SUPPLEMENTARY MATERIAL

Bioactivity-guided isolation of flavonoids from *Cynanchum acutum* L. Subsp. *Sibiricum* (willd.) Rech. f. and investigation of their antiproliferative activity

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Abstract

Cynanchum acutum L. subsp. *sibiricum* (Willd.) Rech. f. was extracted with hexane, acetone, methanol and water individually. A sample was heated in water then extracted with ethyl acetate. Among the extracts, the ethyl acetate extract exhibited the most antiproliferative activity, so isolation of bioactive compounds was carried out from this extract. A new compound, kaempferol-3-*O*-*β*-xylopyranosyl-(1-2)-*β*-rhamnopyranoside **(1)** along with five known compounds, quercetin-3-*O*-*β*-xyloside **(2)**, kaempferol-3-*O*-*β*-glucoside **(3)**, quercetin-3-*O*-*β*-glucoside **(4)**, kaempferol-3-*O*-*β*-rhamnopyranoside **(5),** and kaempferol-3-*O*-*β*-D-neohesperidoside **(6)** were isolated from ethyl acetate extract. The structures were elucidated by spectroscopic techniques, basically 1D-NMR, 2D-NMR and LC-TOF/MS. Antiproliferative effects of isolated compounds were determined by xCELLigence using the HeLa (human uterus carcinoma) cell lines. Compound **2** and compound **5** revealed the good antiproliferative activity against HeLa cell lines.

Keywords: *Cynanchum acutum* subsp. *sibiricum*, antiproliferative activity, flavonoids.

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S1: Experimental

S2: General experimental procedures

NMR spectra were recorded on a spectrometers operation at 400 MHz for ^{1}H and 100 MHz for ^{13}C NMR. Chemical shifts were given in ppm (δ scale), coupling constant *(J)*. Column chromatography was executed on silica (60-230 mesh, Merck). TLC was carried out on analytical alumina plates (60 F_{254}), detection was carried out by spraying with a solution of ceric sulfate and then heating on a hot plate. Isolation of compounds was achieved by Perkin Elmer Series 200 HPLC. Hitachi U-290 UV-Vis spectrometer was used for UV measurements. IR spectra were reported by a Jasco FT/IR-430 spectrometer. HRMS analyses were recorded on Agilent 6210 LC-TOF/MS spectrometer. Commercial reagents were purchased from standard chemical suppliers.

S3: Plant material

The aerial parts of *Cynanchum acutum* L. subsp. *sibiricum* at flowering stage were collected in August from Elazığ / Turkey (15 km west of Baskil town, around Acem çeşmesi, 1400 m, 22.08.2012) and dried at shade. They were carefully placed on the papers with exposure to wind. It was scattered on the paper to avoid the mold. The plant was identified by Dr. Lütfi Behçet. A voucher specimen (L. Behçet 8269) was deposited in the Herbarium of the Faculty of Arts and Sciences, Bingol University.

S4: Extraction and Isolation

Dried and powdered aerial part of each plant material (20 g) was extracted with hexane, acetone, methanol and water individually, each solvent was 50 mL. A sample (30 g) was boiled in water (60 mL) then extracted with ethyl acetate (3 × 20 mL). Totally five extracts were obtained. Antiproliferative activity tests were applied for each extract and the ethyl acetate extract exhibiting the most activity was used for chromatographic techniques to isolate the bioactive compounds.

Dried and powdered aerial part of plant material (1.0 kg) was boiled with distillated water (1 L) for 2h then extracted with ethyl acetate (3 × 100 mL), filtered and solvent was removed by rotary evaporator to yield the extract. The extract was subjected to column chromatography (2.5 × 100) using the silica gel 60 (0.063-0.20 mm) as an absorbent. The column was eluted with solvent systems of increasing polarity from hexane to hexane-EtOAc and EtOAc-MeOH. Five hundred fractions, each 100 ml were collected. Fractions 108-133 (hexane-EtOAc, 2:8) included kaempferol-3-*O*-*β*-rhamnopyranoside **5** (25 mg), fractions 180-210 (hexane-EtOAc, 1:9) yielded to quercetin-3-*O*-*β*-xyloside **2** (25 mg), fractions 220-230 (EtOAc) contained mixture compounds thus preparative HPLC was used to afforded firstly kaempferol-3-*O*-*β*-glucoside **3** (30 mg), and secondly quercetin-3-*O*-*β*-glucoside **4** (15 mg), fractions 320-356 yield to (MeOH-EtOAc, 1:19) kaempferol-3-*O*-*β*-xylopyranosyl-(1-2)-*β*-rhamnopyranoside **(1)** (100 mg), fractions 416-440 (MeOH-EtOAc, 1:9) afforded as kaempferol-3-*O*-neohesperidoside, **6** (50 mg) (Fig. 1). ^{1}H NMR (400 MHz, DMSO-*d6*) of compound **1**: 8.04 (d, *J* = 8.8, H2'), 8.04 (d, *J* = 8.8, H5'), 6.90 (d, *J* = 8.8, H3'), 6.90 (d, *J* = 8.8, H6'), 6.90 (d, *J* = 8.8, H1''), 6.41 (d, J = 1.4, H8), 6.18 (d, J = 1.4, H6), 5.53 (d, *J* = 7.3, H2''), 5.09 (d, *J* = 1.2, H1'''), 3.81 (m, H5'''), 3.76 (m, H2'''), 3.49 (m, H3''), 3.49 (m, H3'''), 3.34 (m, H5''α), 3.31 (m, H4''), 3.18 (m, H4'''), 2.97 (m, H5''β), 0.89 (d, *J* = 6.1, H6'''). ^{13}C NMR (100 MHz, DMSO-*d6*): 160.6 (C2), 133.1 (C3), 177.5 (C4), 161.2 (C5), 99.4 (C6), 165.5 (C7), 94.2 (C8), 156.3 (C9), 104.1 (C10), 121.1 (C1'), 131.1 (C2'), 115.7 (C3'), 156.8 (C4'), 131.1 (C5'), 115.7 (C6'), 115.7 (C1''), 99.8 (C2''), 77.6 (C3''), 70.1 (C4''), 77.3 (C5''α), 66.4 (C5''β), 101.2 (C1'''), 71.0 (C2'''), 70.9 (C3'''), 72.3 (C4'''), 68.9 (C5'''), 17.7 (C6''').

S5: Antiproliferative Assay

xCELLigence assay was used to determine the antiproliferation of extracts (Fig. 2) and isolated compounds. A real-time cell analyzer-single plate (RTCA-SP) instrument (Roche Applied Science, Basel, Switzerland) was employed. The concentrations of 50, 100, 250 µg/mL were used for plant extracts and isolated compounds and recent published method was applied(Okten et al. 2013).

S6: Preperation of stock solution

Stock solutions of the C*ynanchum acutum* subsp. *sibiricum* extracts were prepared in DMSO and diluted with DMEM (+) (Dulbecco's modified eagle medium). The final concentration of DMSO is below 1% in all anticancer activity tests.

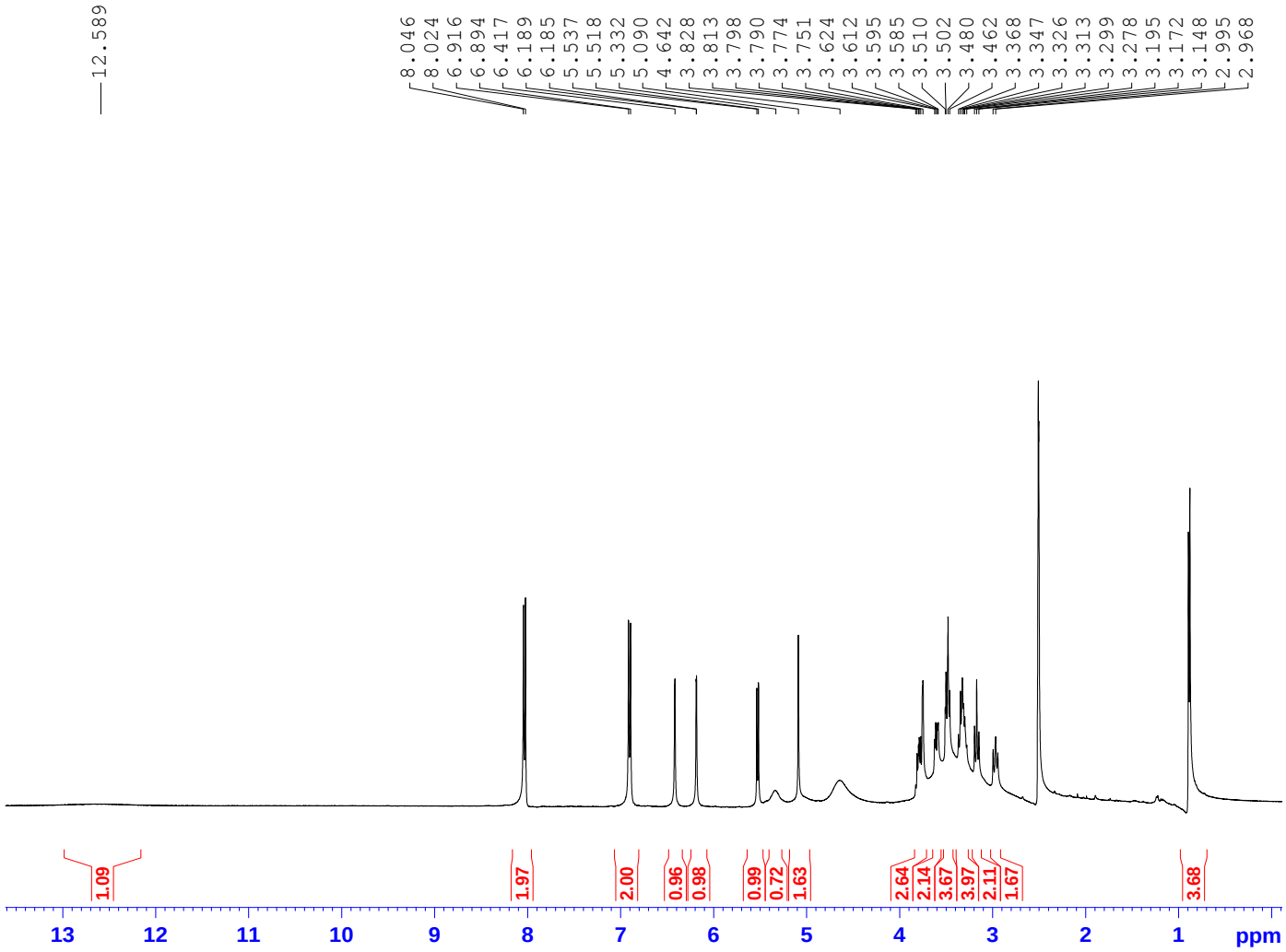
S7: Cell culture

HeLa cells (human cervical cancer cells) were grown in DMEM added 10% fetal bovine serum (FBS) and 2% penicilin-streptomycin. The growth medium was altered every three days.

