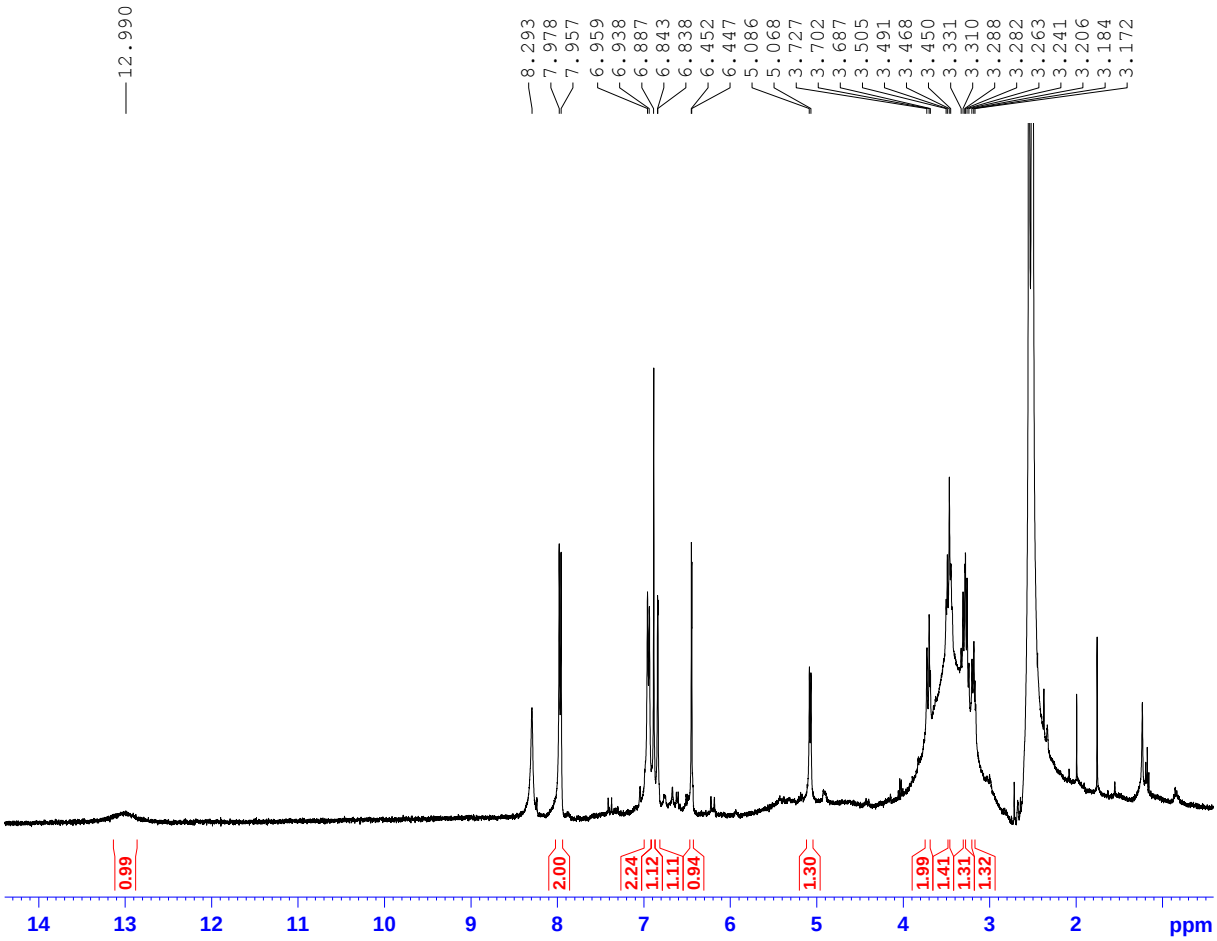
S2: ^{13}C-NMR (100 MHz, DMSO-*d6*) Spectrum of Compound **1** (rosmarinic acid)



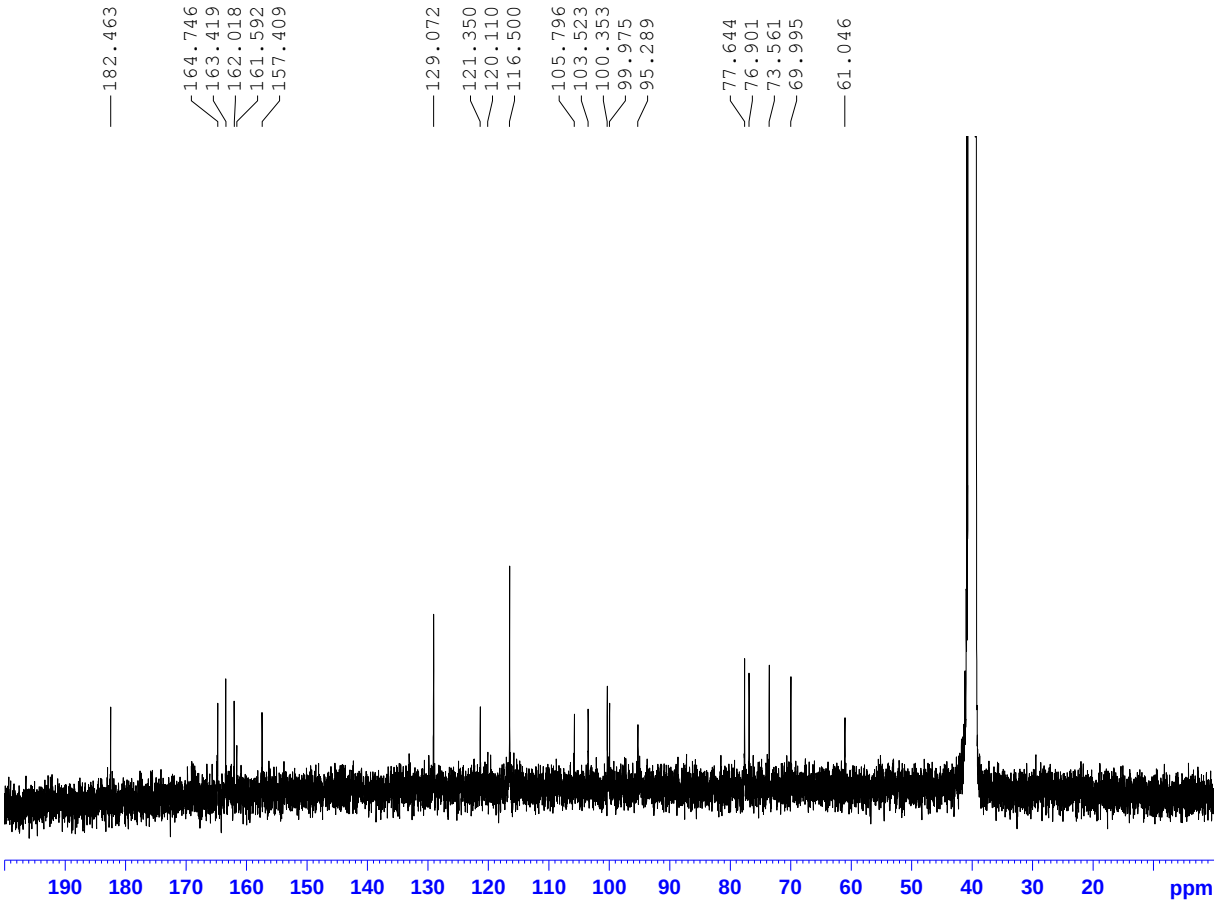
S3: DEPT-135, APT, DEPT-90 NMR (100 MHz, DMSO-*d6*) Spectrum of compound **1** (rosmarinic acid)



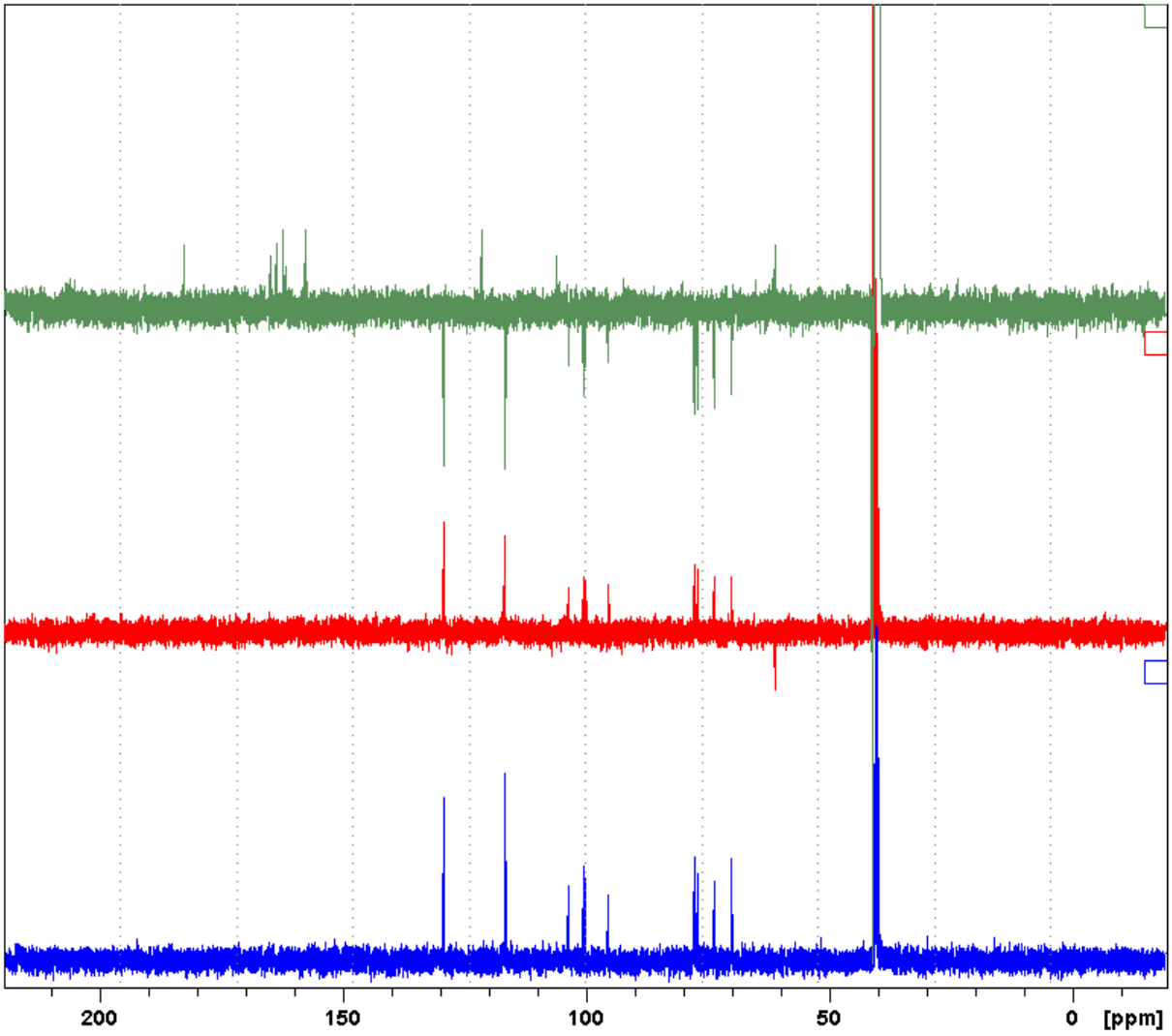
S4: HPLC-QTOF Spectrum of compound **1** (rosmarinic acid)



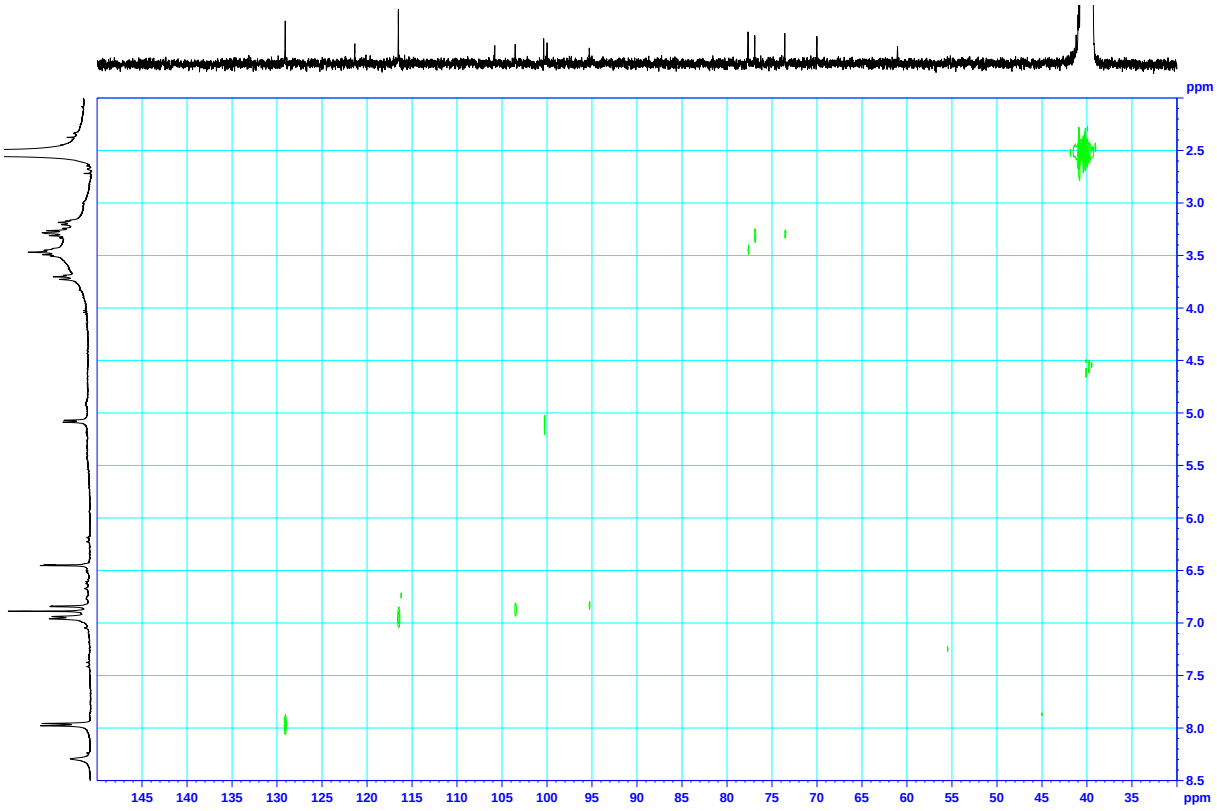
S5: ^{1}H-NMR (400 MHz, DMSO-*d6*) Spectrum of Compound **2** (apigenin 7-*O*-glucoside)