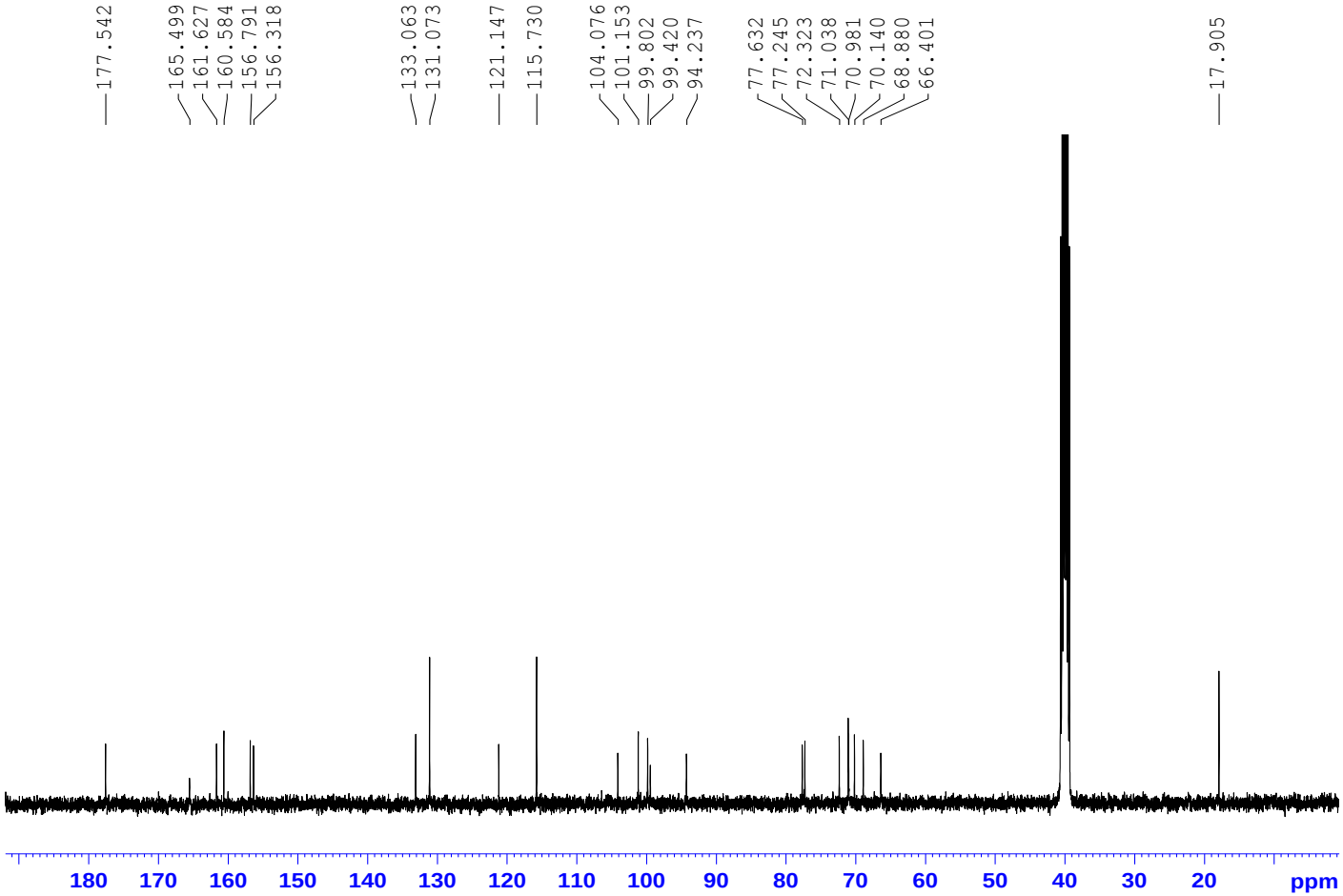
S8: Cell proliferation tests

The xCelligence RTCA SP (Real Time Cell Analyzer Single Plate, Acea Bioscience, Inc.) instrument was used to visualize the antiproliferative effects of the extracts and isolated compounds against to HeLa cells. xCelligence RTCA SP has four different parts: an a single plate station kept in a CO_2 incubator, e-plate 96, an analyzer and a computer with RTCA software. The bottom of E-Plate 96 wells are coated with golden microelectrodes. The RTCA SP system measures impedance differences caused by adherent cells and reports the signals as Cell Index (CI) values a unitless parameter. The higher CI value means the cell population growing and attaching to microelectrodes at the bottom. The cells were cultured in Dulbecco's Modified Eagle Medium-high glucose (with 10% FBS and 2% penicillin-streptomycin) at 37 °C, 95% humidity and 5% CO_2 atmosphere. To analyse the antiproliferative effects of the extracts and isolated molecules, HeLa cells were detached from the culture flask with 10 mL trypsin/EDTA solution. After cell detachment, 10 mL of DMEM (+) was added to this cell suspension and gently mixed with a sterile pipette. Then the cell suspension was partitioned into two different falcon tubes and centrifuged at 600 rpm for 5 minutes. At this time, 50

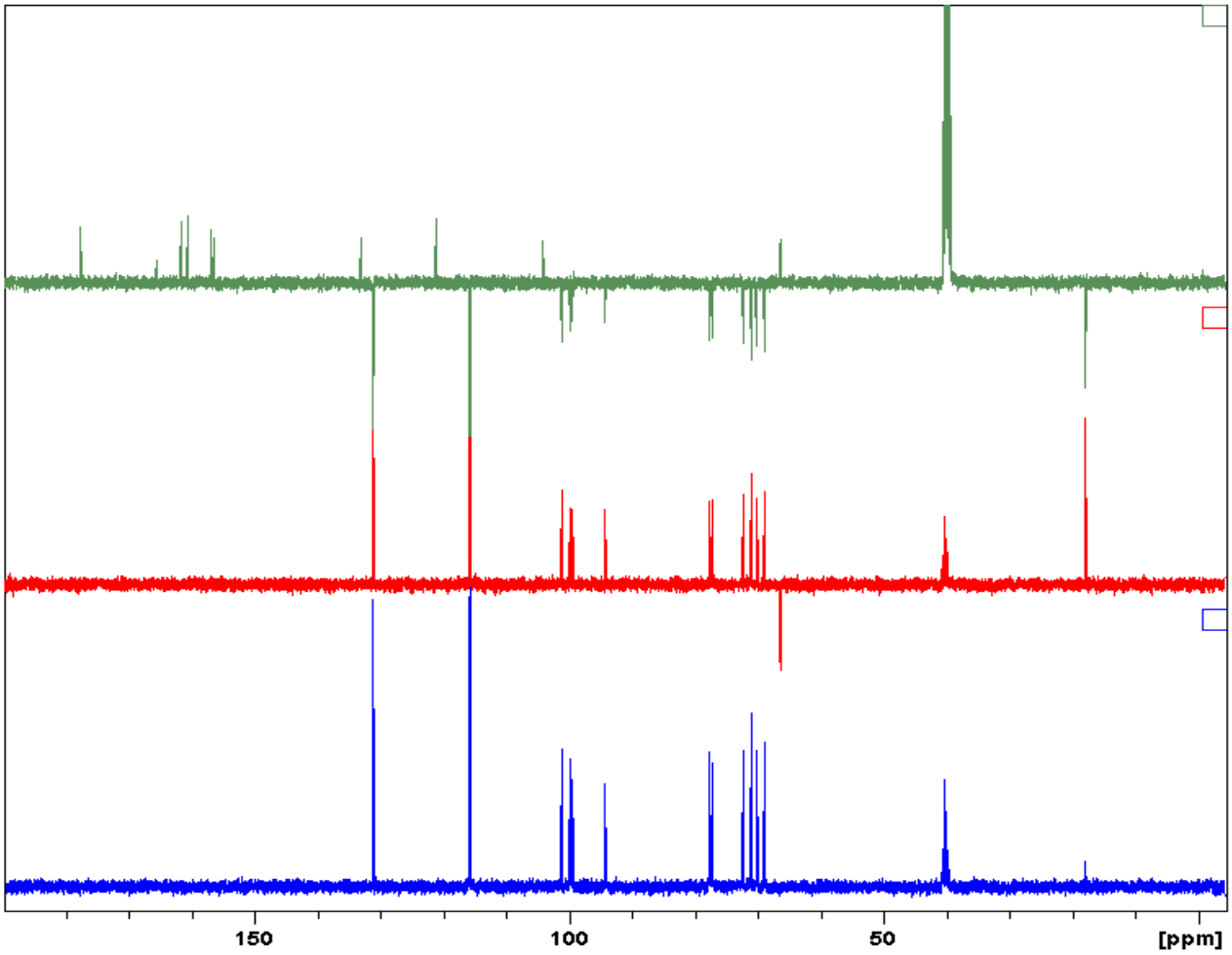
µL DMEM(+) was added to each well of the plate and left in the steril cabinet for 15 min and in the CO_2 incubator for another 15 min to permit the electrodes to equilibrate with the medium. After this step, the E-Plate 96 was entereded to the xCelligence RTCA SP station and a background measurement (Step 1) was performed for 1 minute. After step 1, the plate was ejected from the RTCA station and 100 µL HeLa cell suspension was added to each well (25000 cells 100 μL^{-1}) except for three. The cell suspension was not added to three wells. These wells were left blank to control if there would be a cell index (CI) measured due to the culture medium. The plate was left in the steril cabinet for 30 min for the cell attachment to the electrodes. After 30 min E-plate 96 was inserted into the station and second measurement (Step 2) lasting 80 min was started. At the end of Step 2, the plate was ejected from the RTCA SP station and placed into the steril cabinet. The sample solutions were added to the wells at three different concentrations (100, 50 and 10 µg mL^{-1}). The final volume of the plate wells were set to 200 µL with DMEM(+).After this period, E-plate 96 was placed into the RTCA station. The last measurement (Step 3, 48 h) was started.



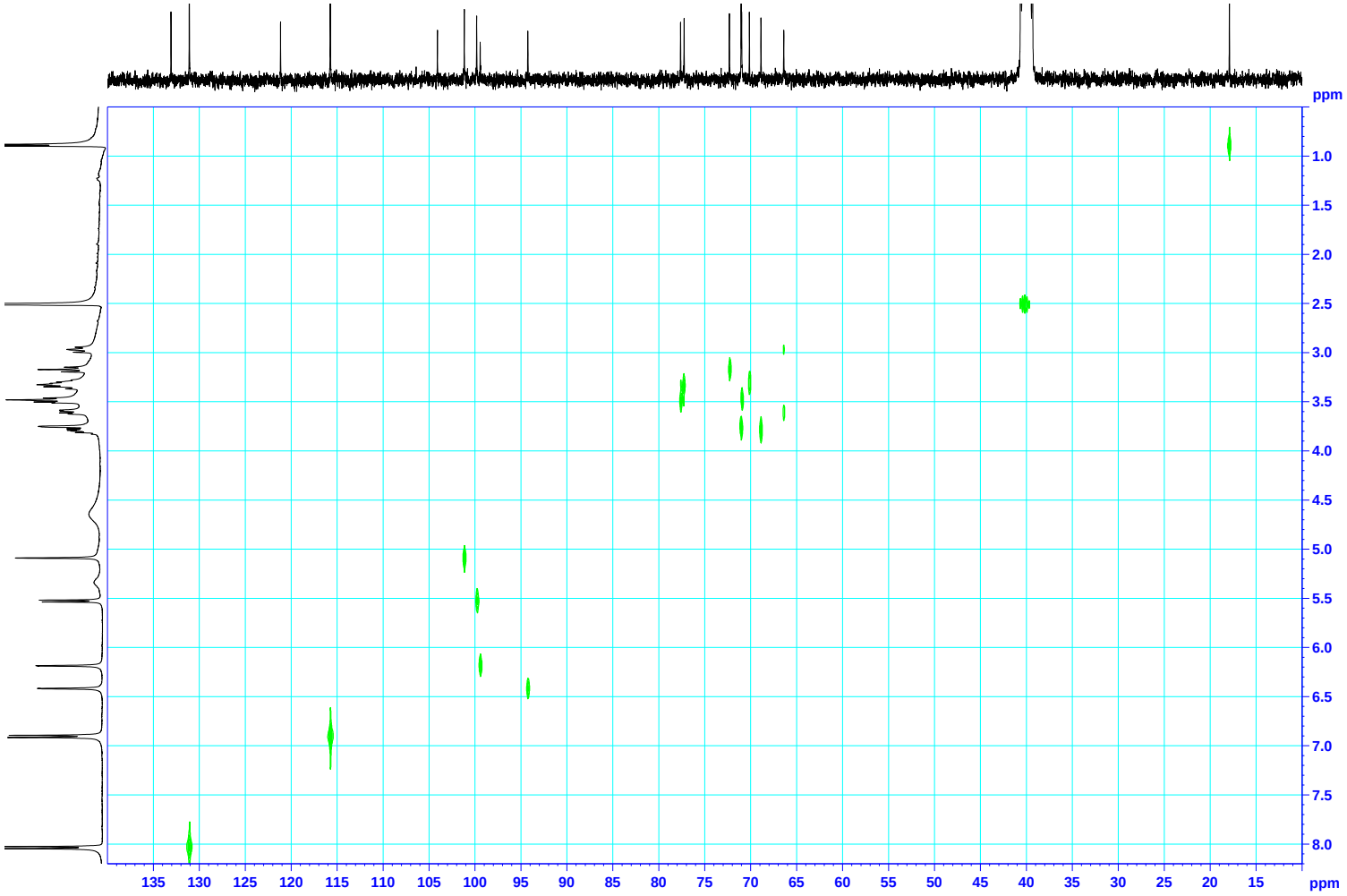
S9: ^{1}H-NMR (400 MHz, DMSO-*d6*) spectrum of kaempferol-3-O-*β*-xylopyranosyl-(1-2)-*β*-rhamnopyranoside (**1**)



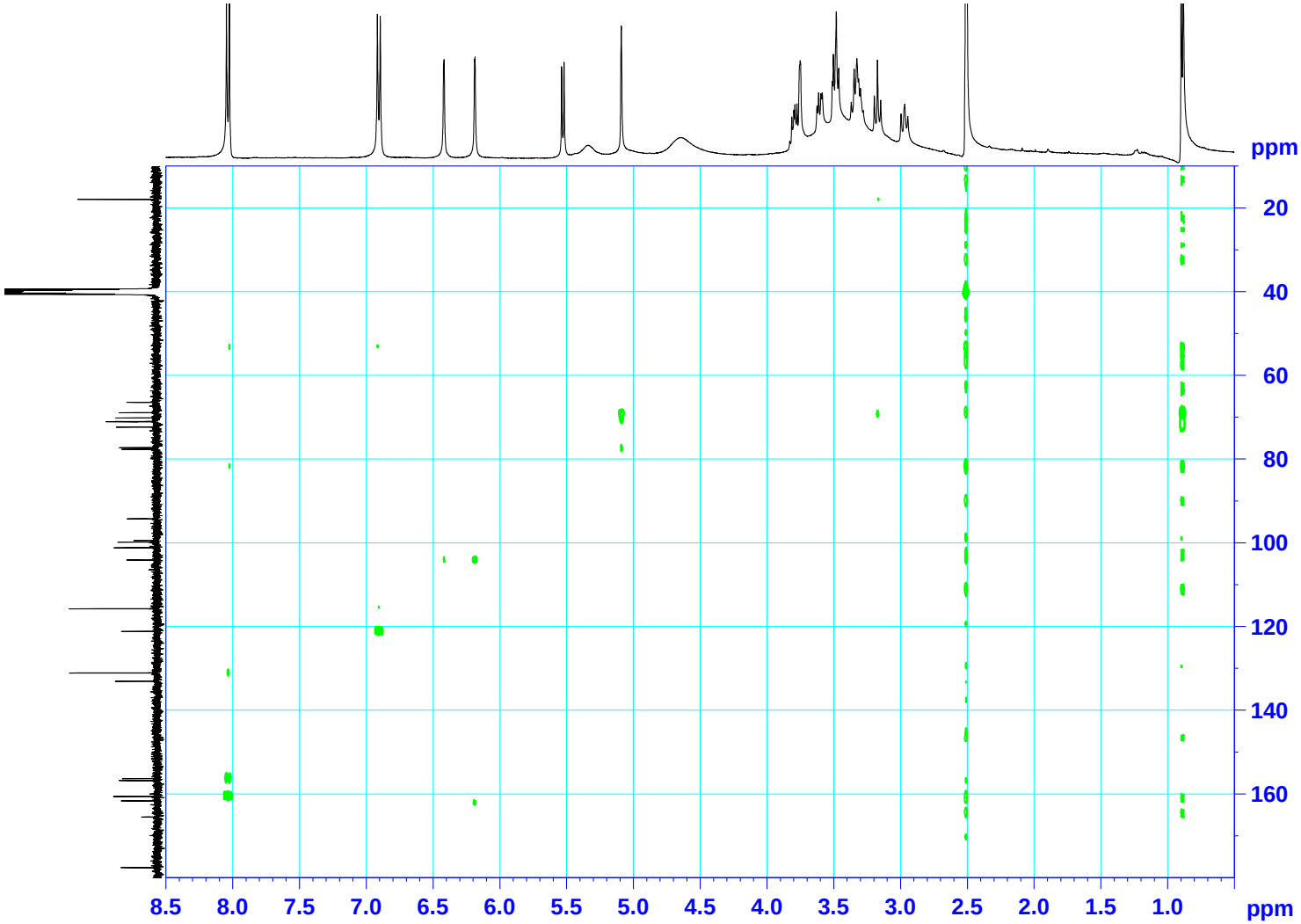
S10: ^{13}C-NMR (100 MHz, DMSO-*d6*) spectrum of kaempferol-3-O-*β*-xylopyranosyl-(1-2)-*β*-rhamnopyranoside (**1**)



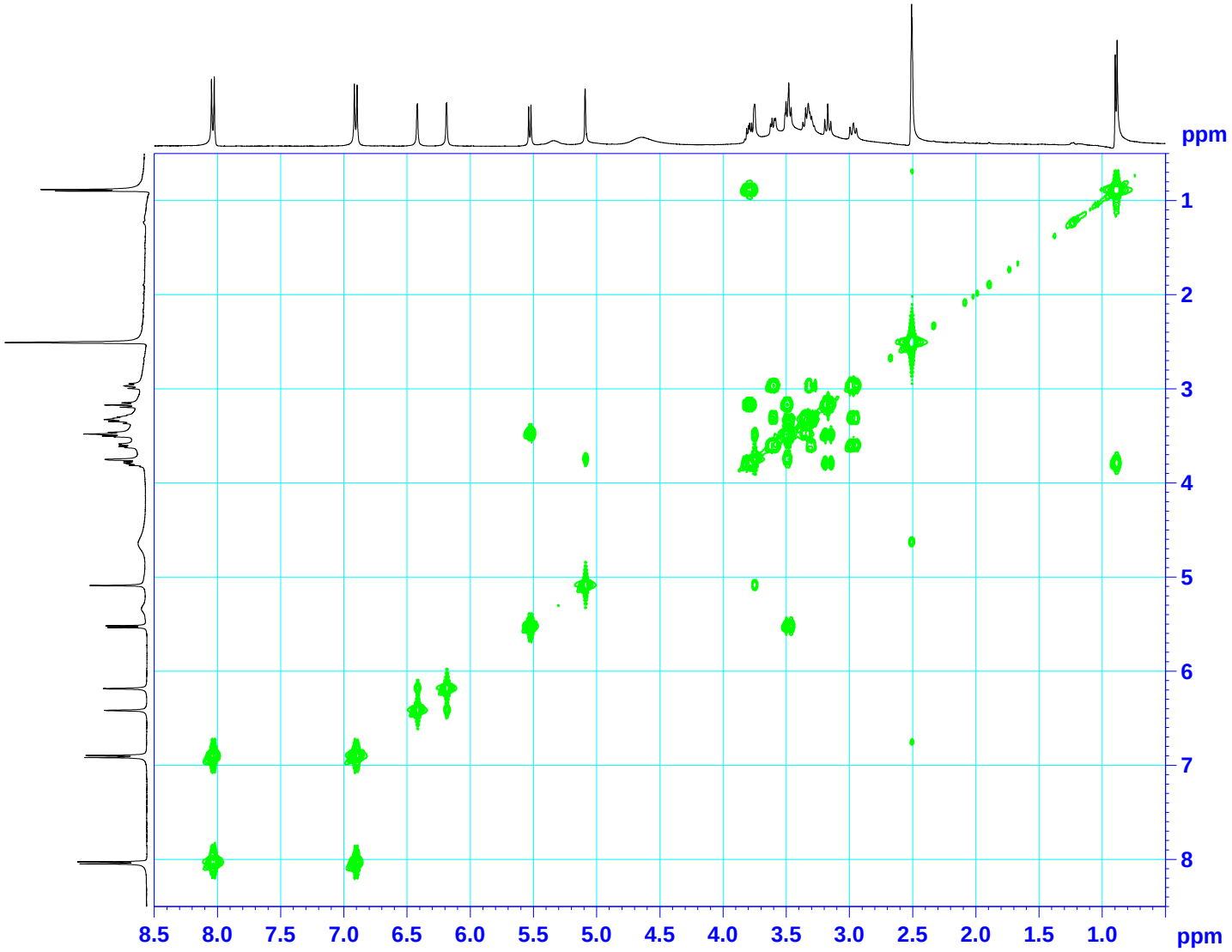
S11: APT, DEPT-135, DEPT-90 NMR (100 MHz, DMSO-*d6*) spectrum of kaempferol-3-O-*β*-xylopyranosyl-(1-2)-*β*-rhamnopyranoside (**1**)



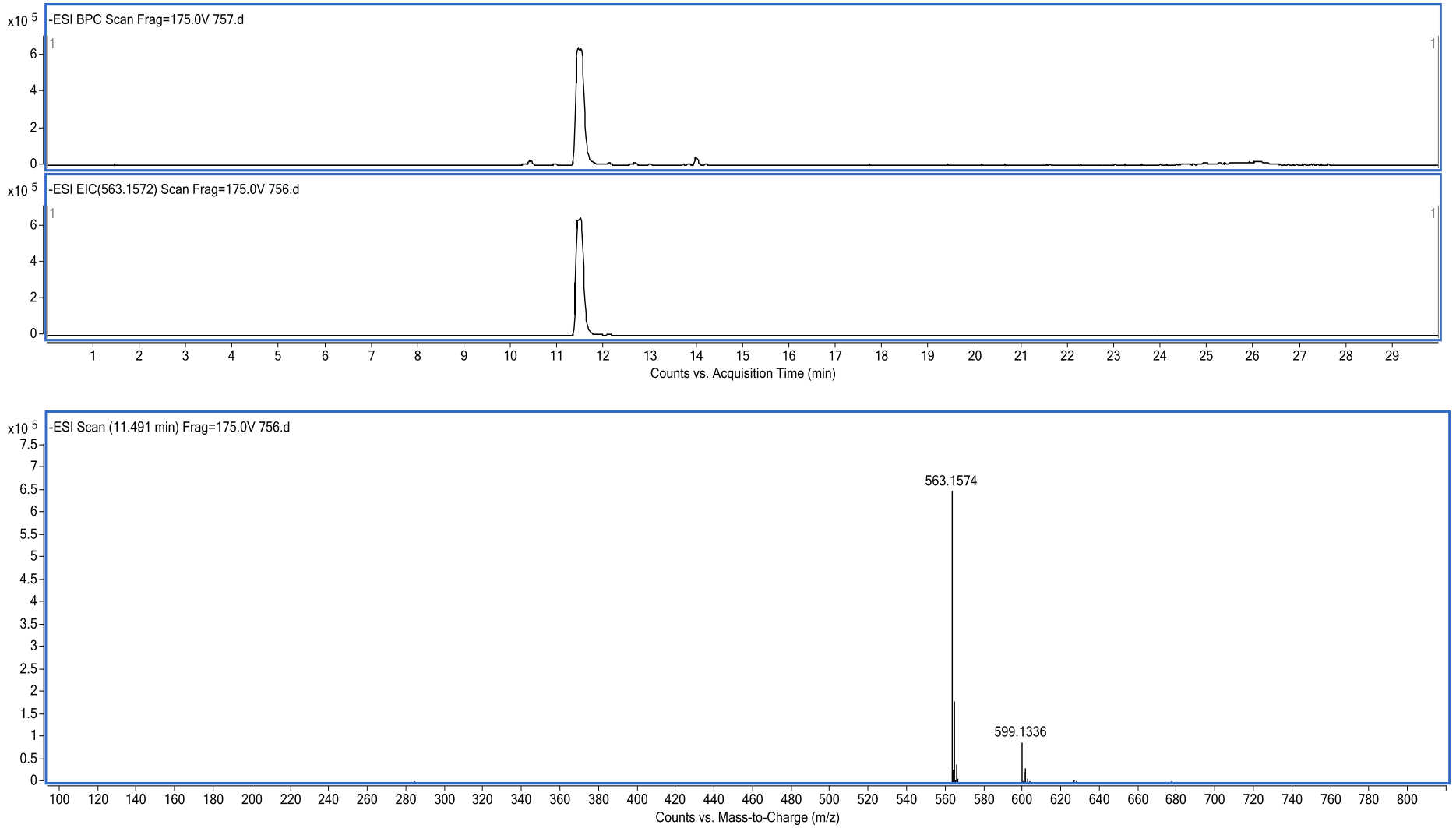
S12: HETCOR (400 MHz, DMSO-*d6*) spectrum of kaempferol-3-O-*β*-xylopyranosyl-(1-2)-*β*-rhamnopyranoside (**1**)