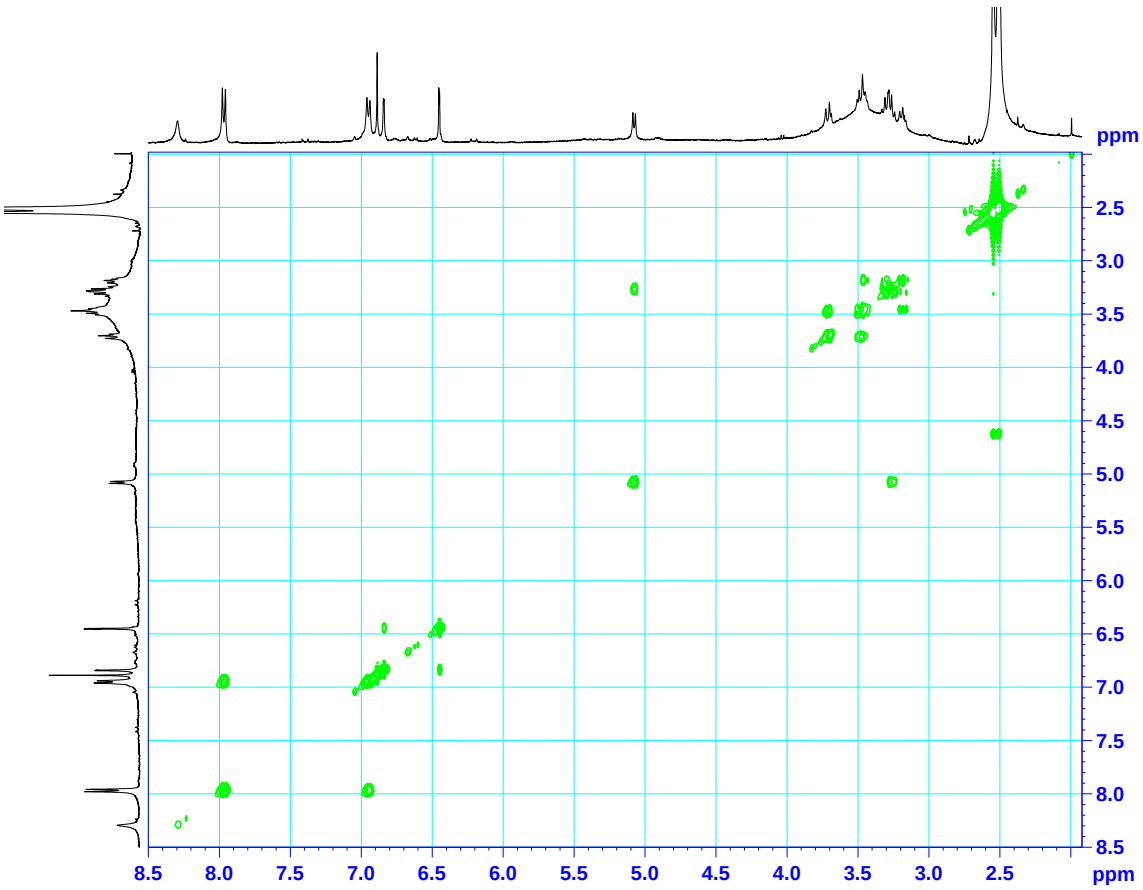
S6: ^{13}C-NMR (100 MHz, DMSO-*d6*) Spectrum of Compound **2** (apigenin 7-*O*-glucoside)



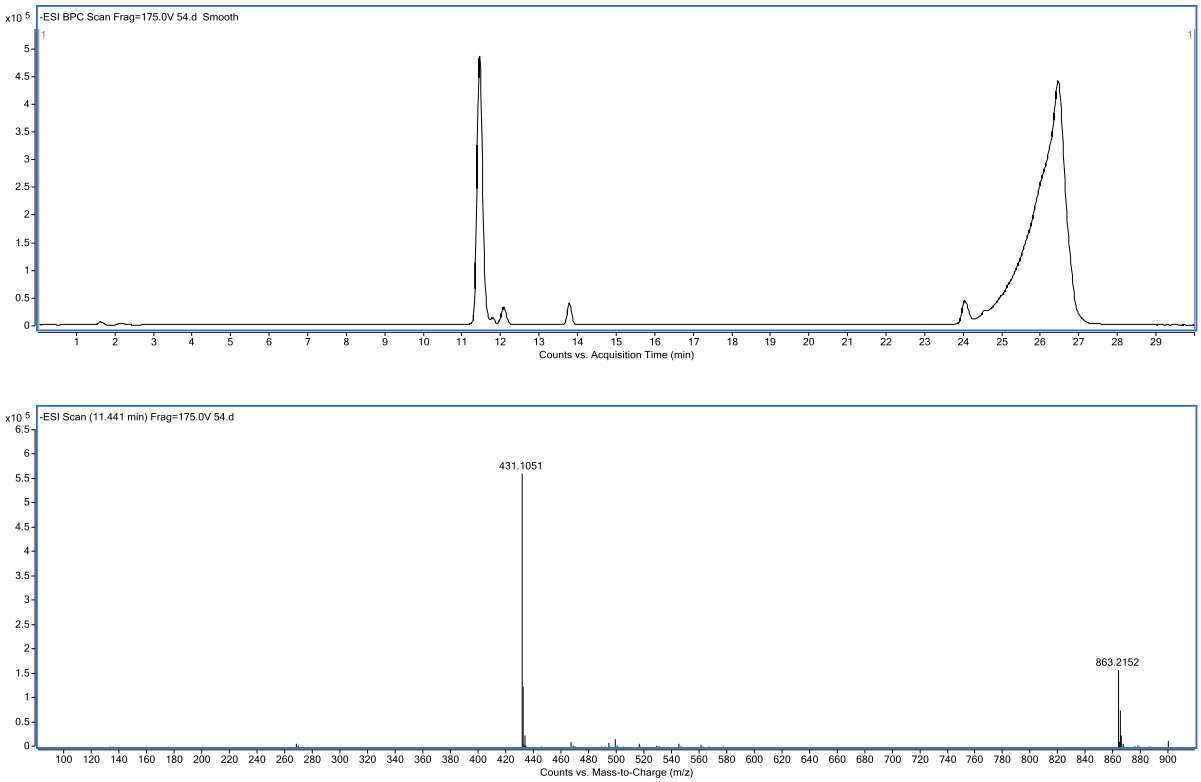
S7: APT, DEPT-90 NMR (100 MHz, DMSO-*d6*) Spectrum of compound **2** **(**apigenin 7-*O*-glucoside)



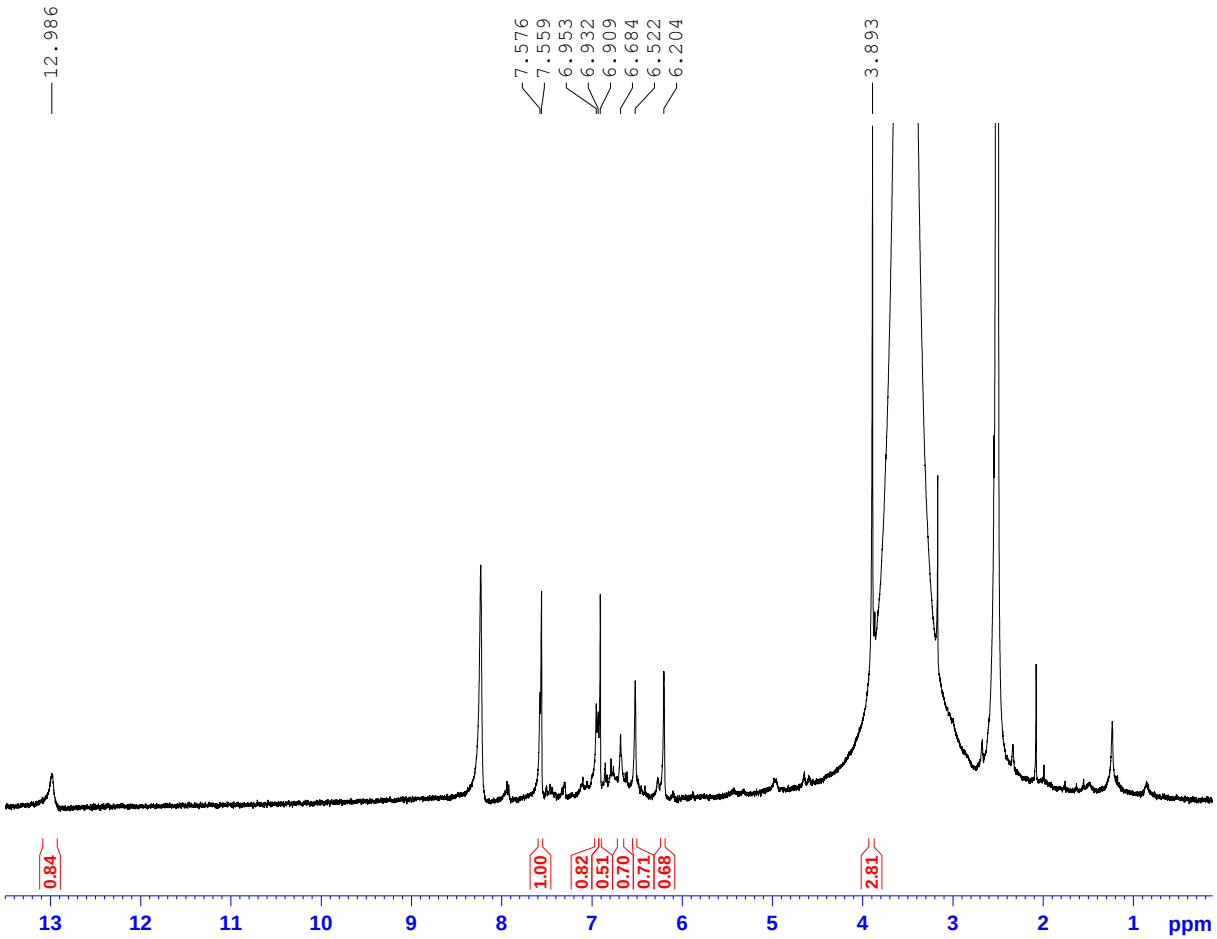
S8: HETCOR (400 MHz, DMSO-*d6*) Spectrum of compound **2 (**apigenin 7-*O*-glucoside)



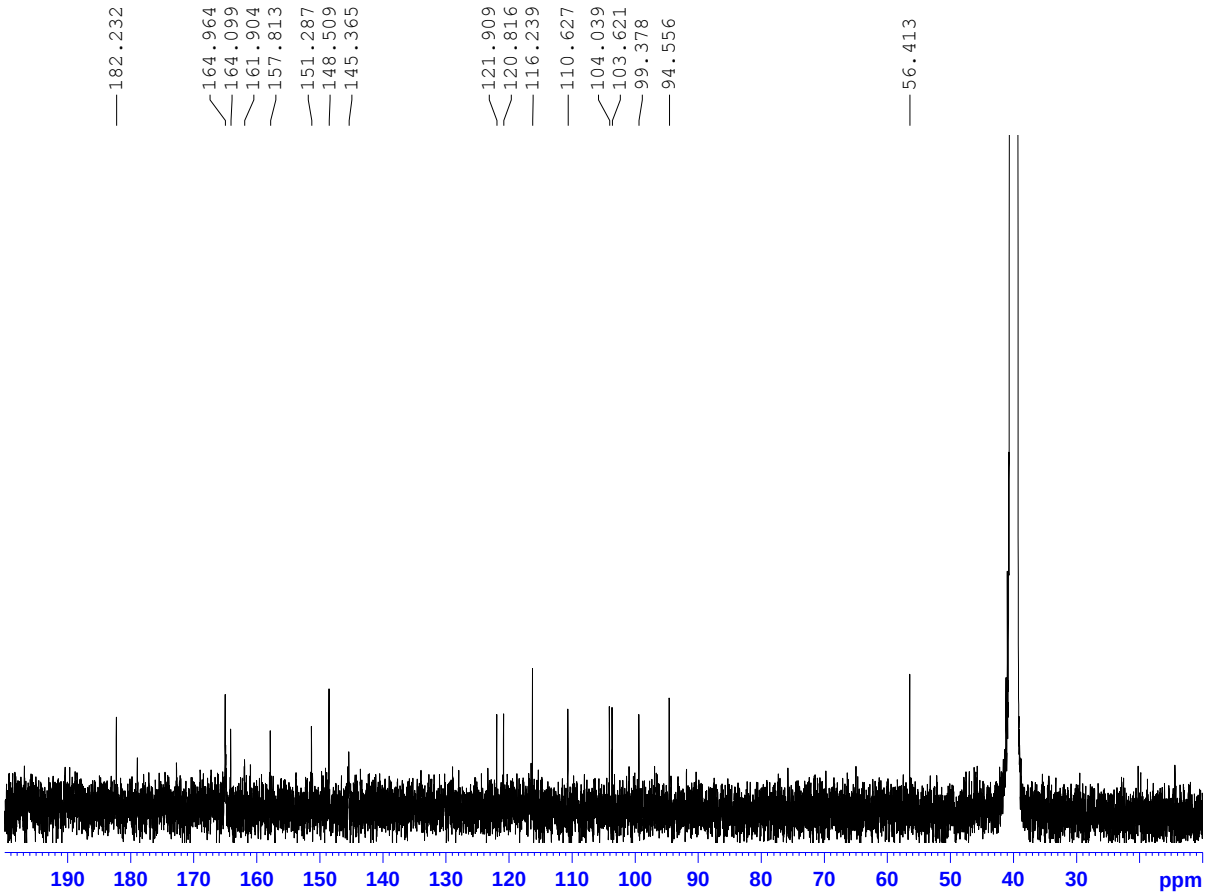
S9: COSY-90 (400 MHz, DMSO-*d6*) Spectrum of compound **2** (apigenin 7-*O*-glucoside)



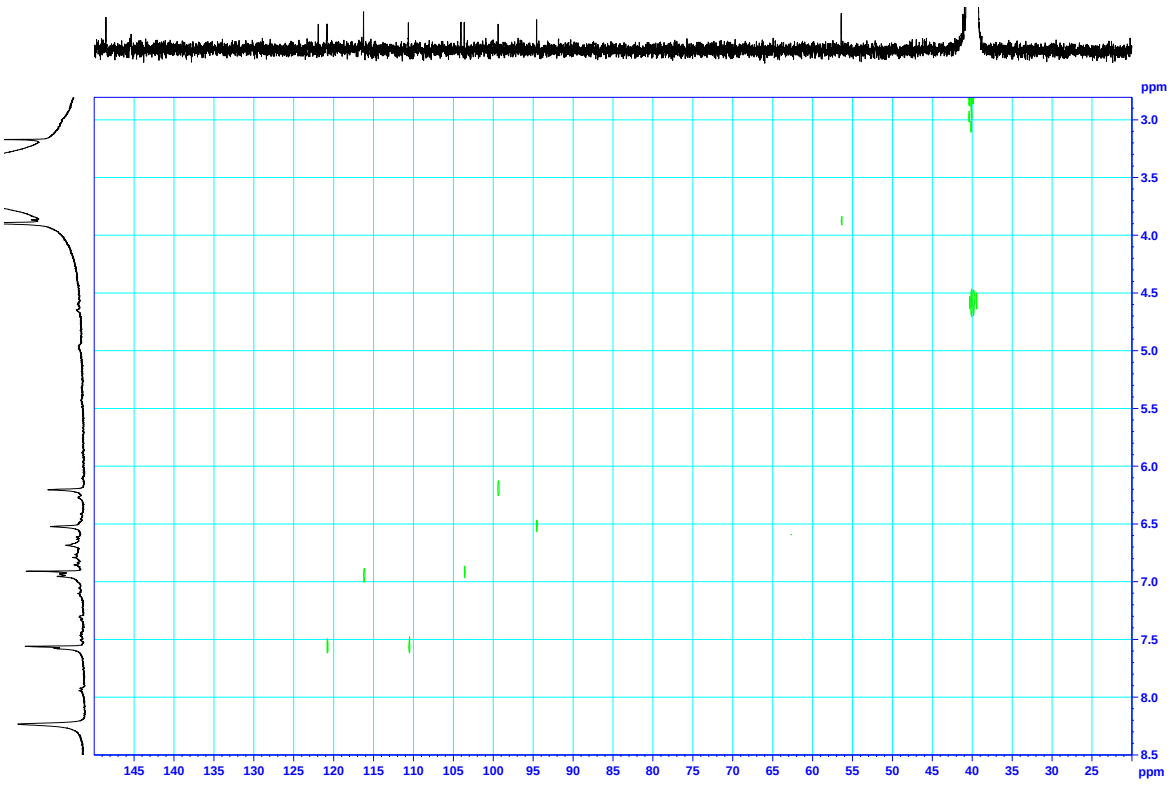
S10: HPLC-QTOF Spectrum of compound **2** (apigenin 7-*O*-glucoside)