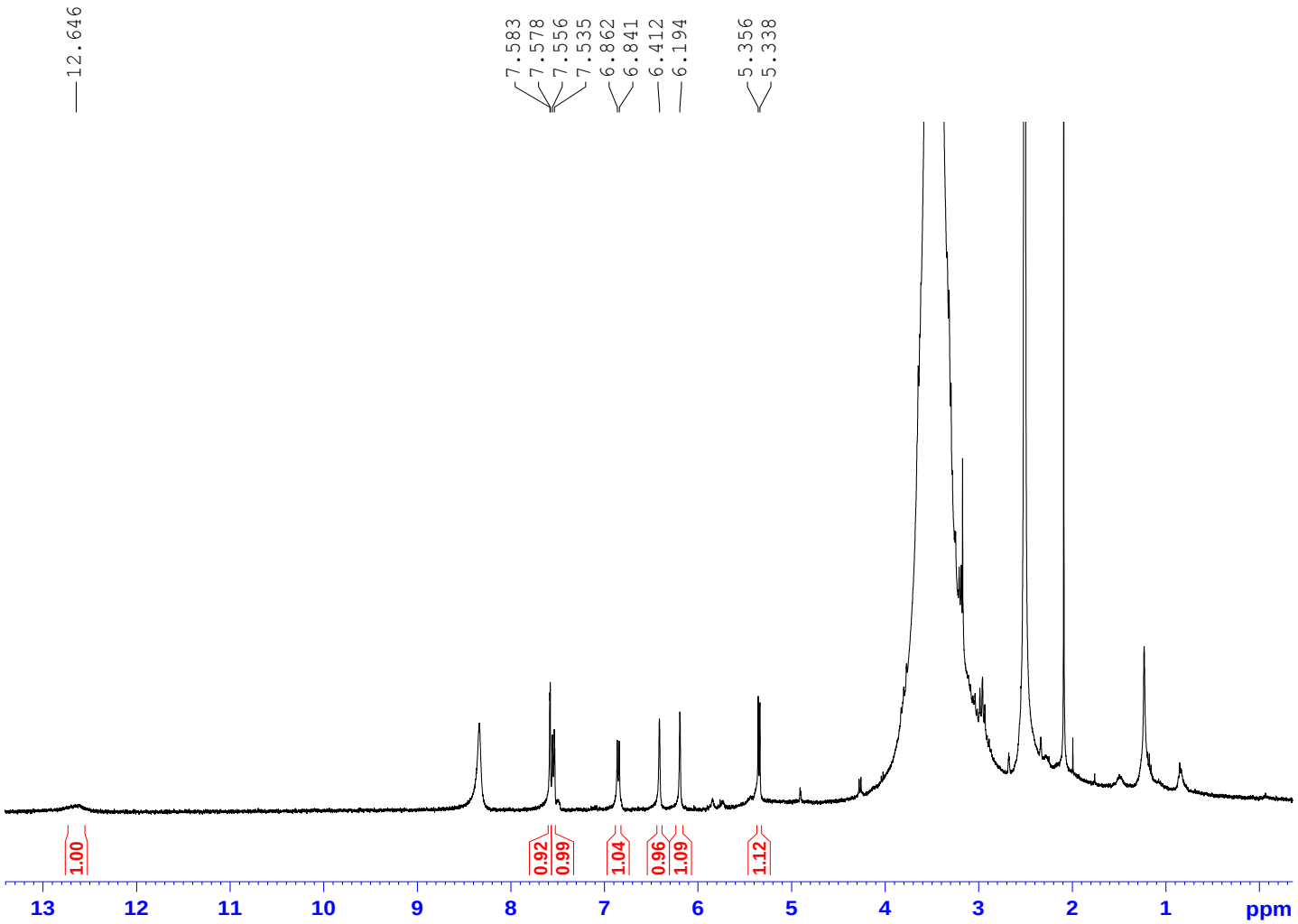
S13: HMBC (400 MHz, DMSO-*d6*) spectrum of kaempferol-3-O-*β*-xylopyranosyl-(1-2)-*β*-rhamnopyranoside (**1**)



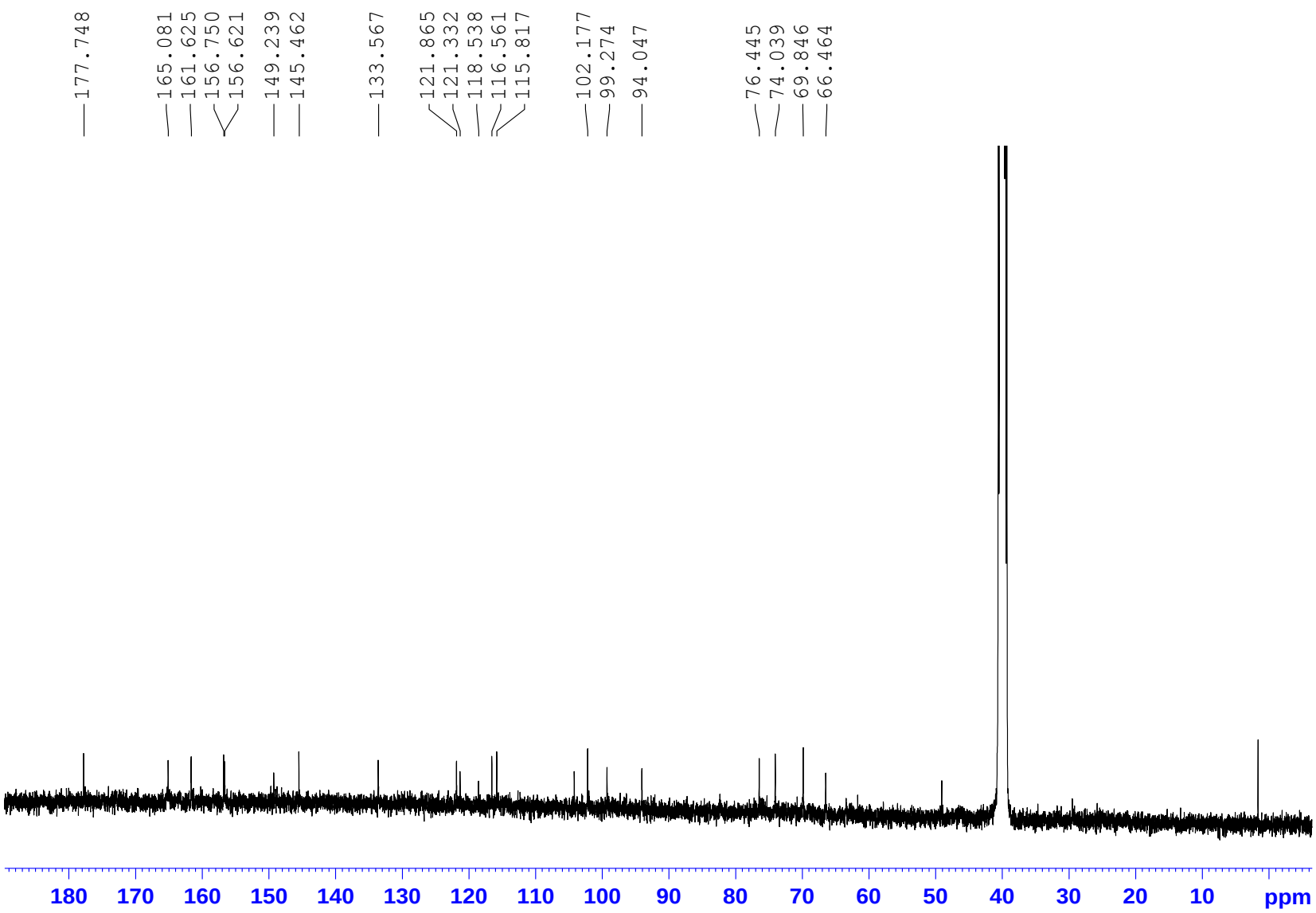
S14: COSY-90 (400 MHz, DMSO-*d6*) spectrum of kaempferol-3-O-*β*-xylopyranosyl-(1-2)-*β*-rhamnopyranoside (**1**)



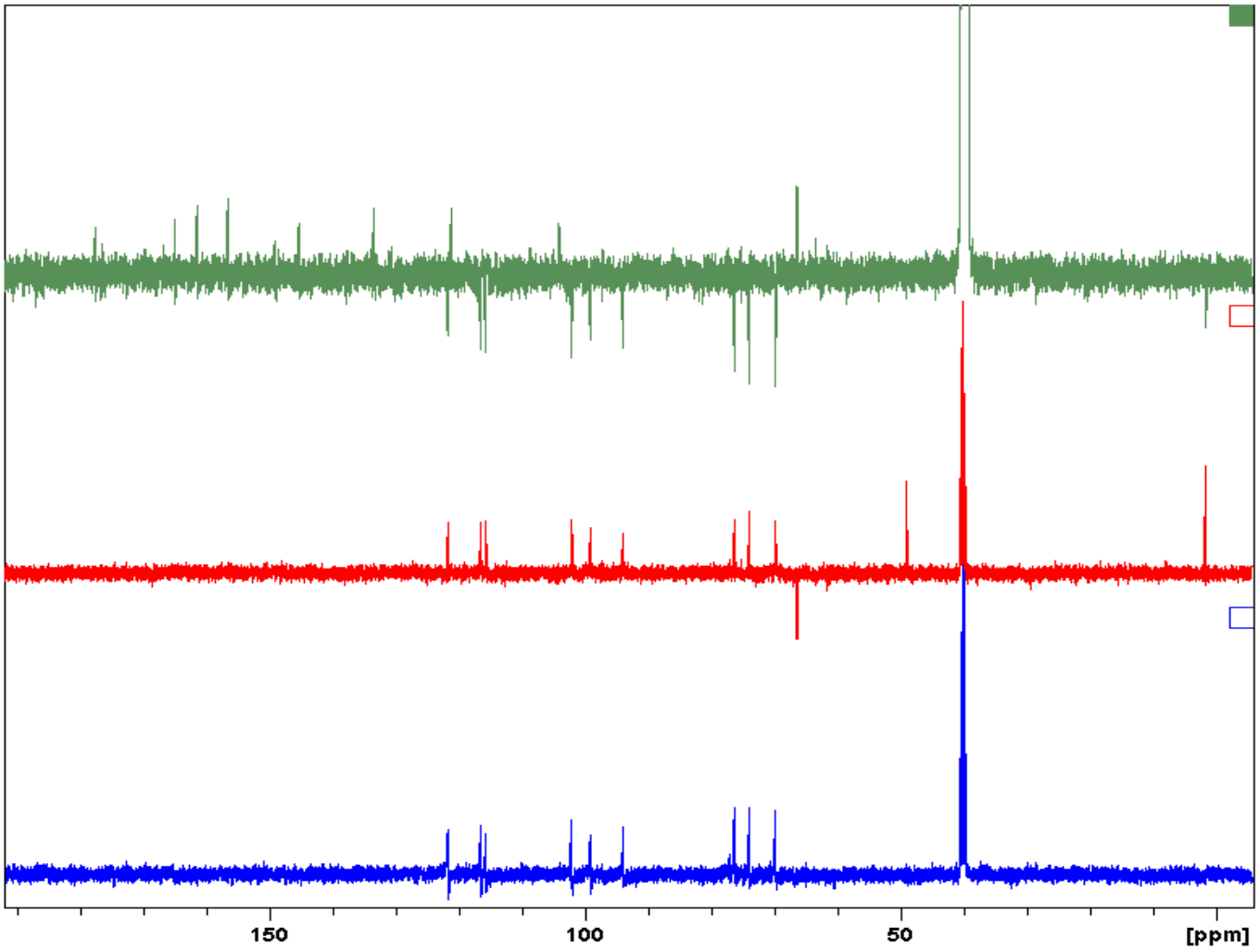
S15: LC-TOF/MS spectrum of kaempferol-3-O-*β*-xylopyranosyl-(1-2)-*β*-rhamnopyranoside (**1**)