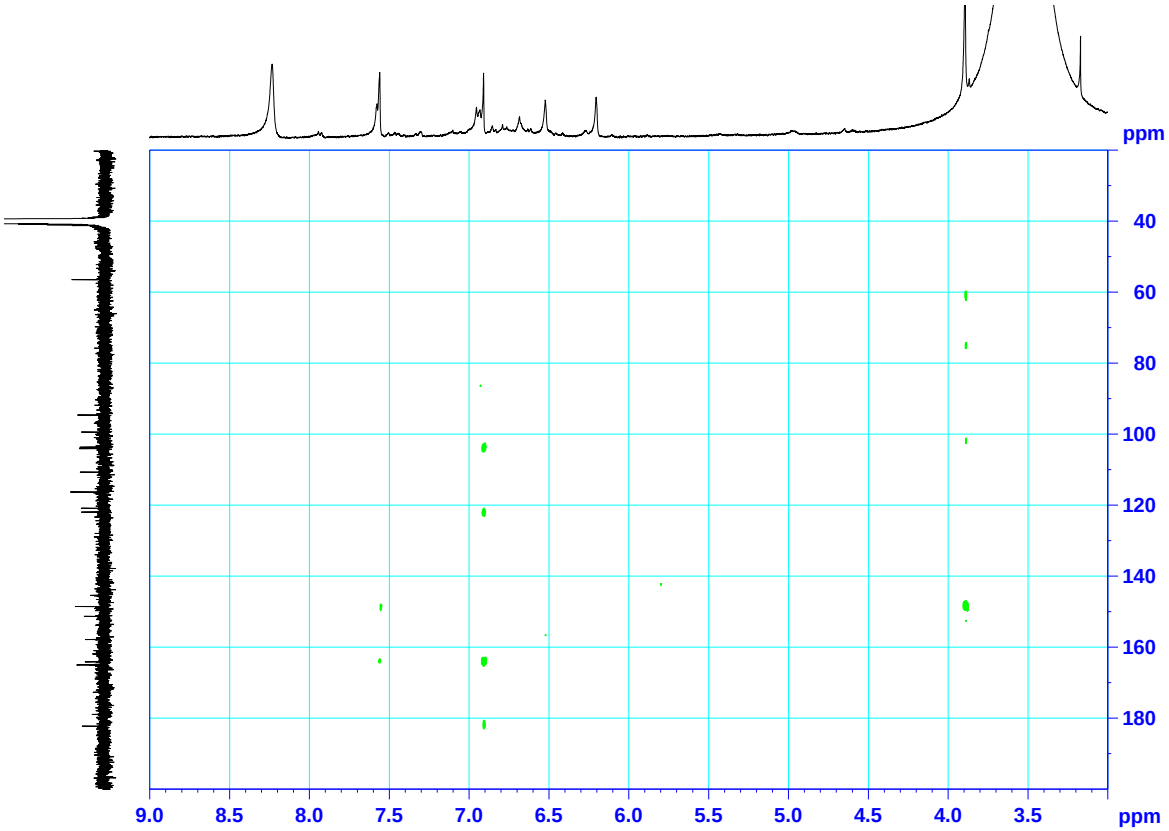
S11: ^{1}H-NMR (400 MHz, DMSO-*d6*) Spectrum of Compound **3** (chrysoeriol)



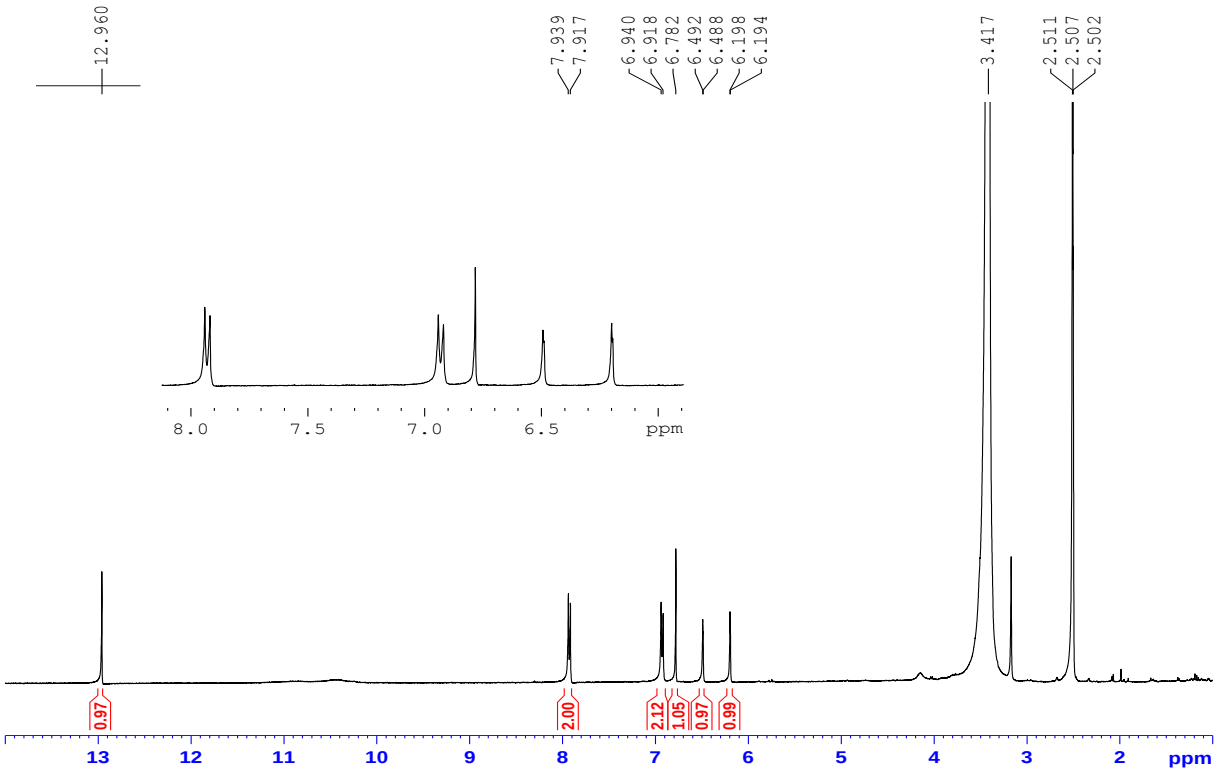
S12: ^{13}C-NMR (100 MHz, DMSO-*d6*) Spectrum of Compound **3** (chrysoeriol)



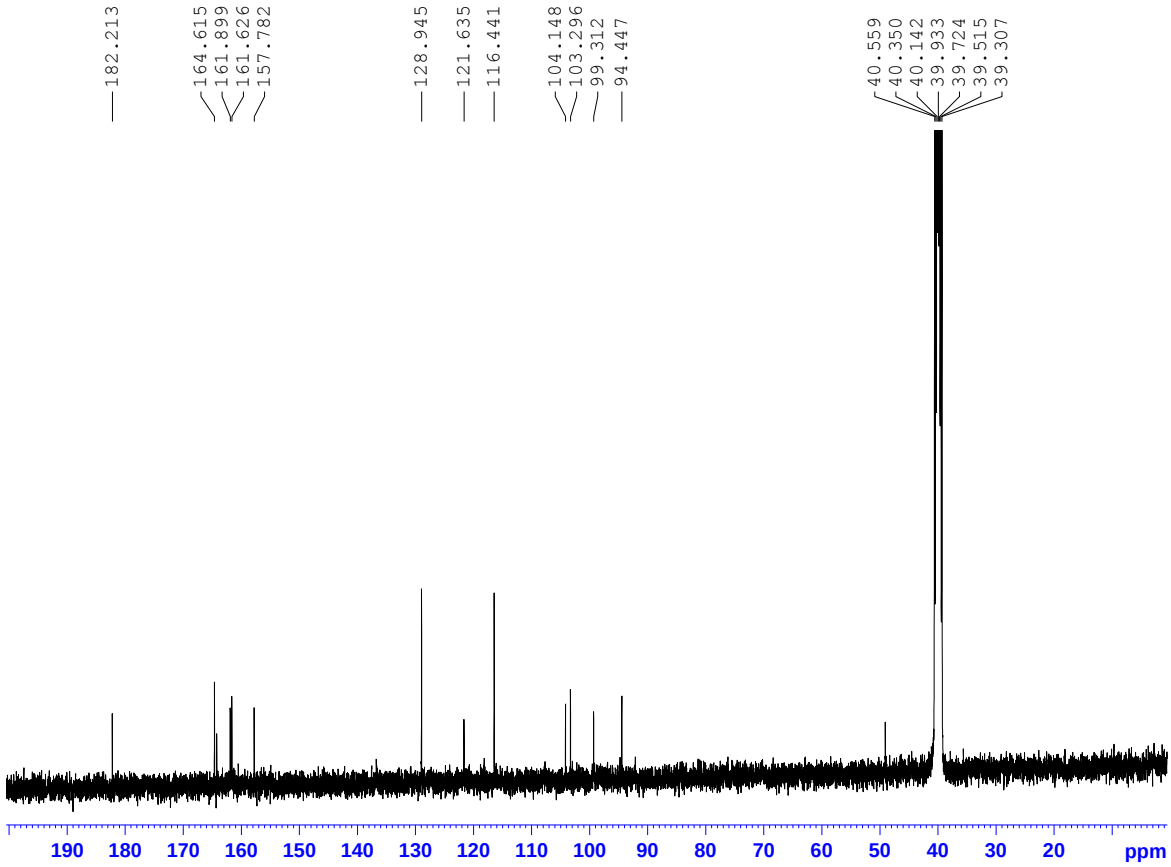
S13: HETCOR (400 MHz, DMSO-*d6*) Spectrum of compound **3 (**chrysoeriol)



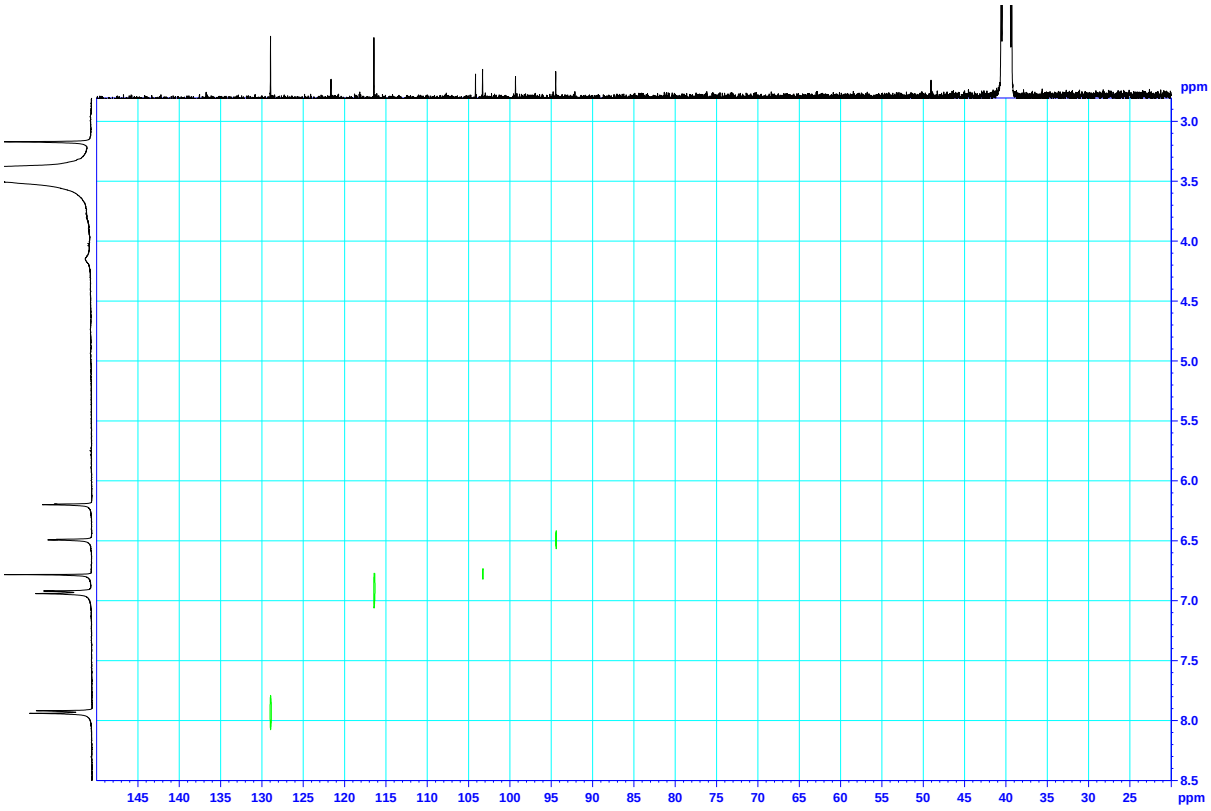
S14: HMBC (400 MHz, DMSO-*d6*) Spectrum of compound **3 (**chrysoeriol)



S15: ^{1}H-NMR (400 MHz, DMSO-*d6*) Spectrum of Compound **4** (apigenin)



S16: ^{13}C-NMR (100 MHz, DMSO-*d6*) Spectrum of Compound **4** (apigenin)



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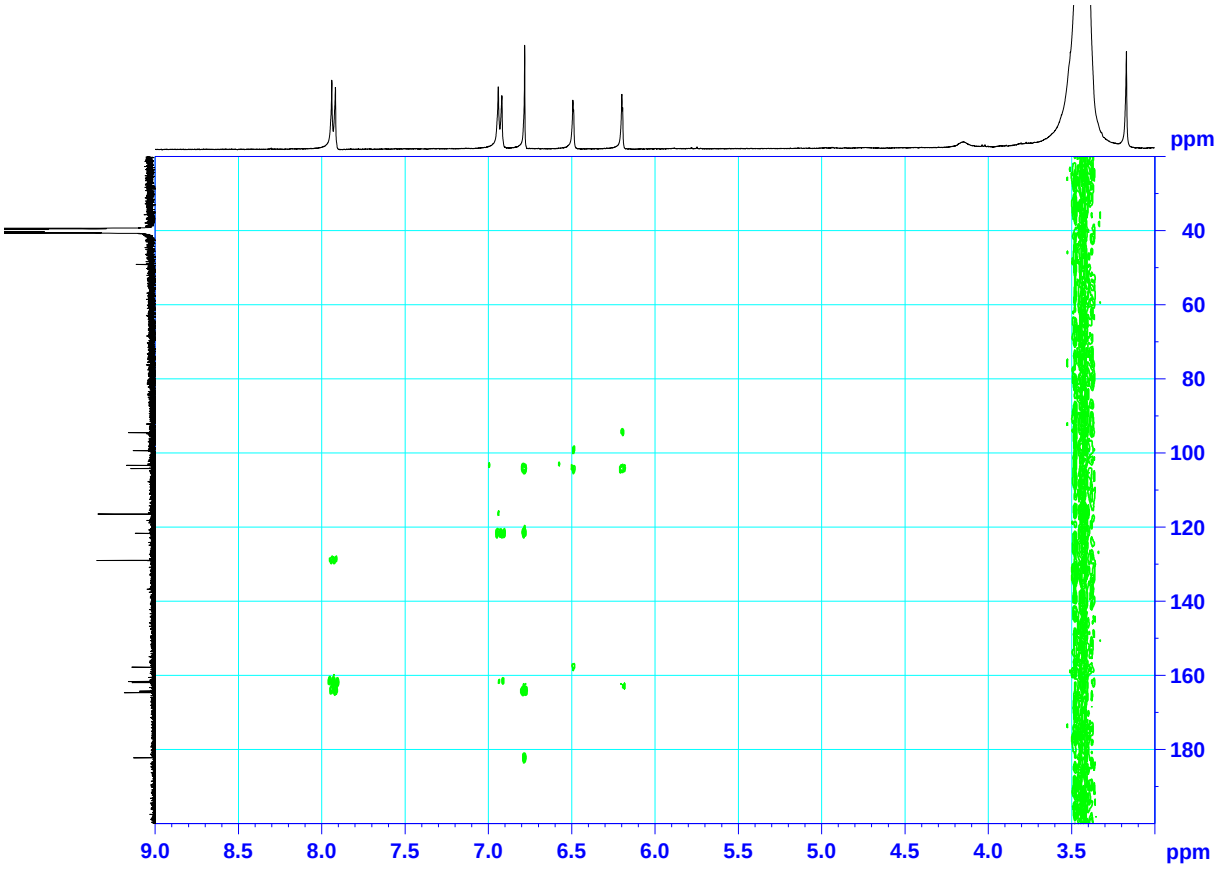
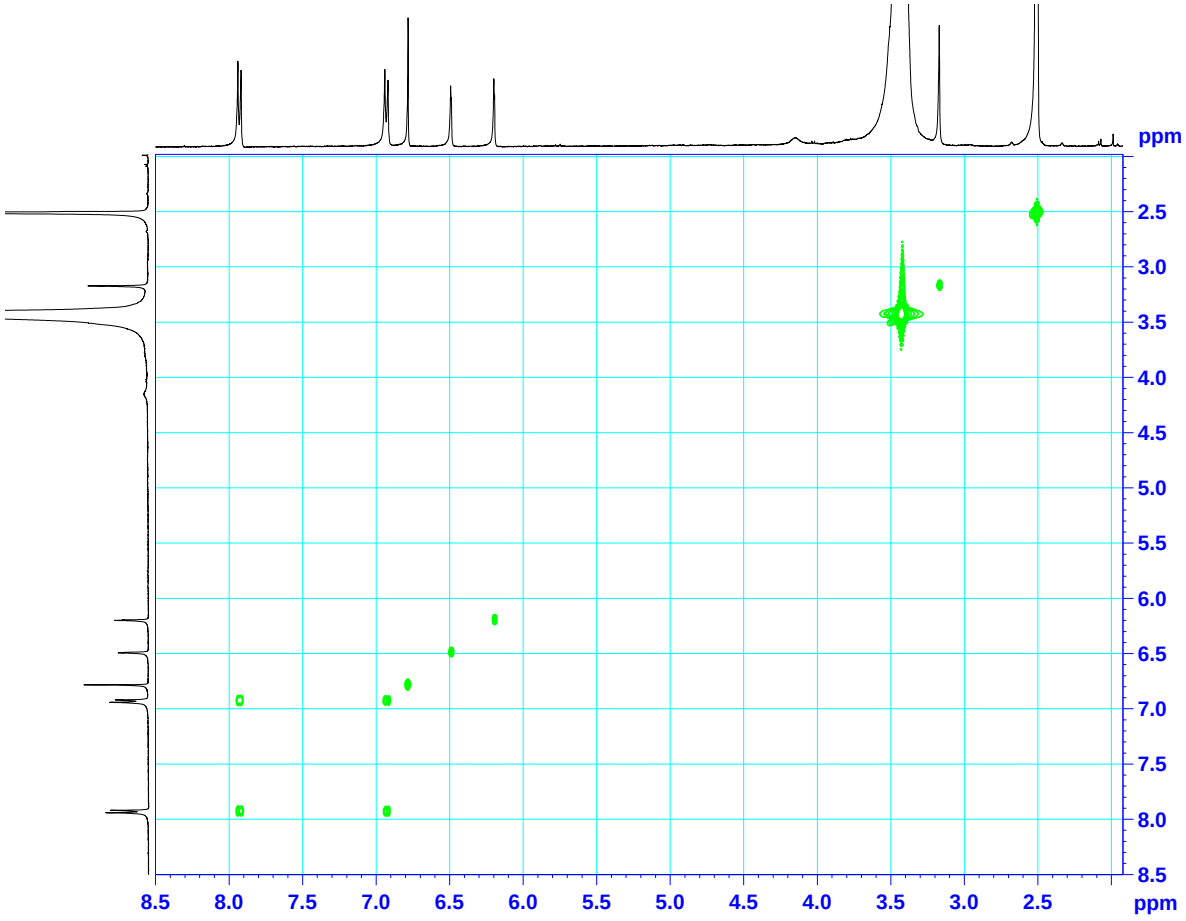
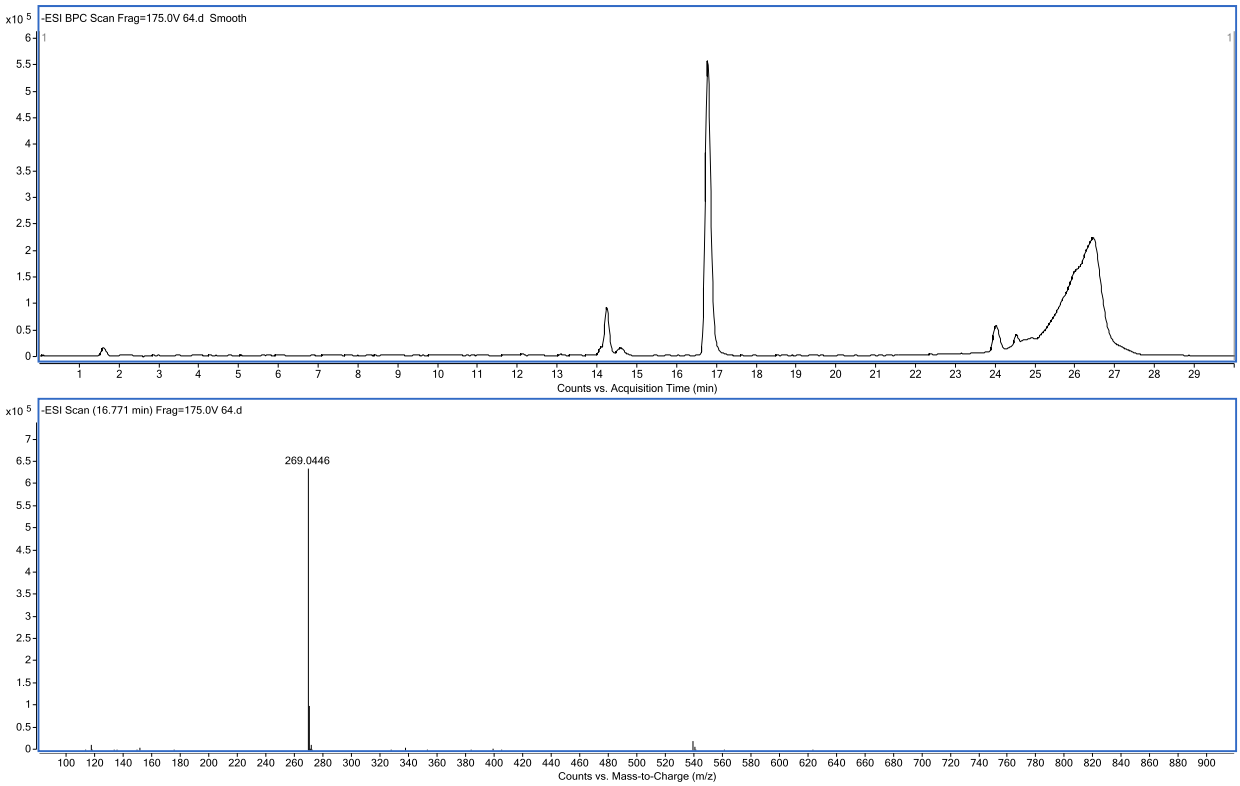


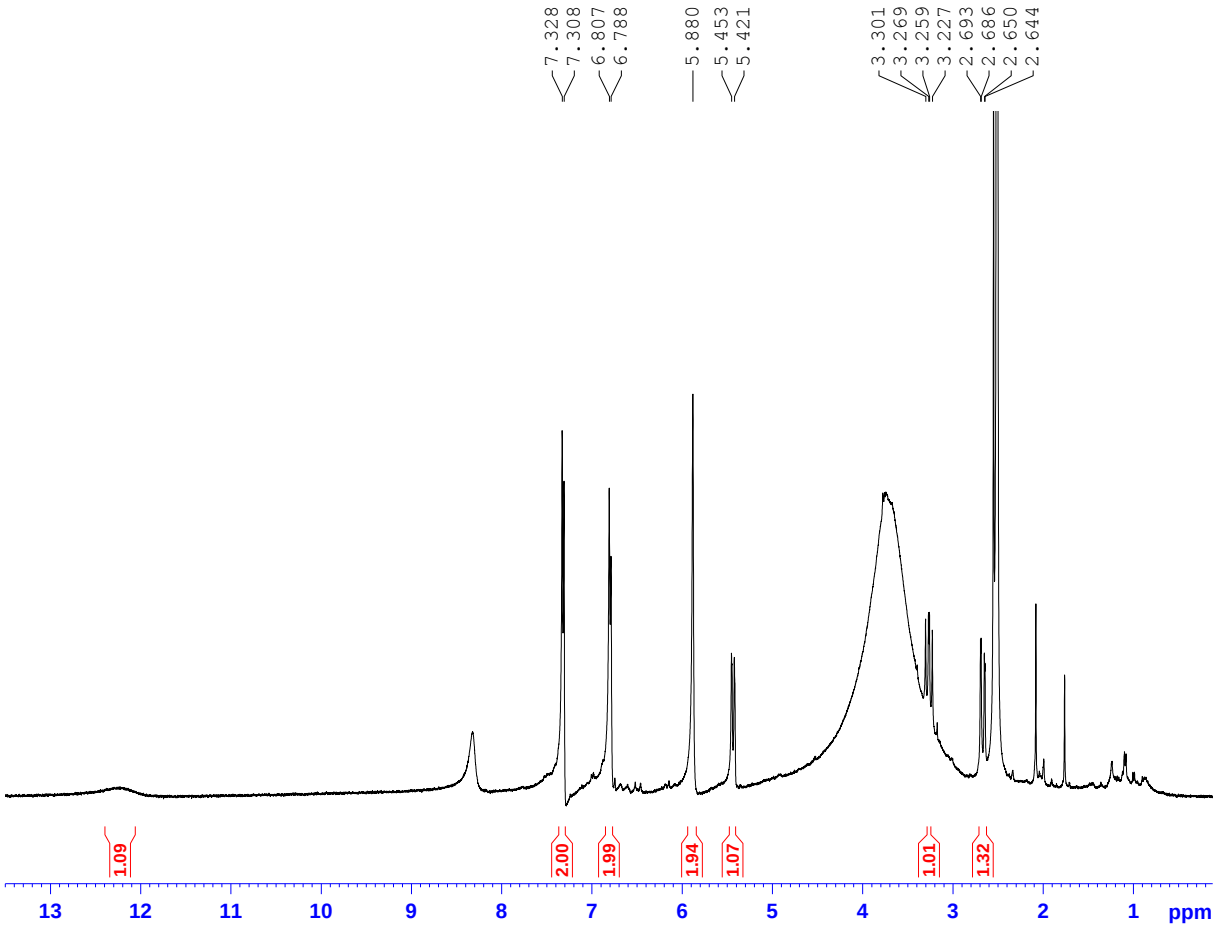
S18: HMBC (400 MHz, DMSO-*d6*) Spectrum of compound **4 (**Apigenin)



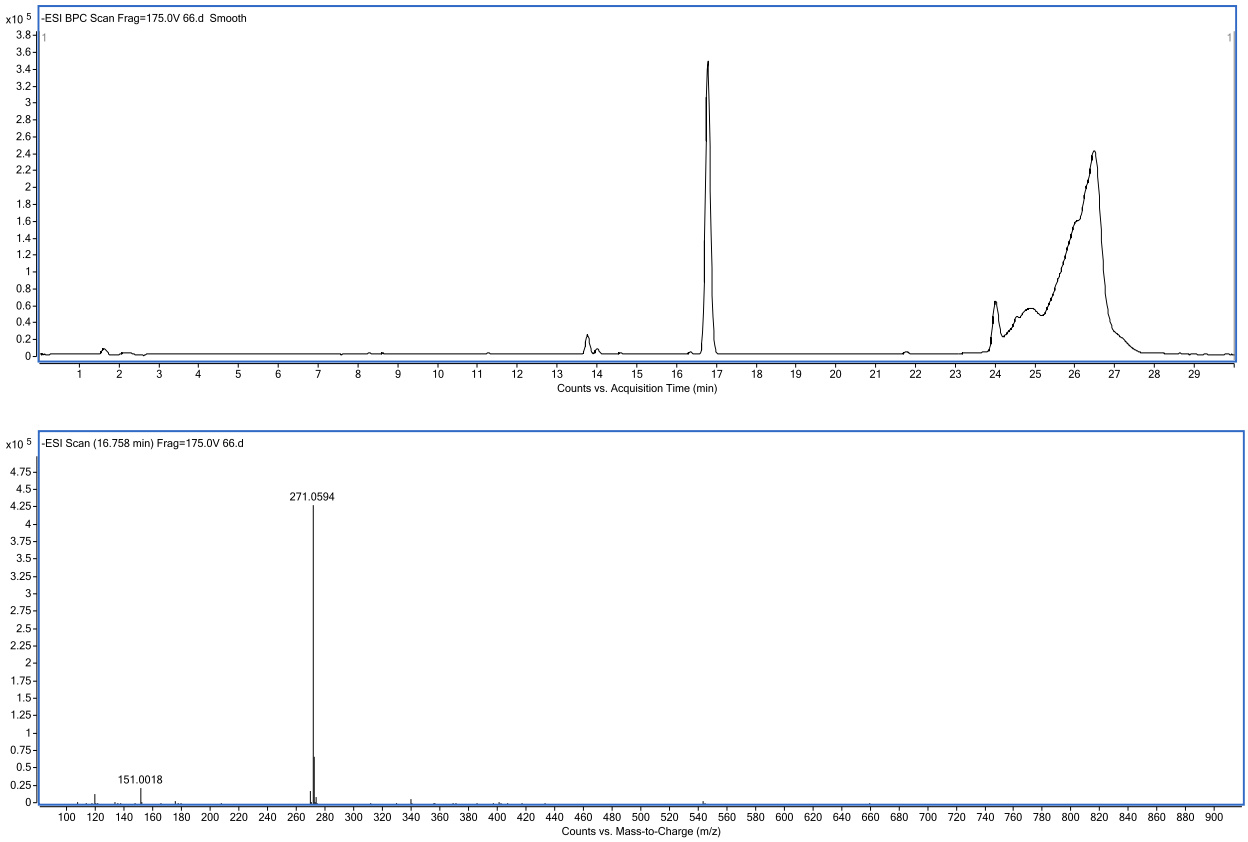
S19: COSY-90 (400 MHz, DMSO-*d6*) Spectrum of compound **4** (apigenin)



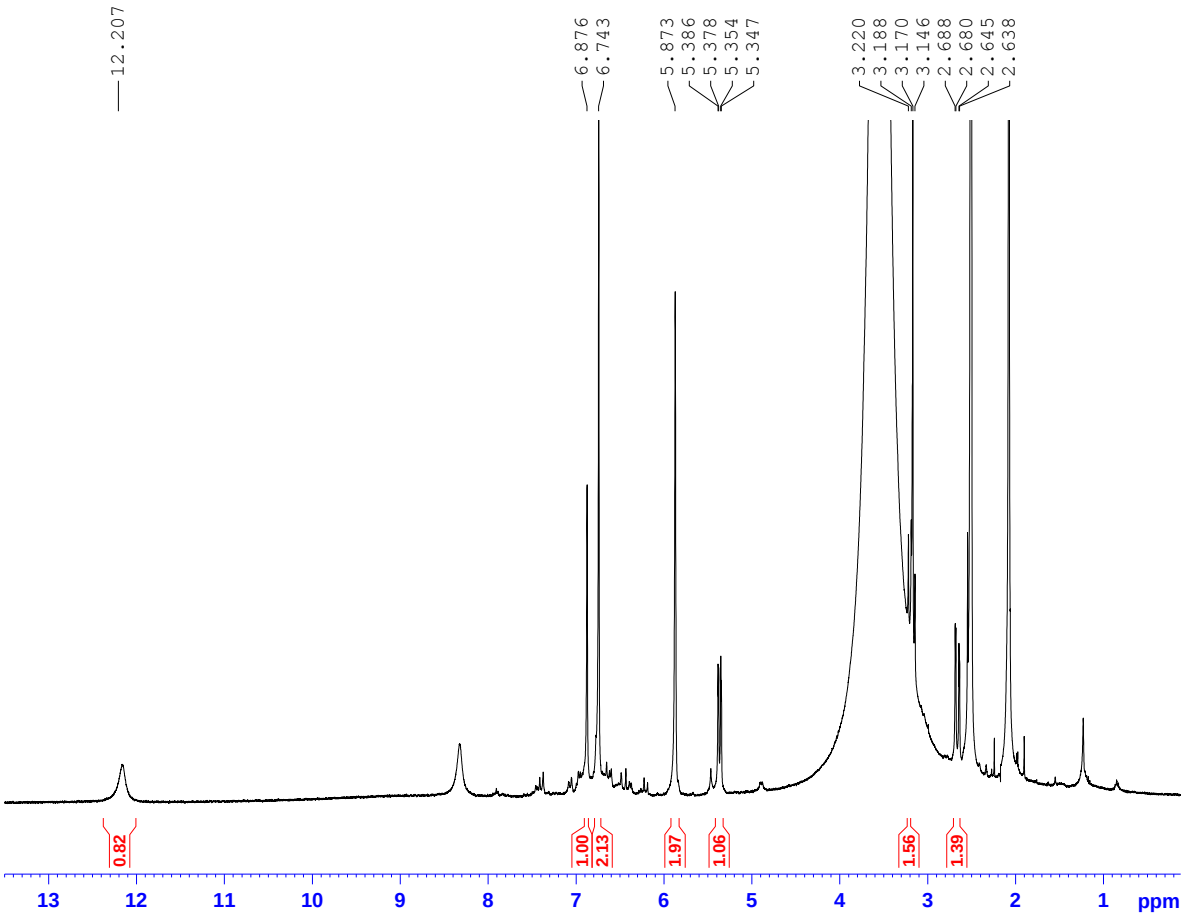
S20: HPLC-QTOF Spectrum of compound **4** (apigenin)