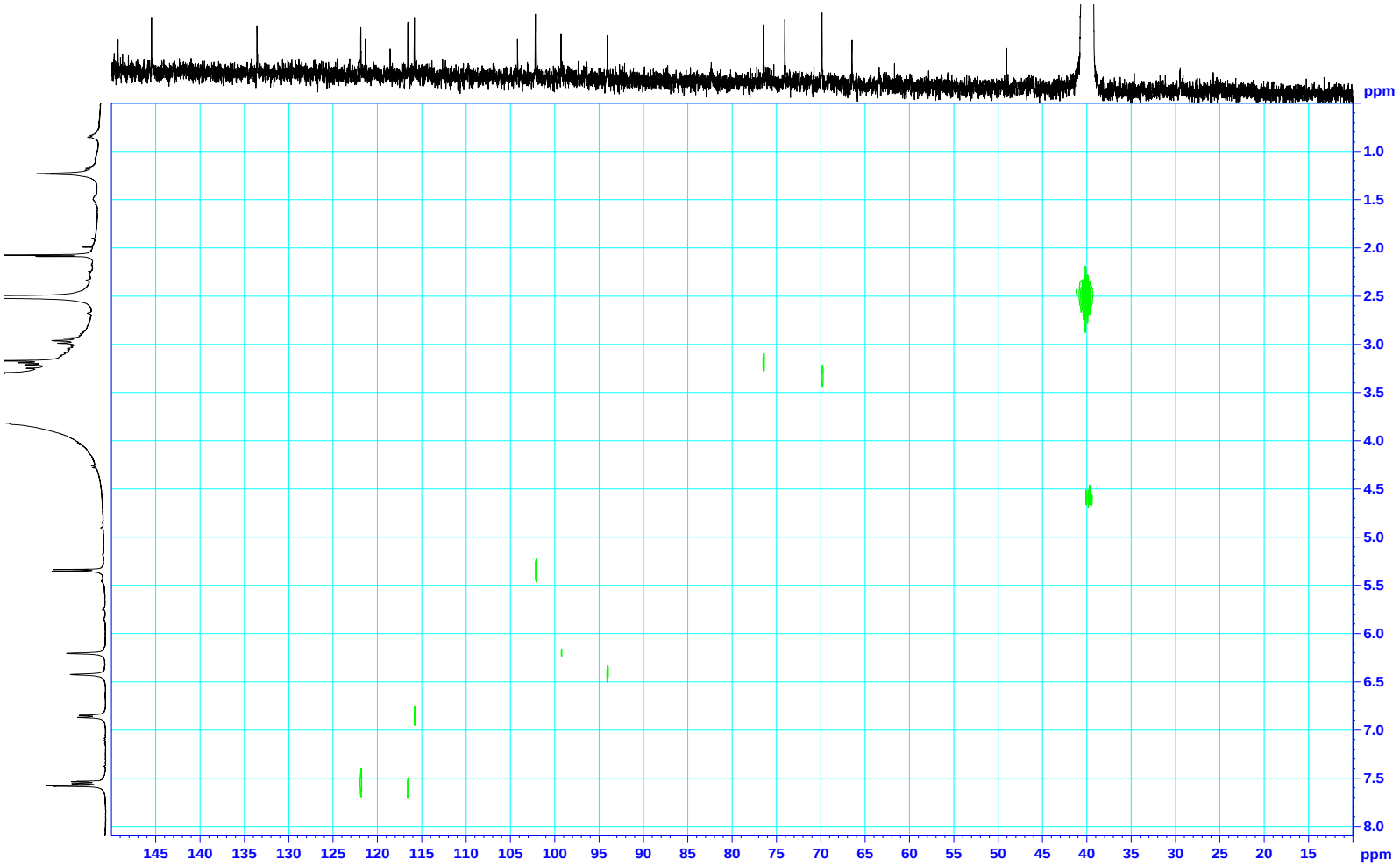
S16: ^{1}H-NMR (400 MHz, DMSO-*d6*) spectrum of quercetin-3-*O*-*β*-xyloside (**2**)



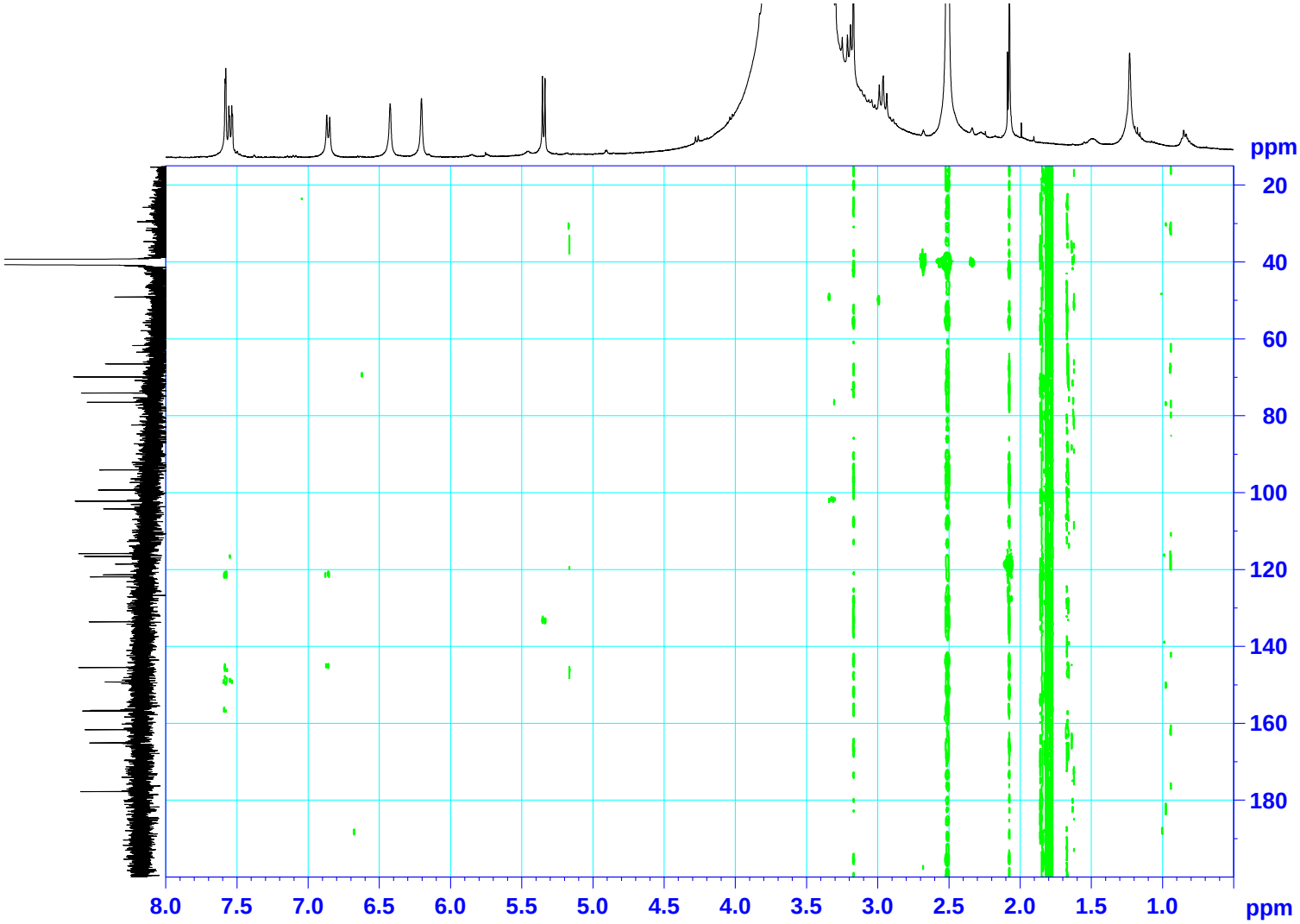
S17: ^{13}C-NMR (100 MHz, DMSO-*d6*) spectrum of quercetin-3-*O*-*β*-xyloside (**2**)



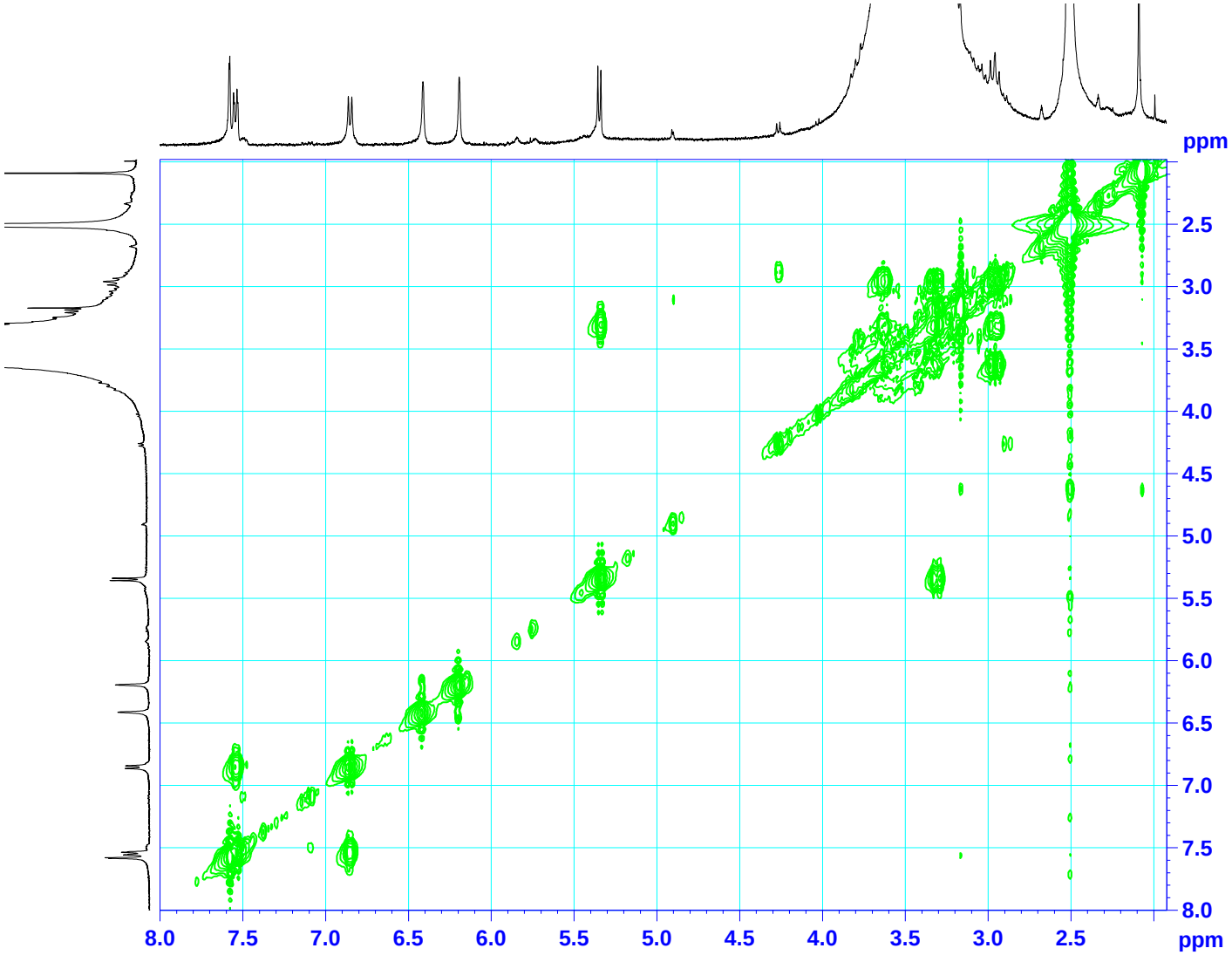
S18: APT, DEPT-135, DEPT-90 NMR (100 MHz, DMSO-*d6*) spectrum of quercetin-3-*O*-*β*-xyloside (**2**)



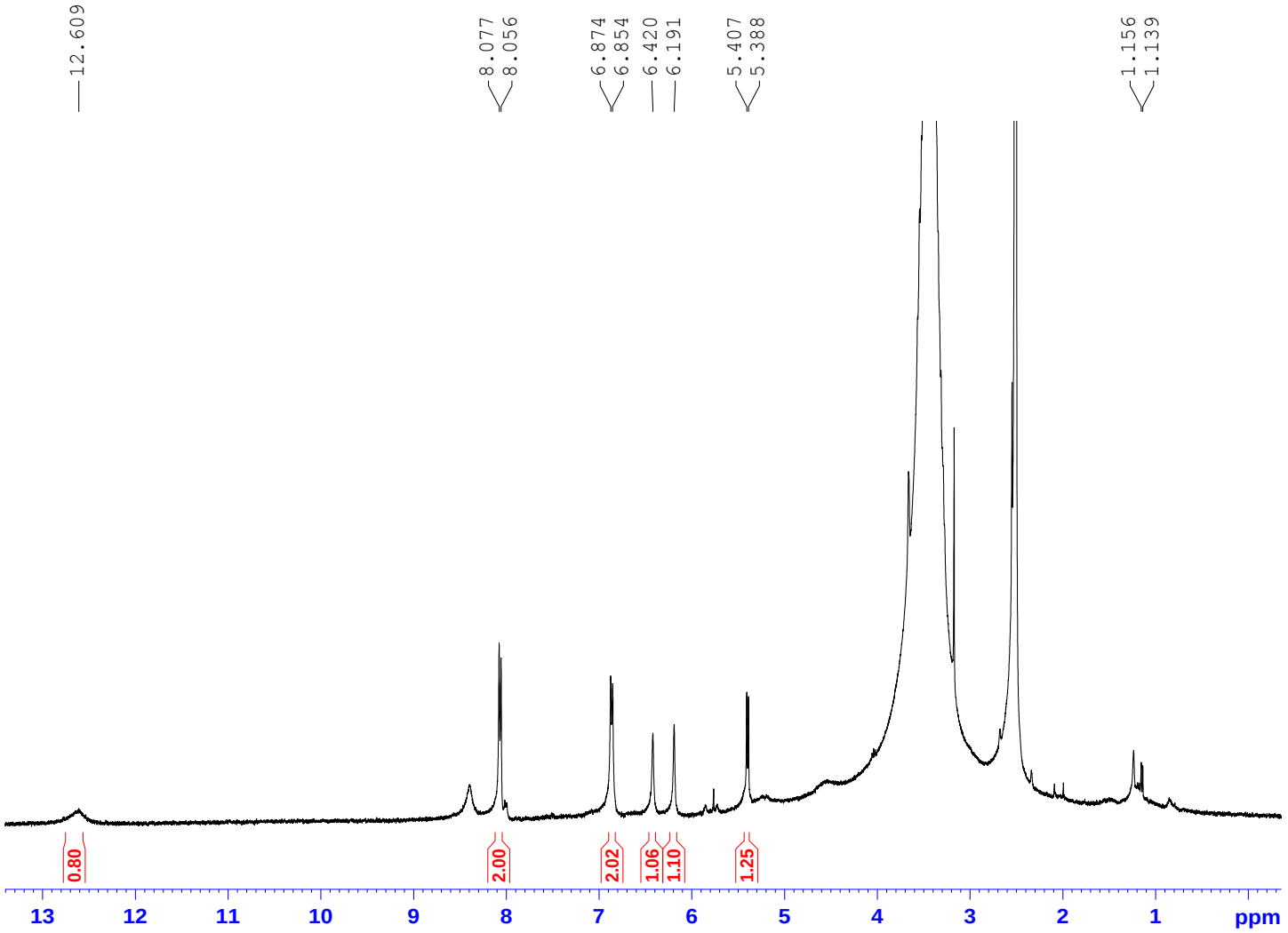
S19: HETCOR (400 MHz, DMSO-*d6*) spectrum of quercetin-3-*O*-*β*-xyloside (**2**)



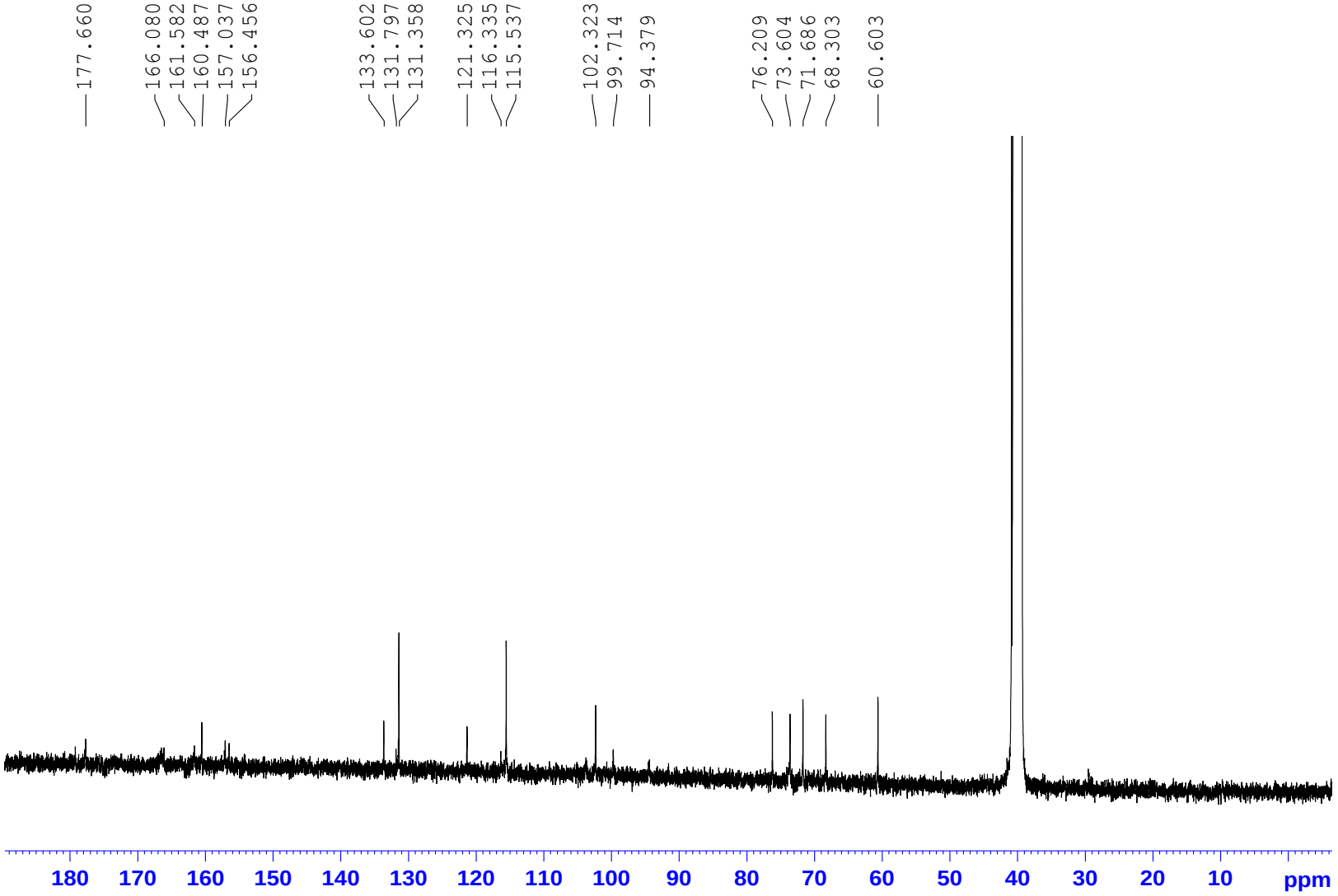
S20: HMBC (400 MHz, DMSO-*d6*) spectrum of quercetin-3-*O*-*β*-xyloside (**2**)



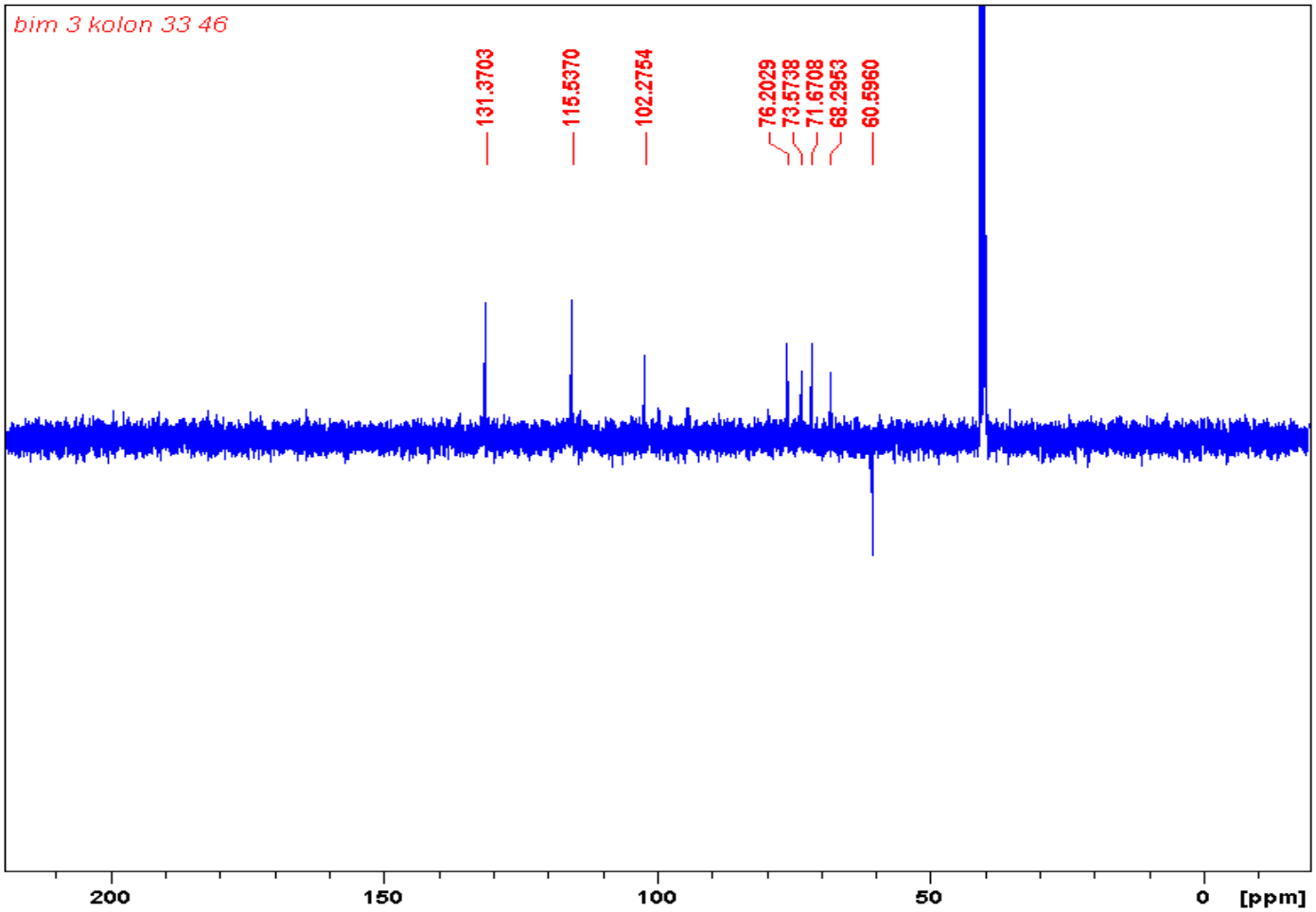
S21: COSY-90 (400 MHz, DMSO-*d6*) spectrum of quercetin-3-*O*-*β*-xyloside (**2**)



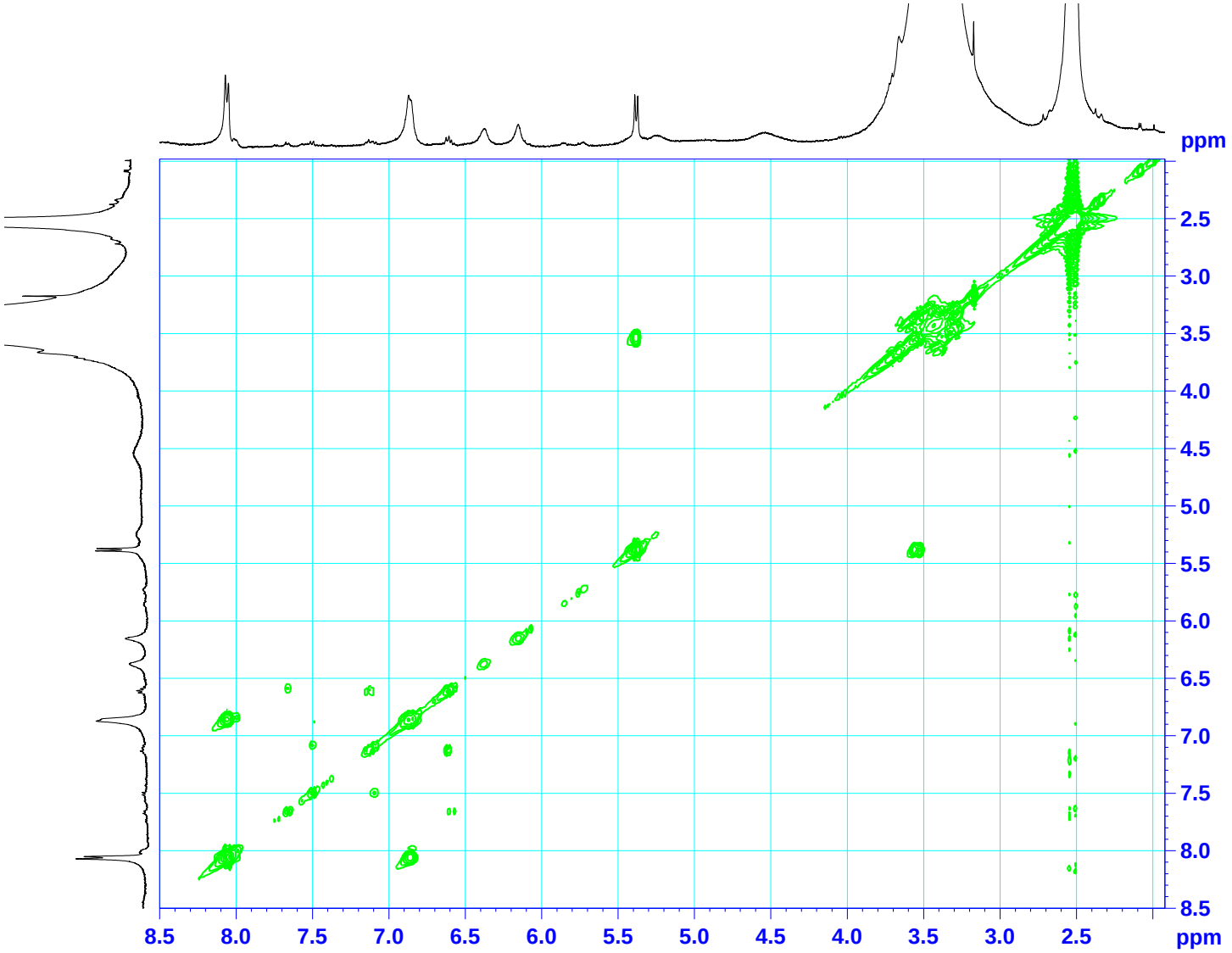
S22: ^{1}H-NMR (400 MHz, DMSO-*d6*) spectrum of kaempferol-3-*O*-*β*-glucoside (**3**)