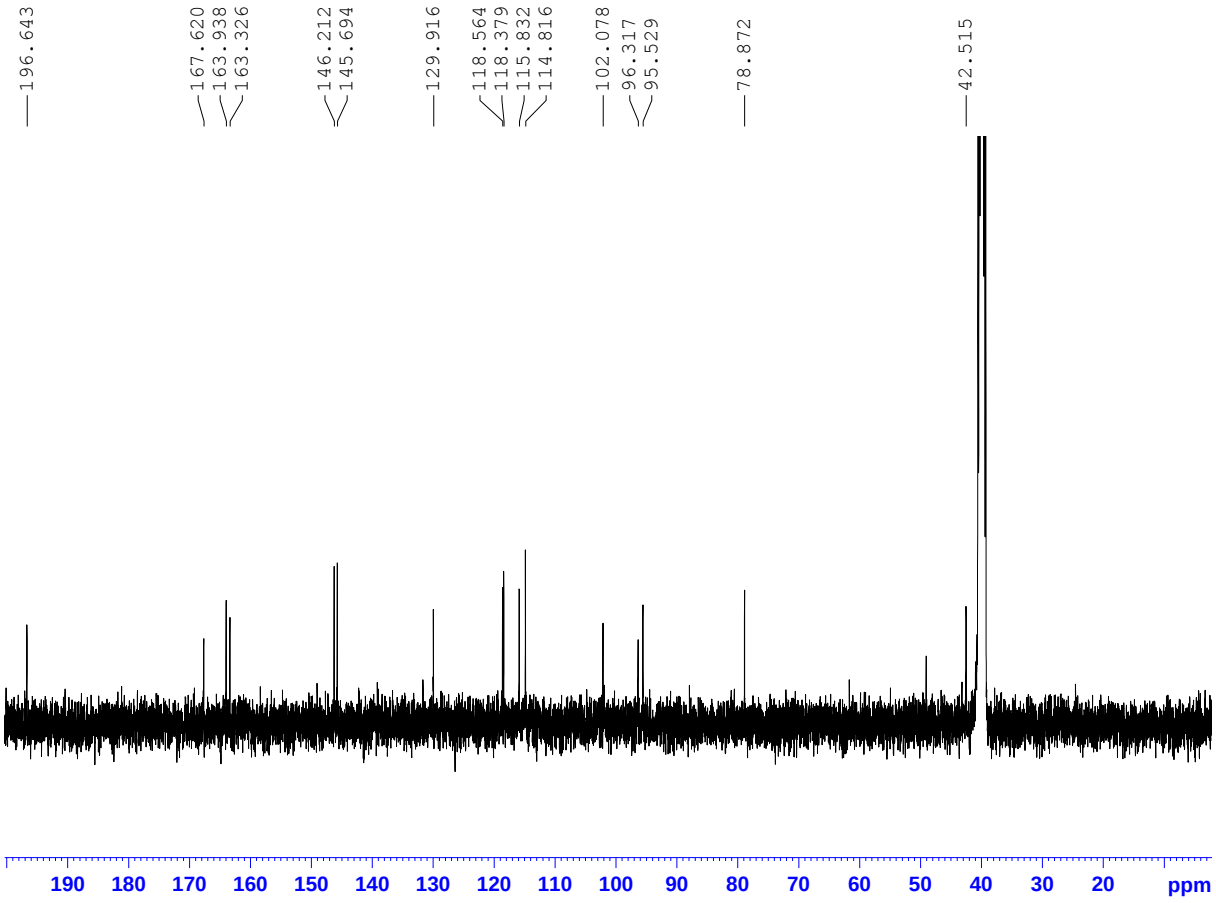
S21: ^{1}H-NMR (400 MHz, DMSO-*d6*) Spectrum of Compound **5** (naringenin)



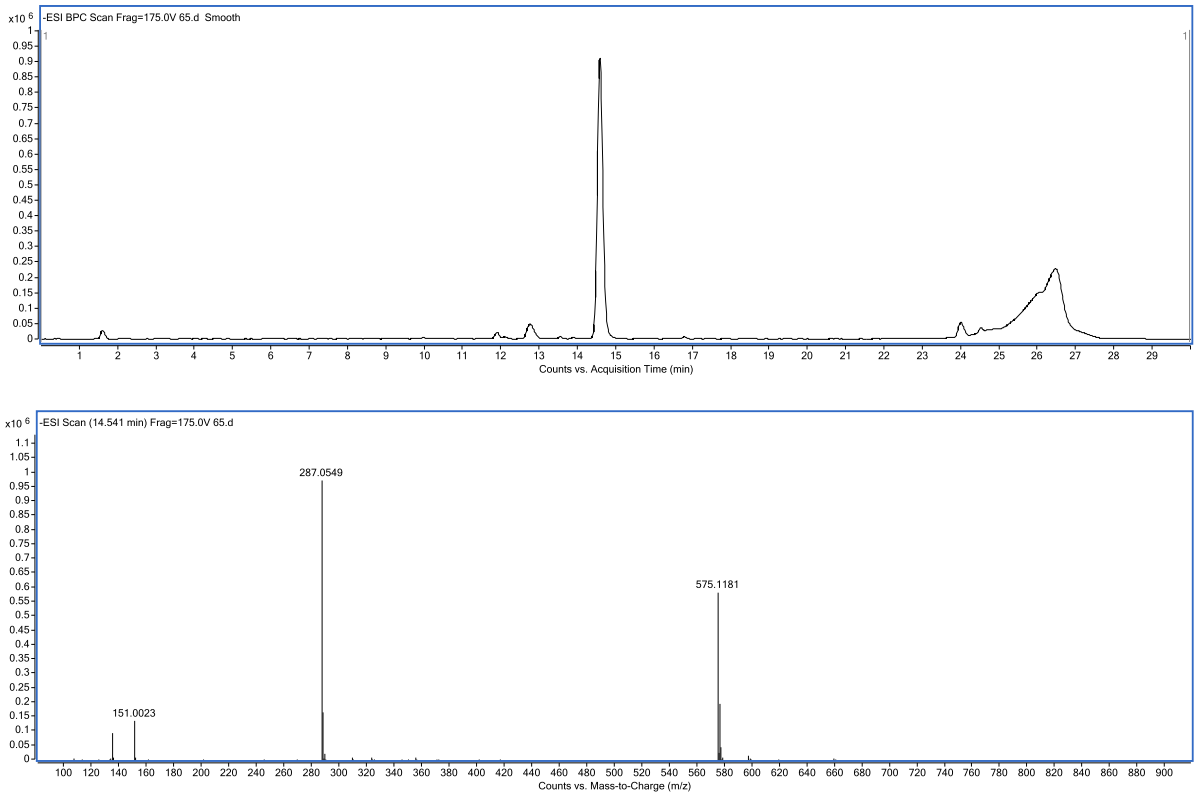
S22: HPLC-QTOF Spectrum of compound **5** (naringenin)



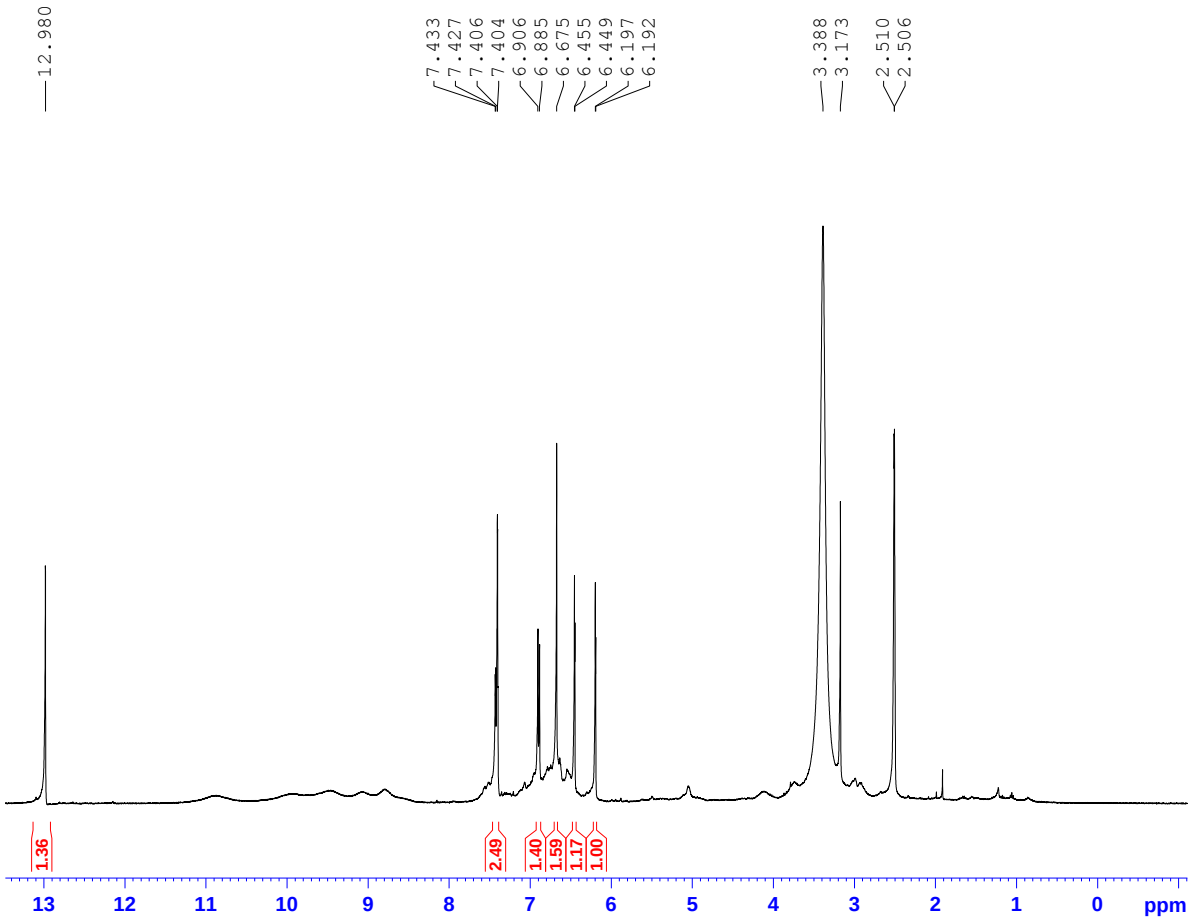
S23: ^{1}H-NMR (400 MHz, DMSO-*d6*) Spectrum of Compound **6** (eriodictyol)



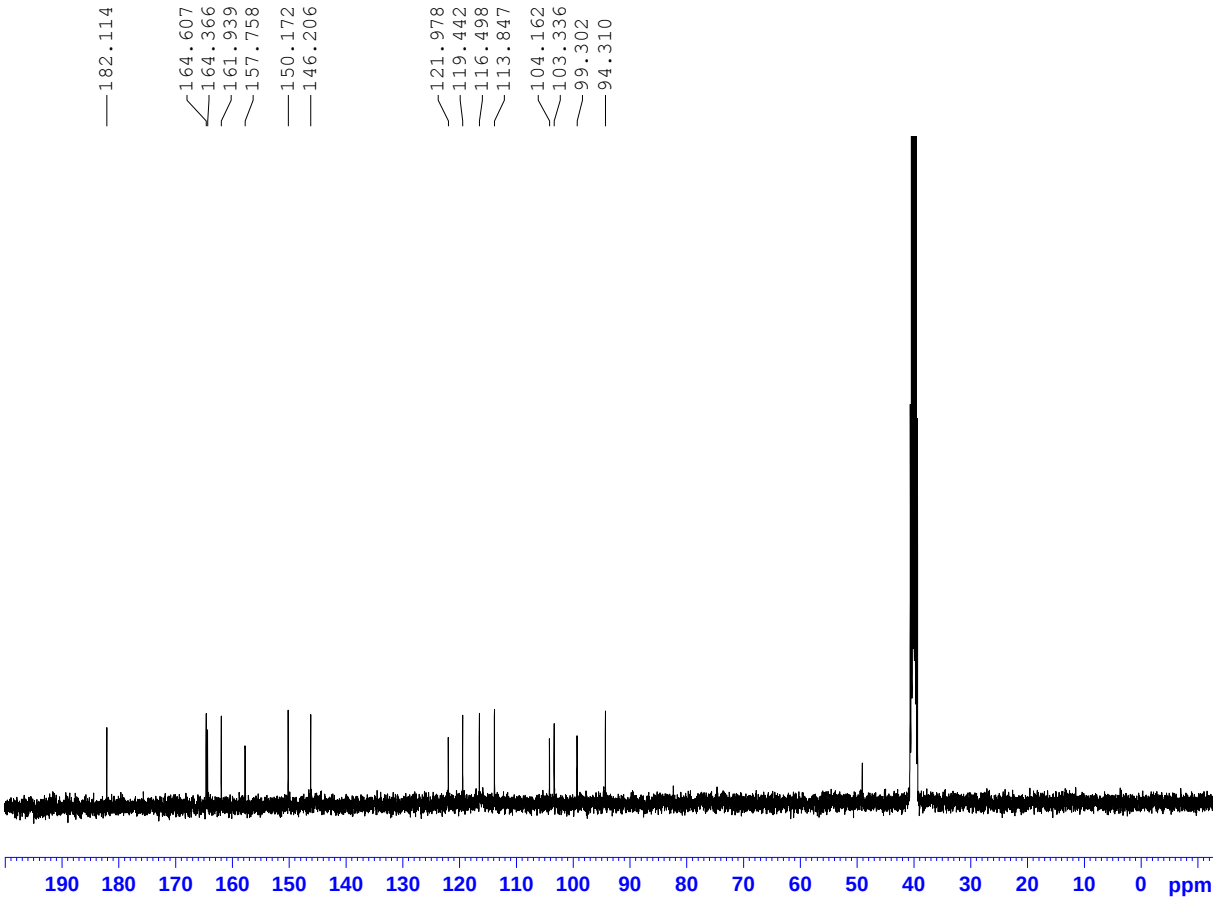
S24: ^{13}C-NMR (100 MHz, DMSO-*d6*) Spectrum of Compound **6** (eriodictyol)