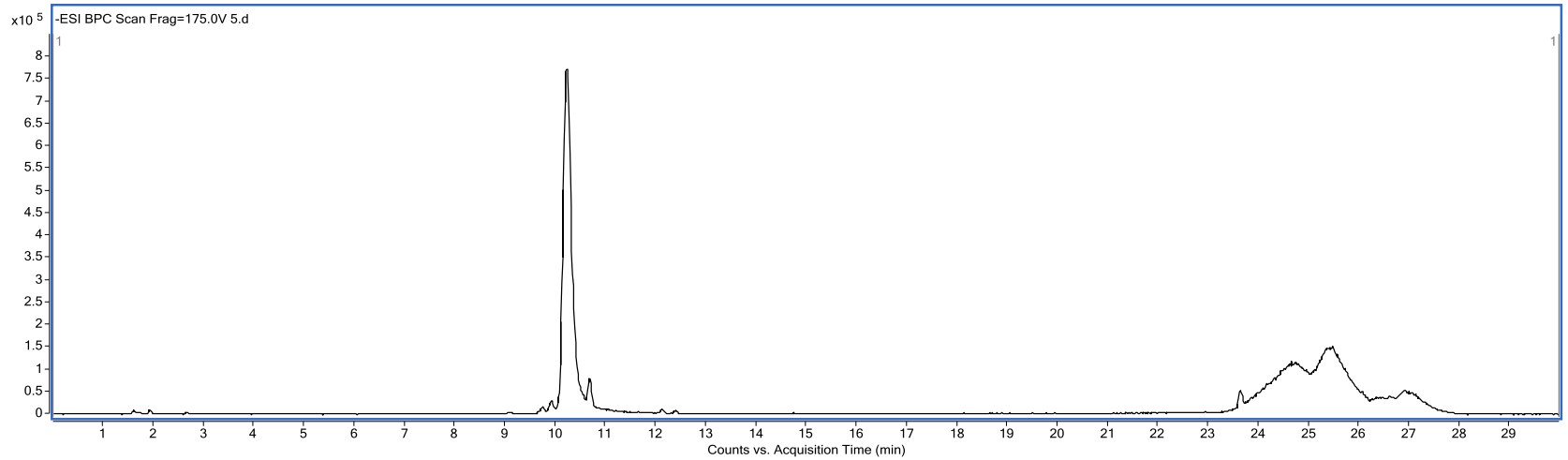
S23: ^{13}C-NMR (100 MHz, DMSO-*d6*) spectrum of kaempferol-3-*O*-*β*-glucoside (**3**)

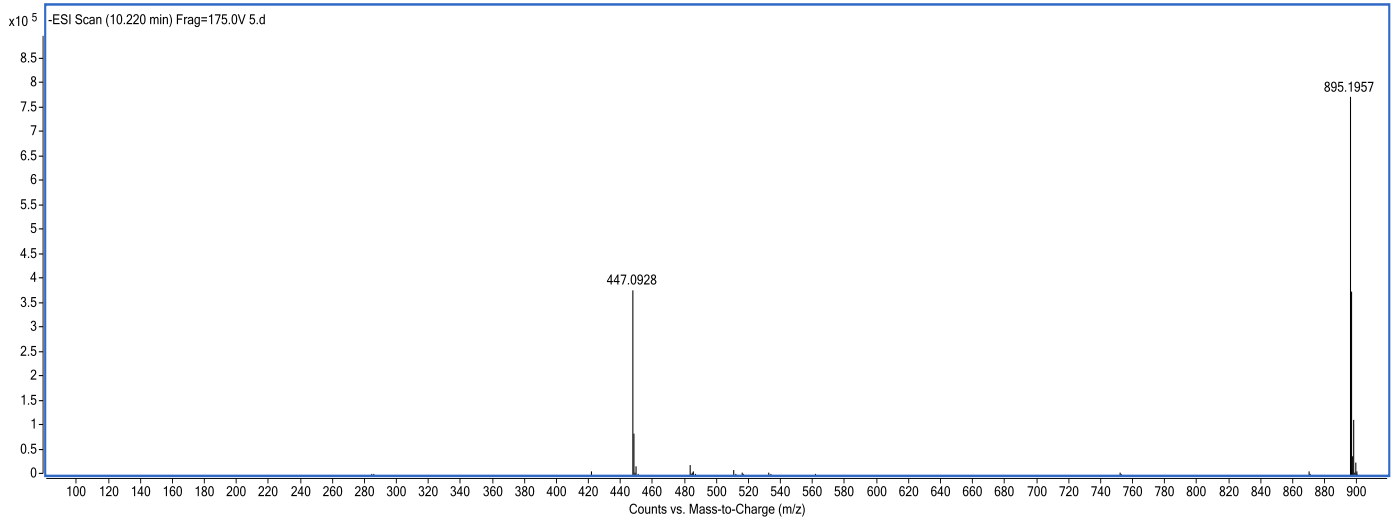


S24: DEPT-135 NMR (100 MHz, DMSO-*d6*) spectrum of kaempferol-3-*O*-*β*-glucoside (**3**)

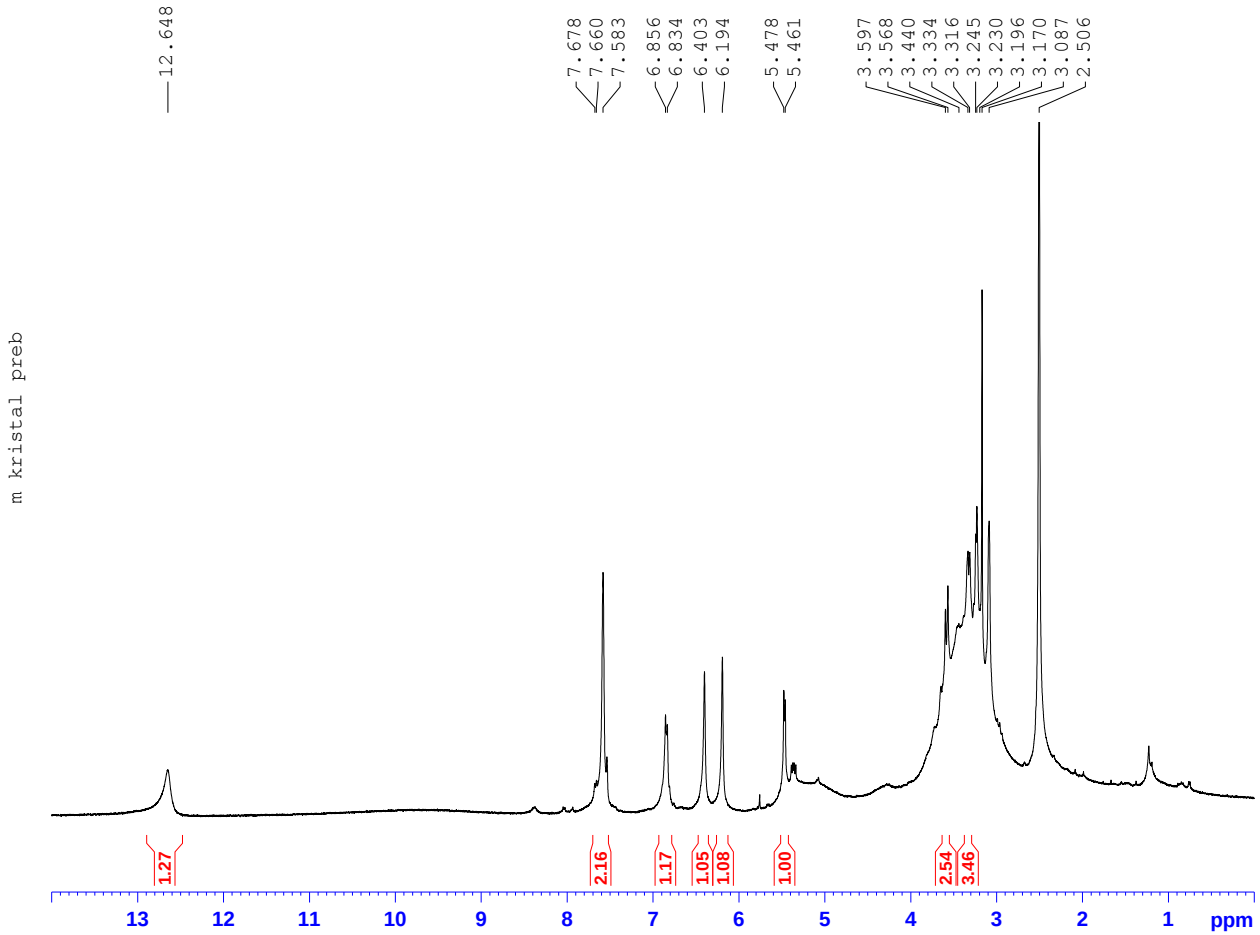


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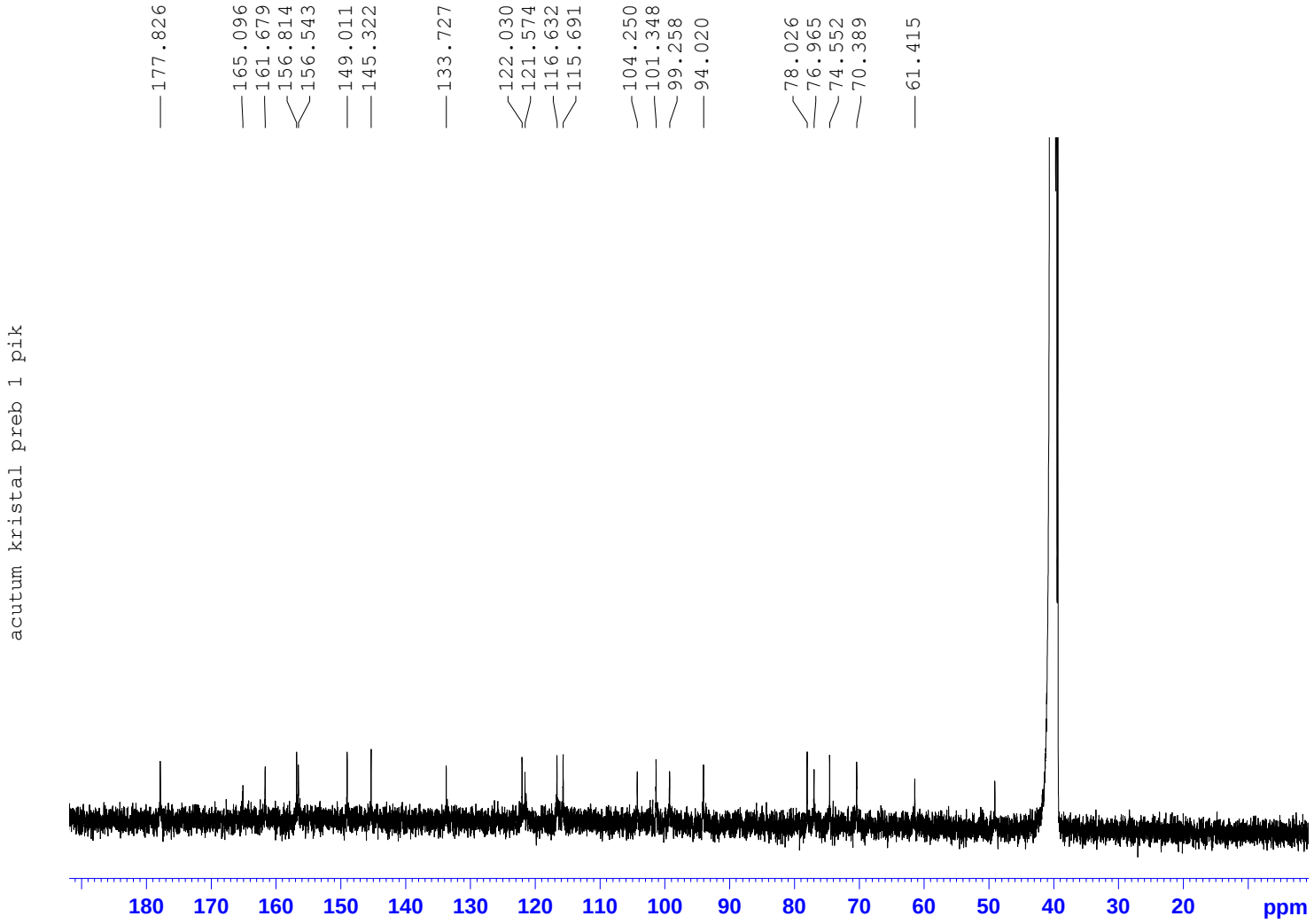