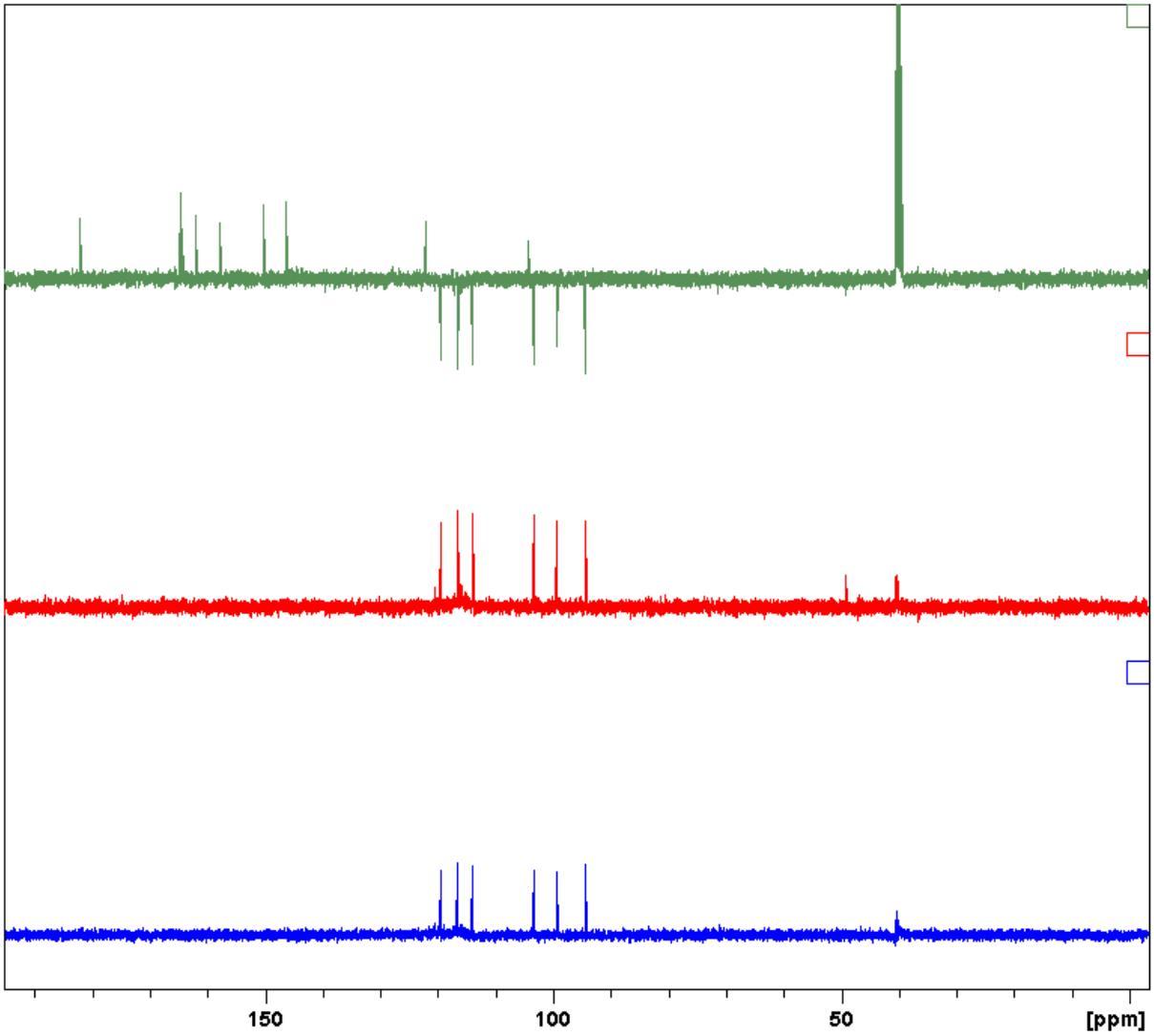
S25: HPLC-QTOF Spectrum of compound **6** (eriodictyol)



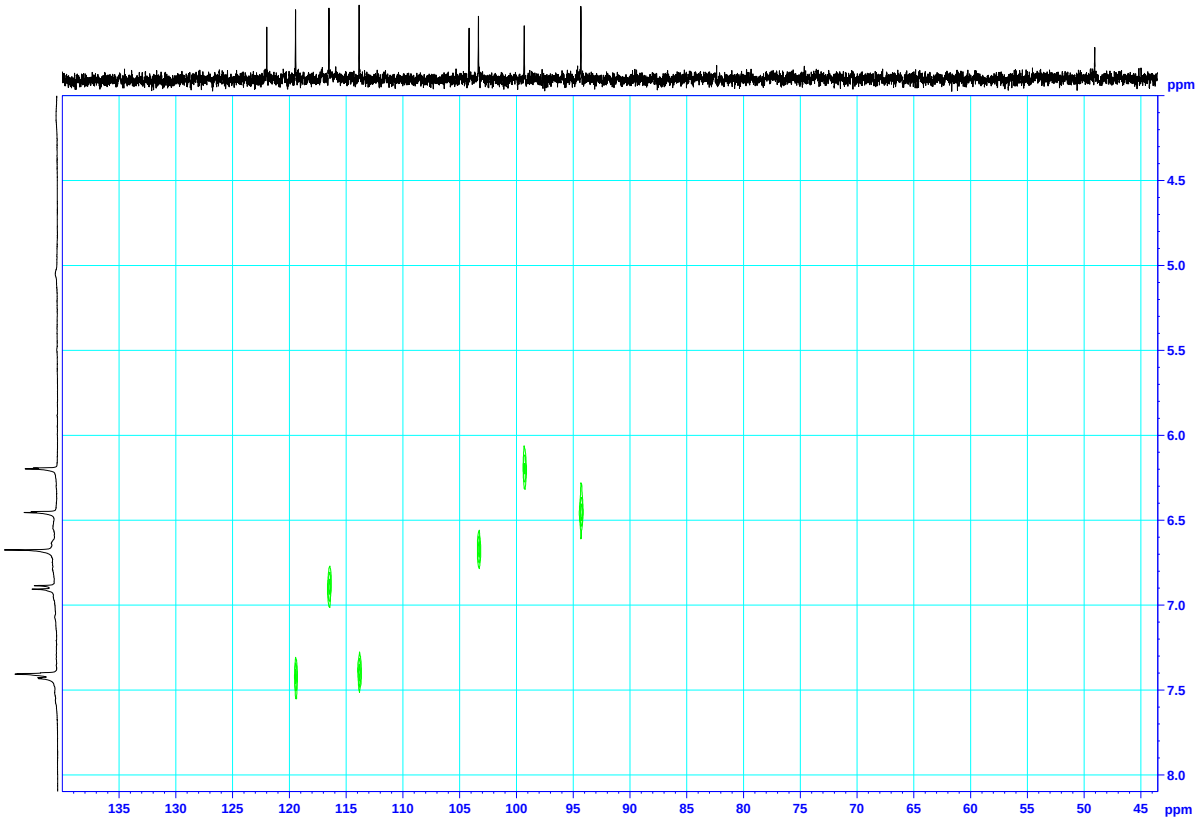
S26: ^{13}C-NMR (100 MHz, DMSO-*d6*) Spectrum of Compound **7** (luteolin)



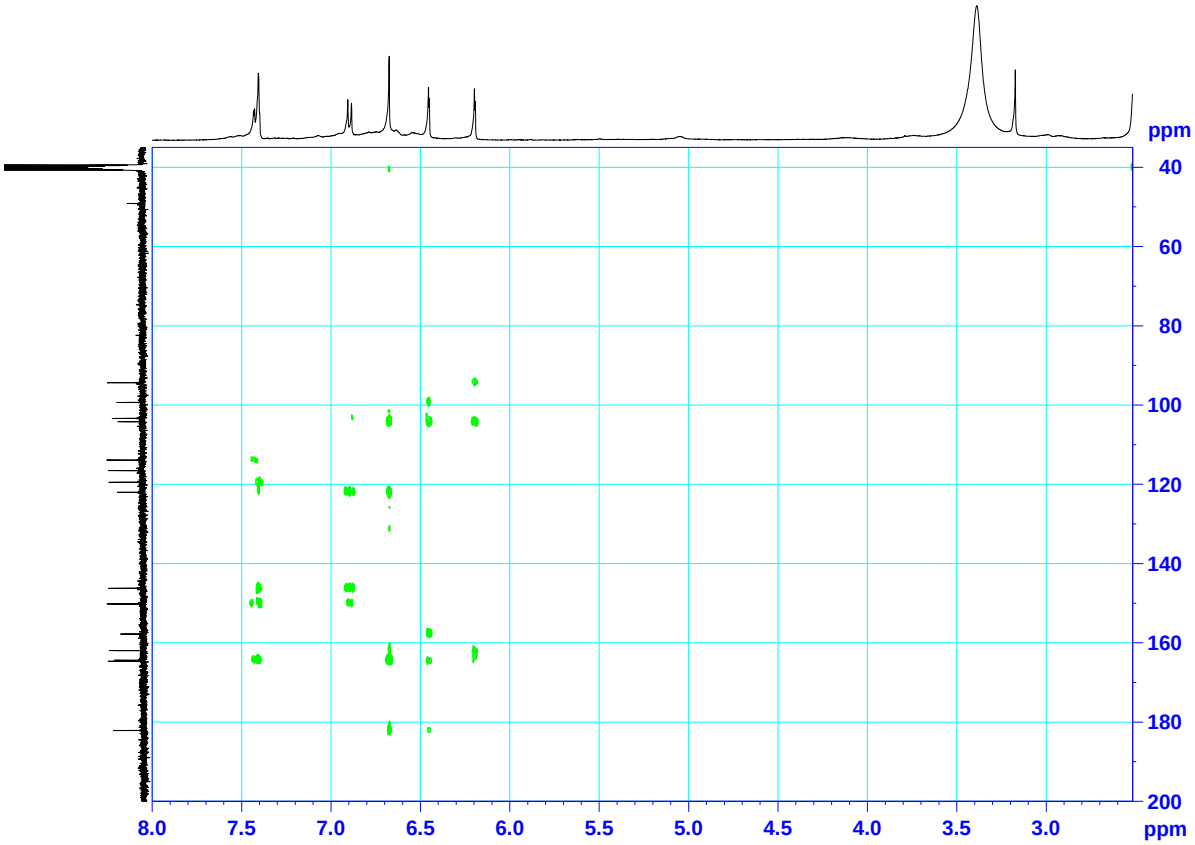
S27: ^{13}C-NMR (100 MHz, DMSO-*d6*) Spectrum of Compound **7** (luteolin)



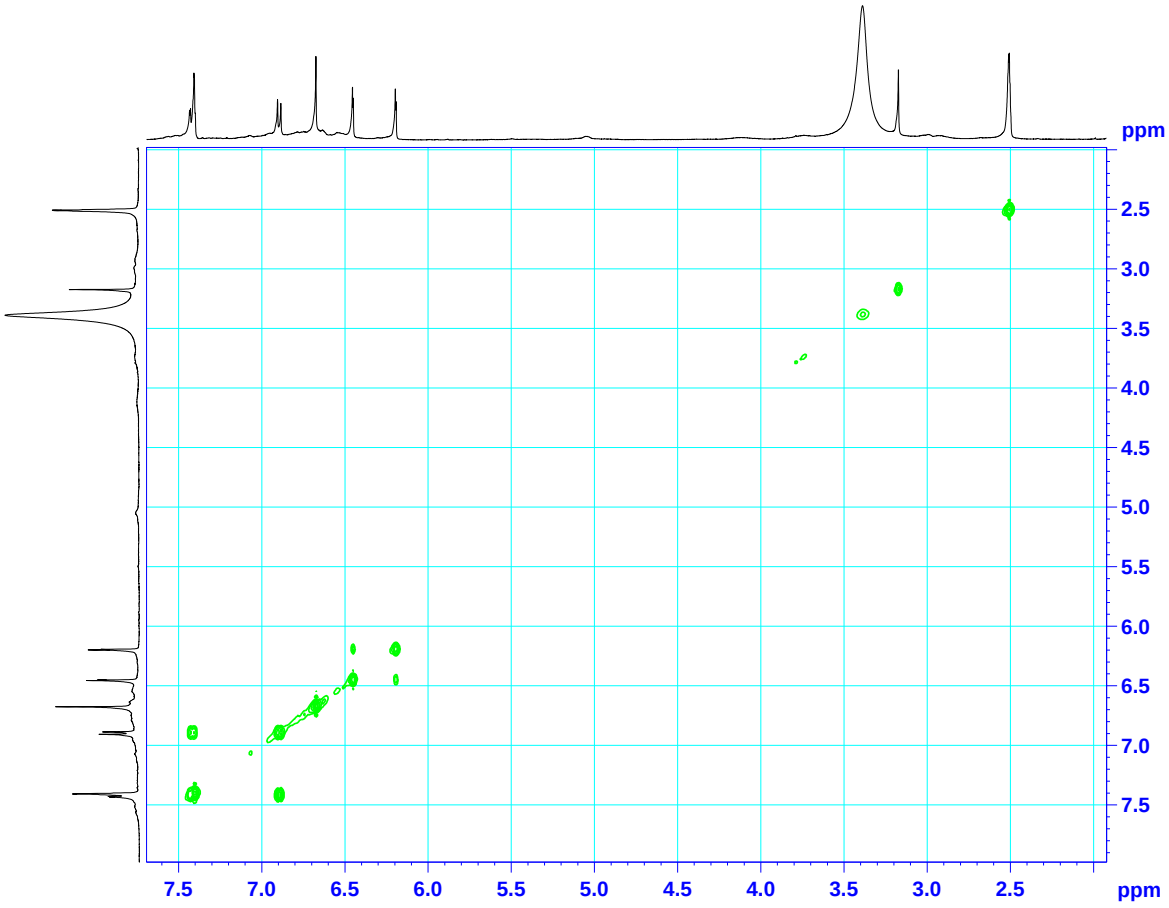
S28: APT, DEPT-90 NMR (100 MHz, DMSO-*d6*) Spectrum of compound **7** **(**luteolin)



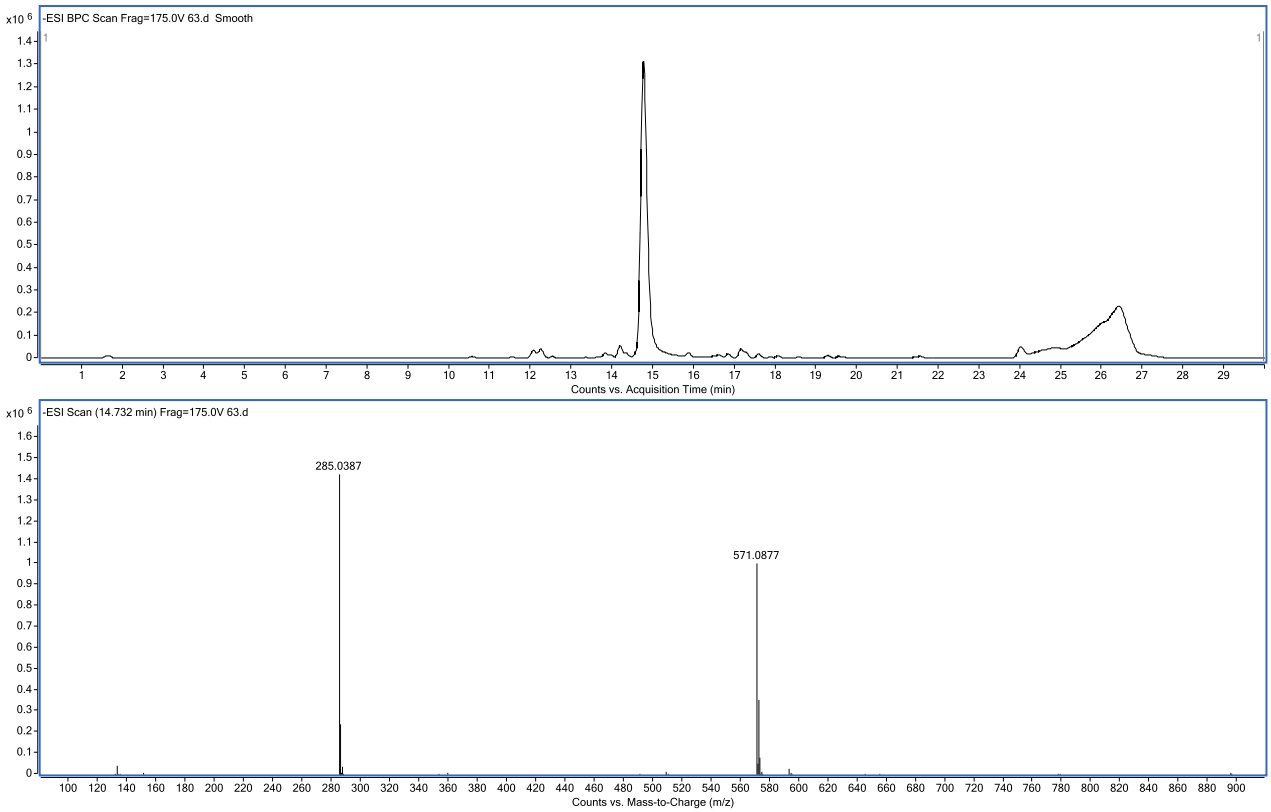
S29: HETCOR (400 MHz, DMSO-*d6*) Spectrum of compound **7 (**luteolin)



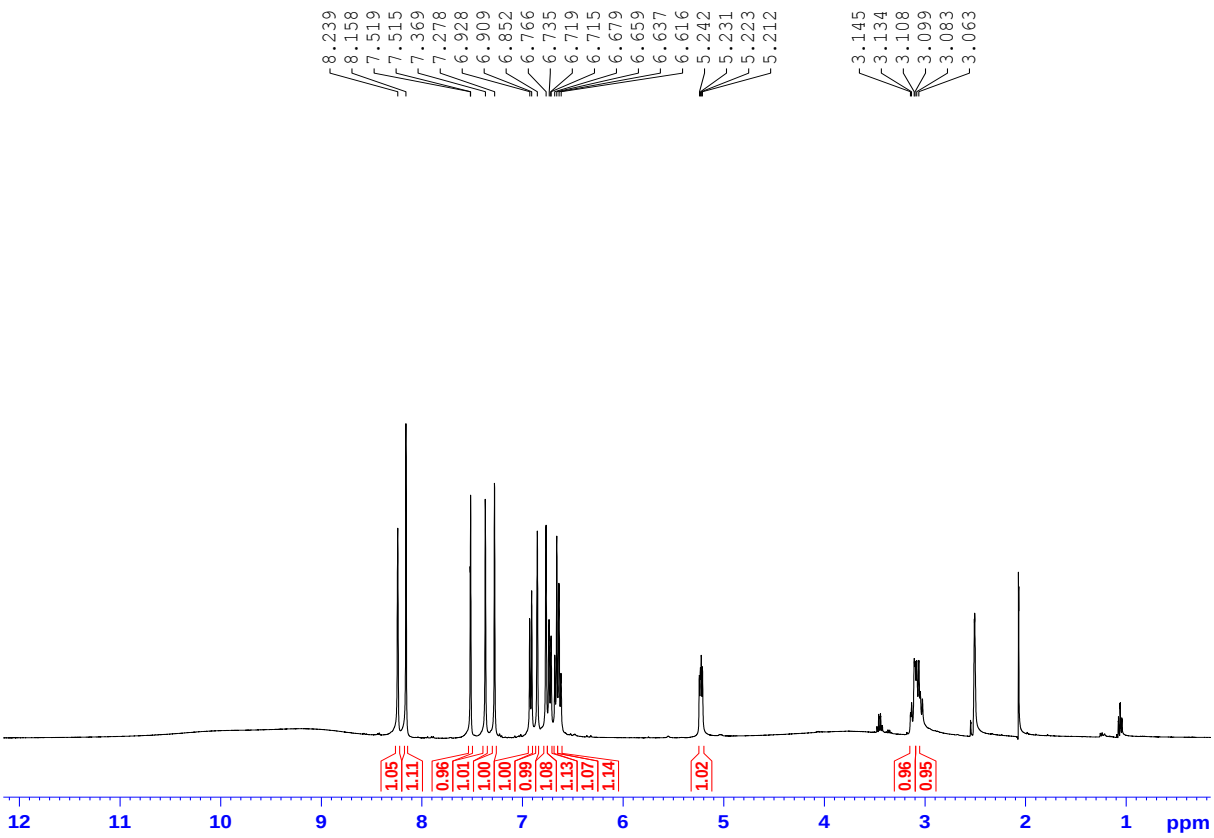
S30: HMBC (400 MHz, DMSO-*d6*) Spectrum of compound **7** (luteolin)



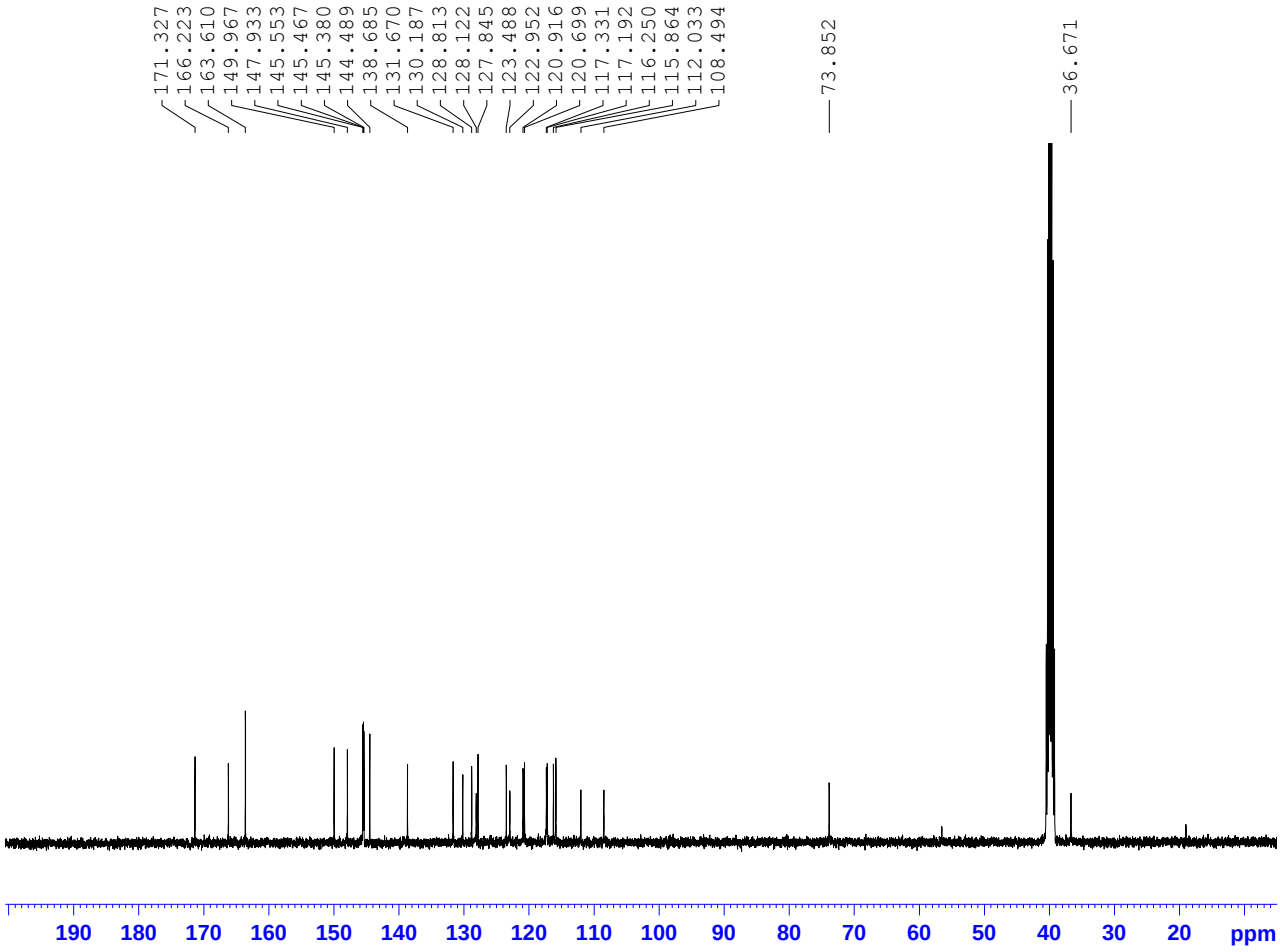
S31: COSY-90 (400 MHz, DMSO-*d6*) Spectrum of compound **7** (luteolin)



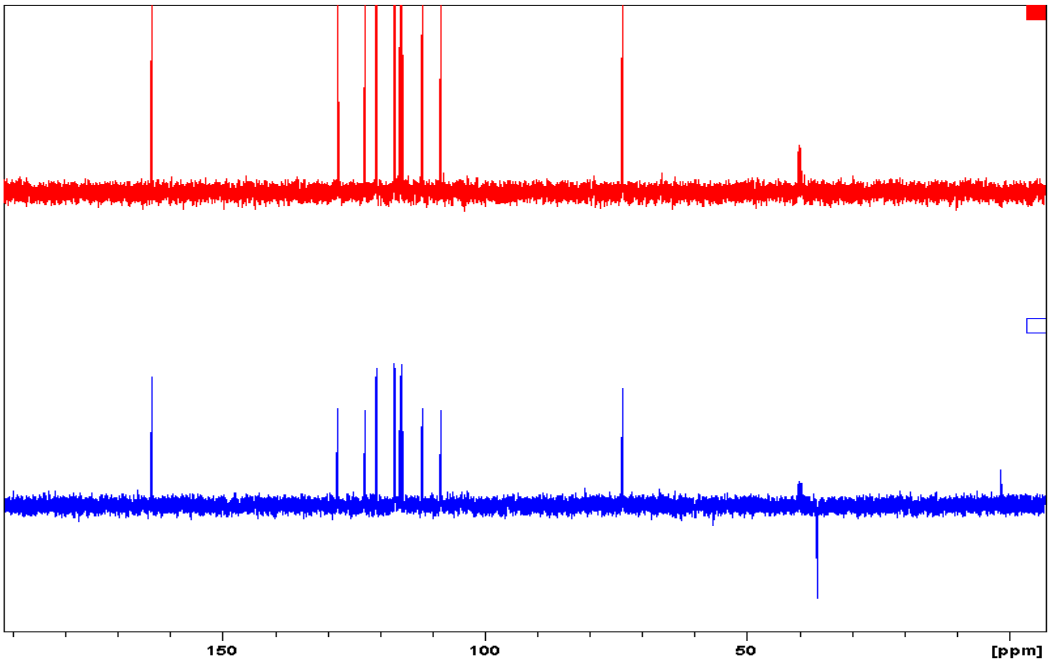
S32: HPLC-QTOF Spectrum of compound **7** (luteolin)



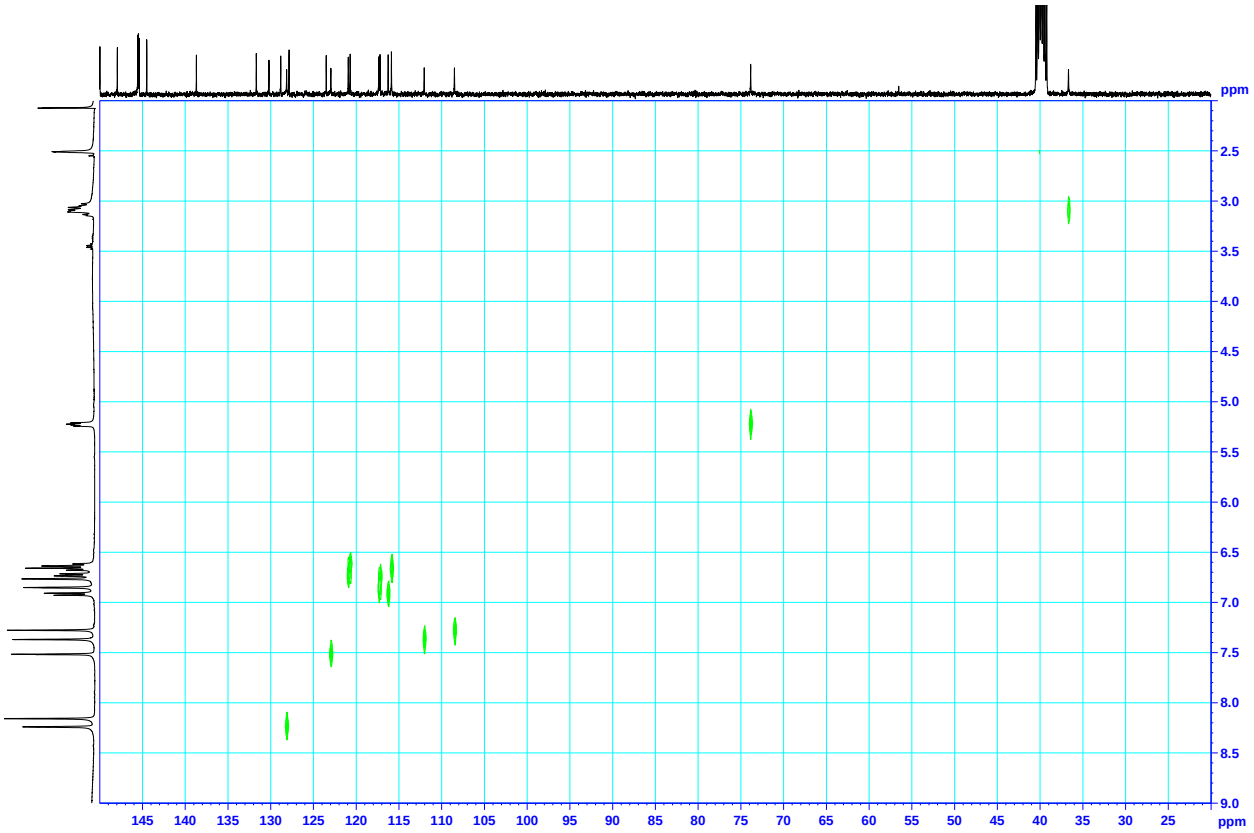
S33: ^{1}H-NMR (400 MHz, DMSO-*d6*) Spectrum of Compound **8** (globoidnan A)



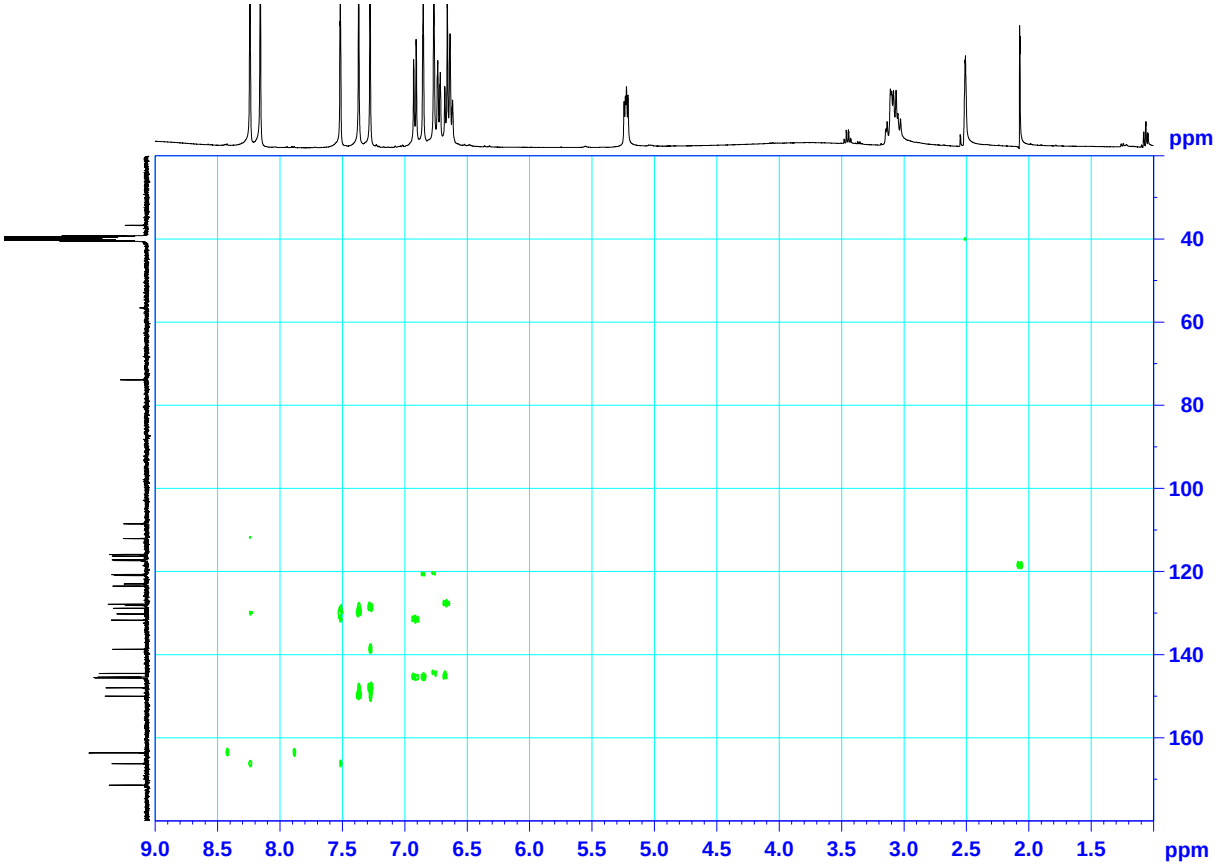
S34: ^{13}C-NMR (100 MHz, DMSO-*d6*) Spectrum of Compound **8** (globoidnan A)



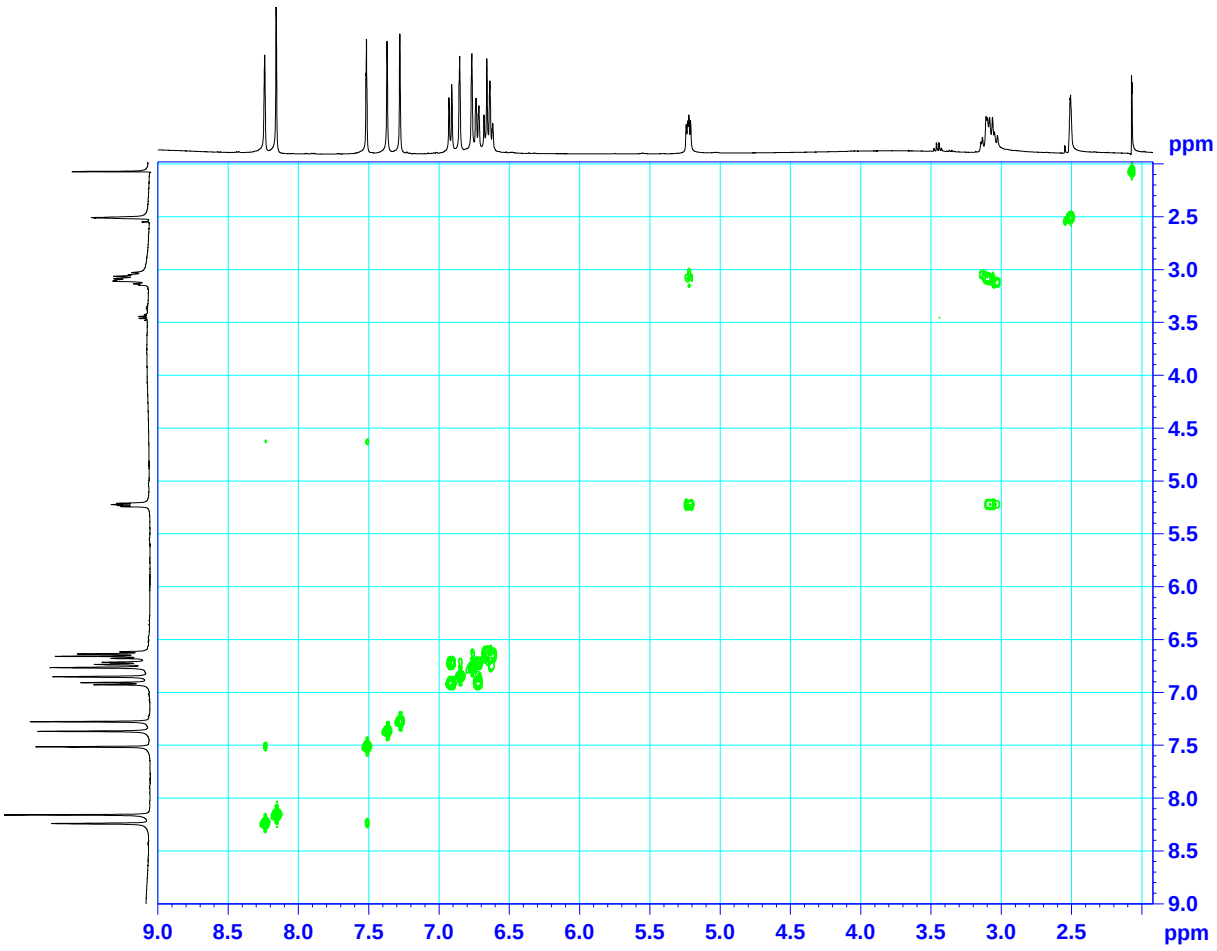
S35:, DEPT-90 NMR (100 MHz, DMSO-*d6*) Spectrum of compound **8 (**globoidnan A)



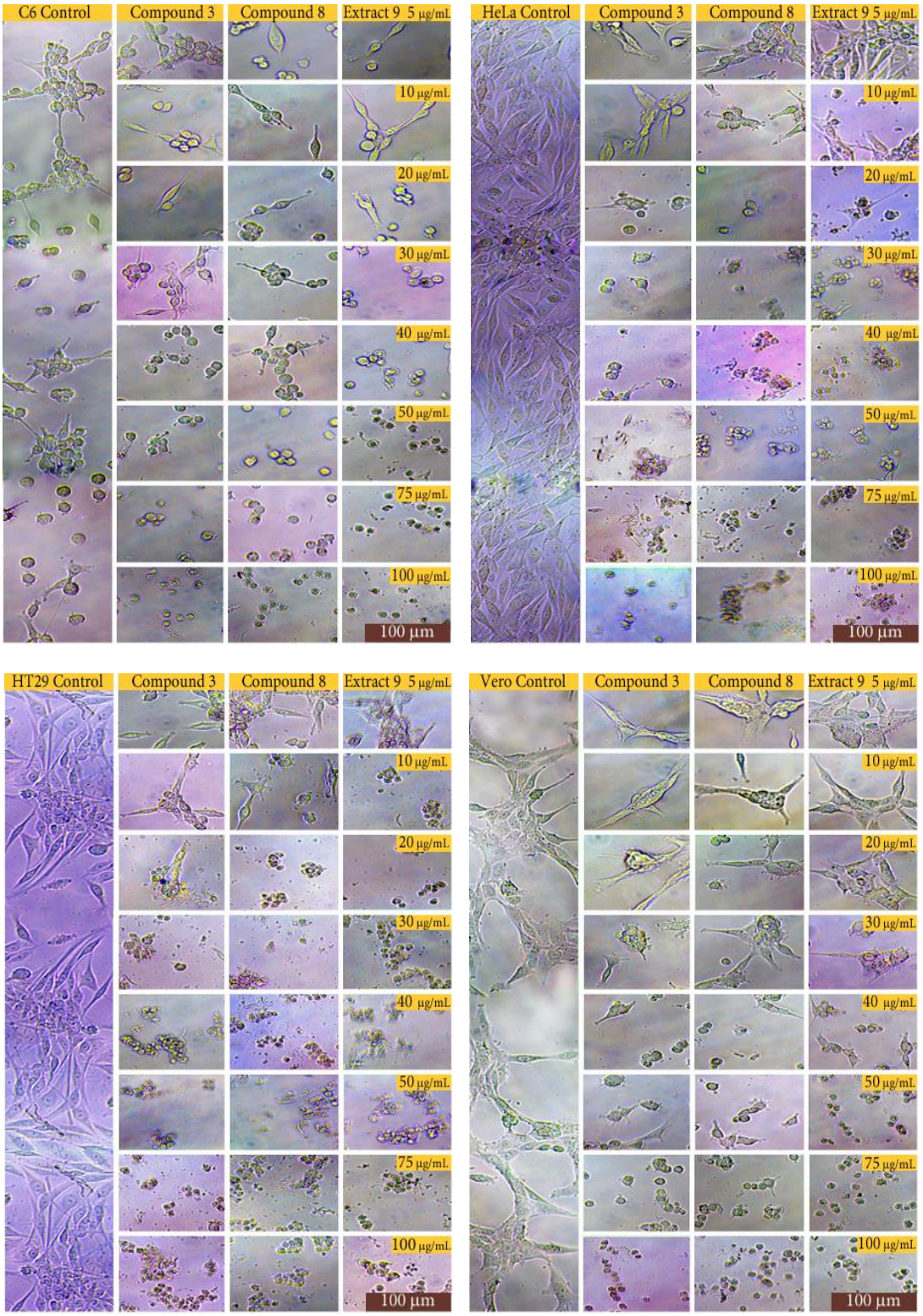
S36: HETCOR (400 MHz, DMSO-*d6*) Spectrum of compound **8 (**globoidnan A)



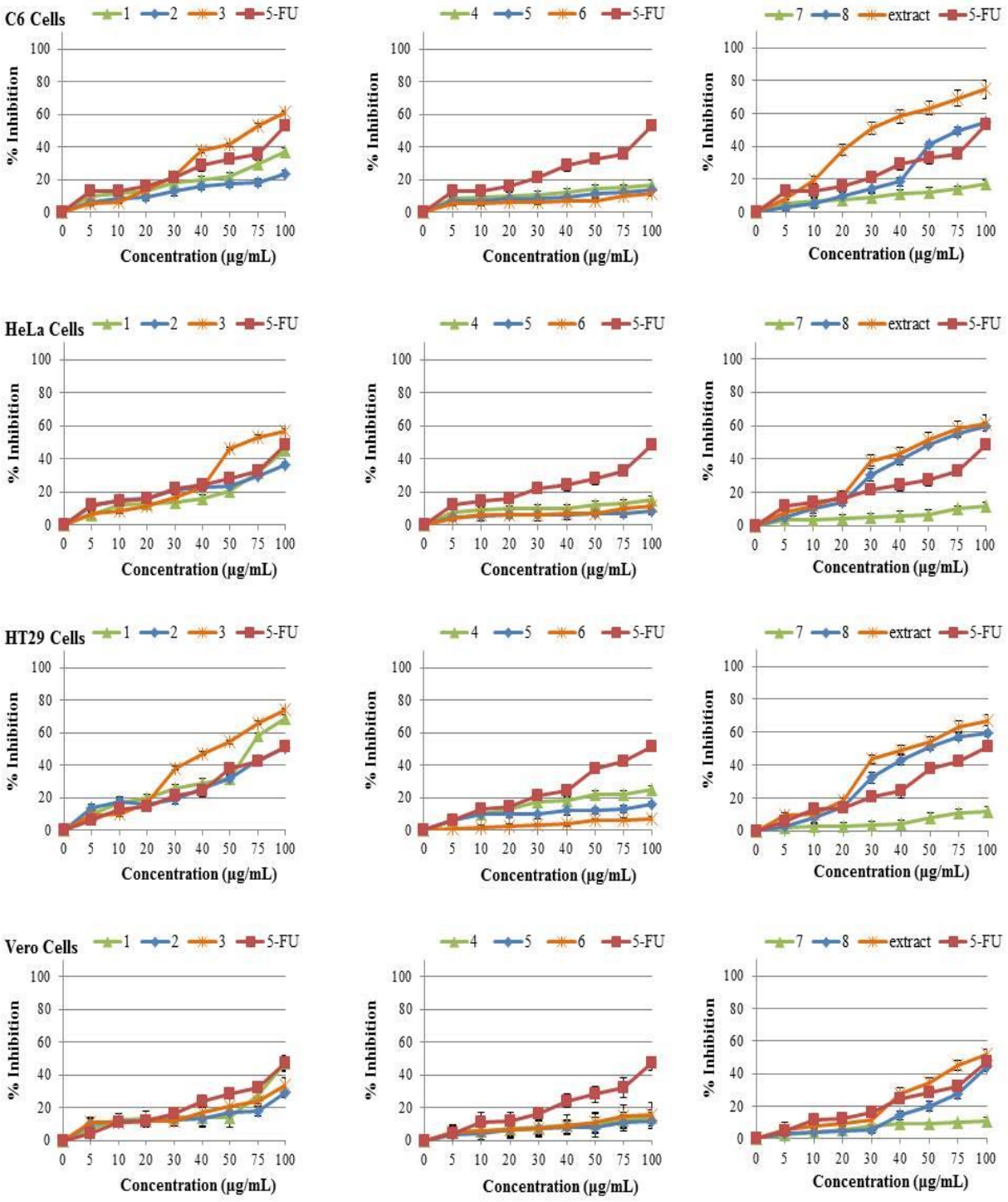
S37: HMBC (400 MHz, DMSO-*d6*) Spectrum of compound **8 (**globoidnan A)



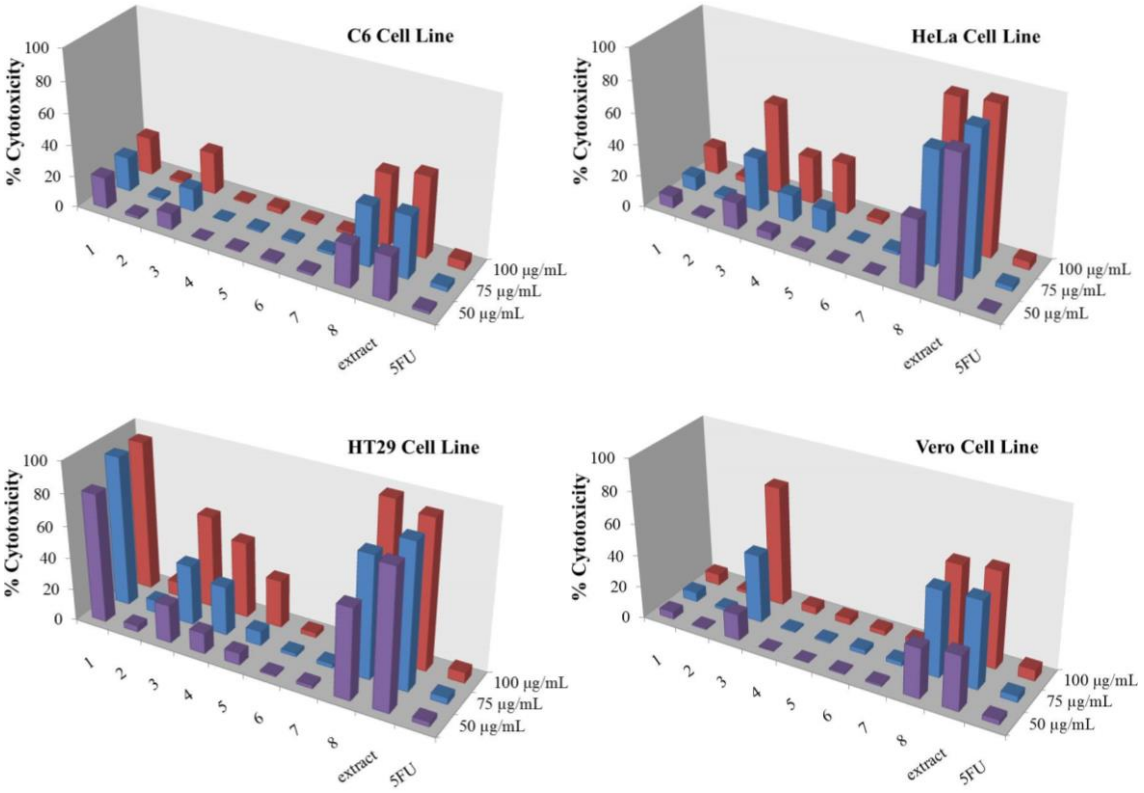
S38: COSY-90 (400 MHz, DMSO-*d6*) Spectrum of compound **8 (**globoidnan A)



S39. C6, HeLa, HT29, and Vero cell images were visualized by digital camera attached inverted microscope (Leica IL10, Germany) at 24 h after treatment with the compounds. DMSO treated cells as controls. All scales 100 µm.



S40. Antiproliferative effect of compounds, extract and 5-FU on C6, HeLa and HT29 and Vero cell lines



S41. Cytotoxicity of isolated compounds, extract and 5-FU on C6, HeLa, HT29 and Vero cell lines