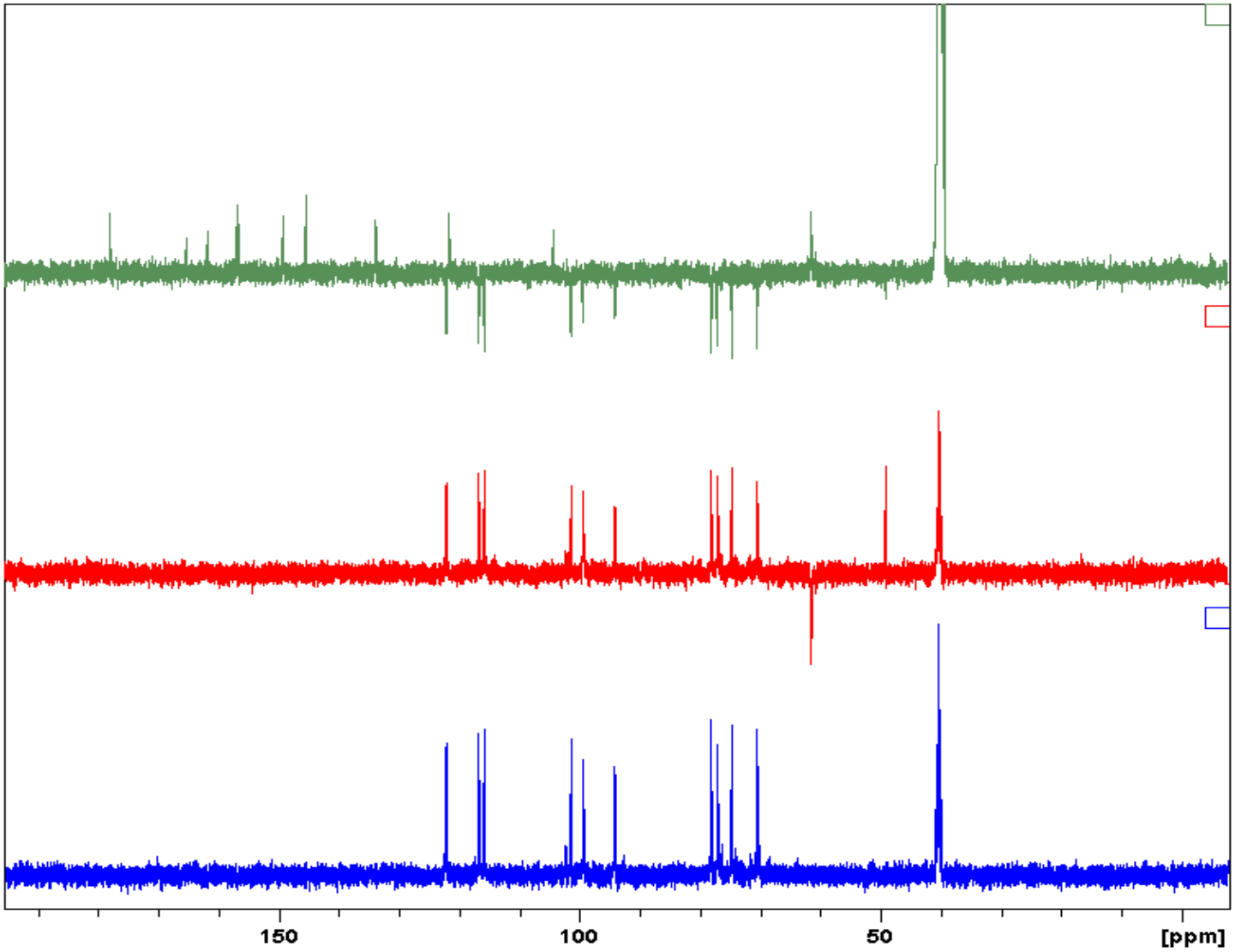
S26: LC-TOF/MS spectrum of kaempferol-3-*O*-*β*-glucoside (**3**)



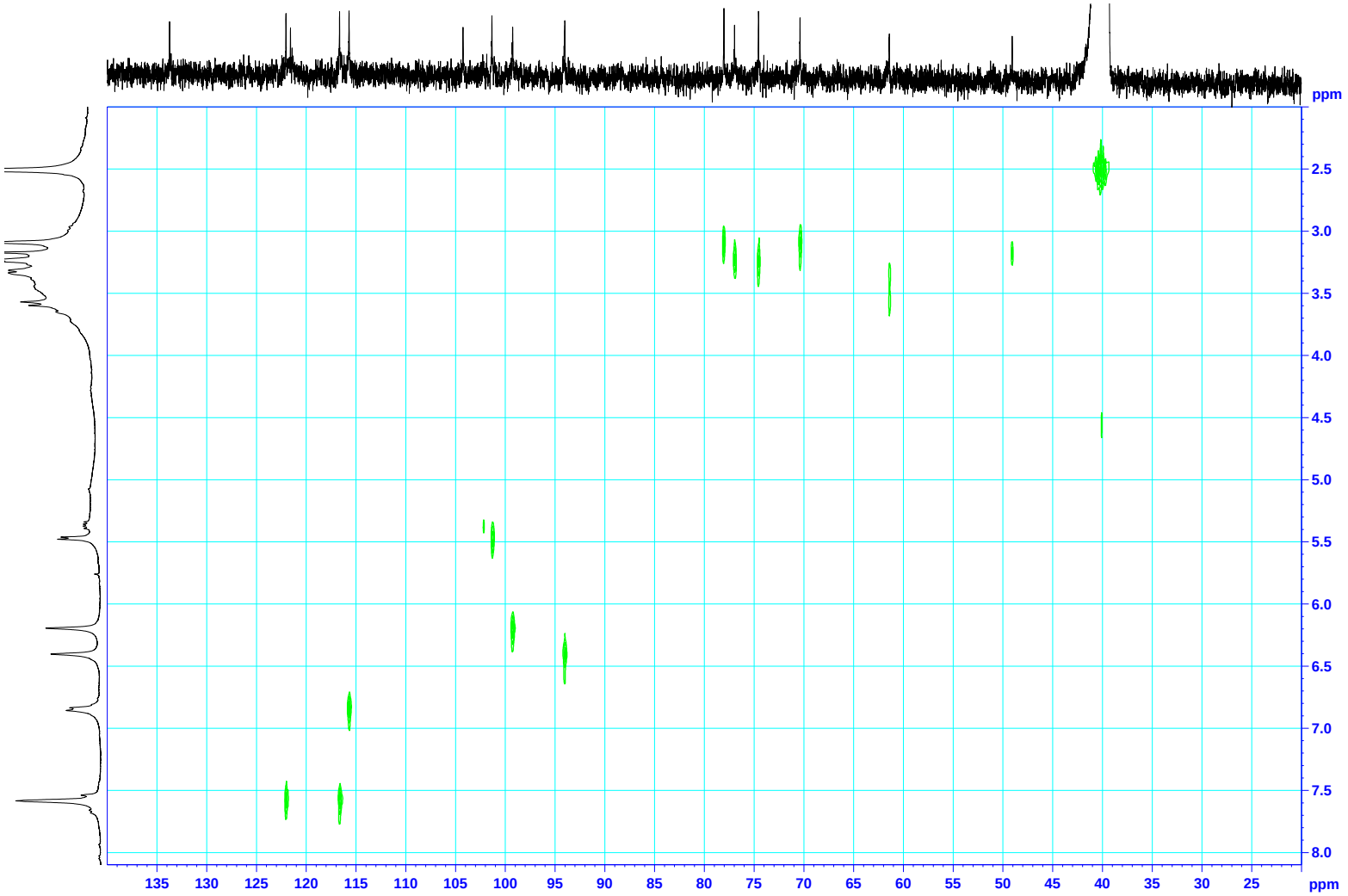
S27: ^{1}H-NMR (400 MHz, DMSO-*d6*) spectrum of quercetin-3-*O*-*β*-glucoside (**4**)



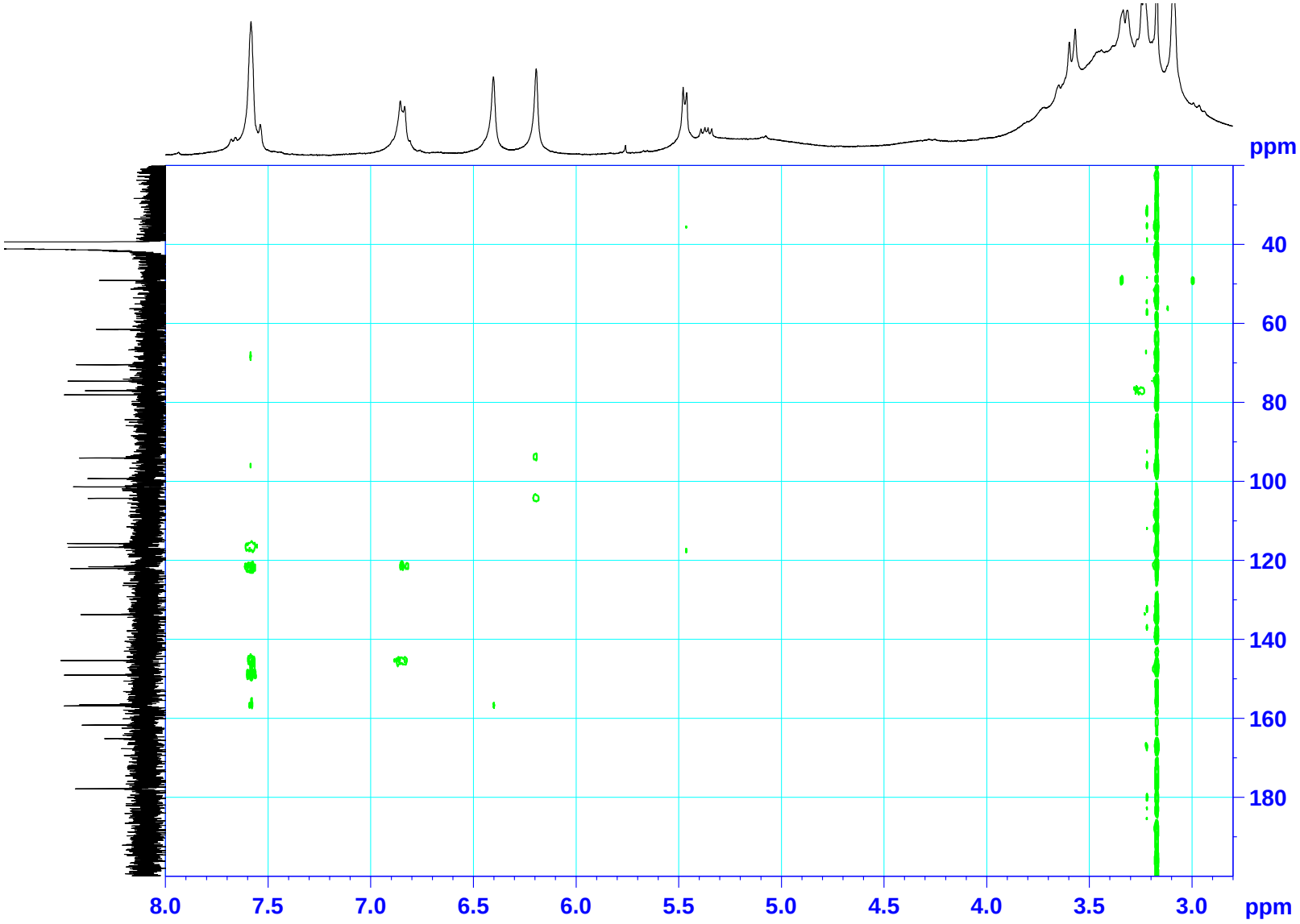
S28: ^{13}C-NMR (100 MHz, DMSO-*d6*) spectrum of quercetin-3-*O*-*β*-glucoside (**4**)



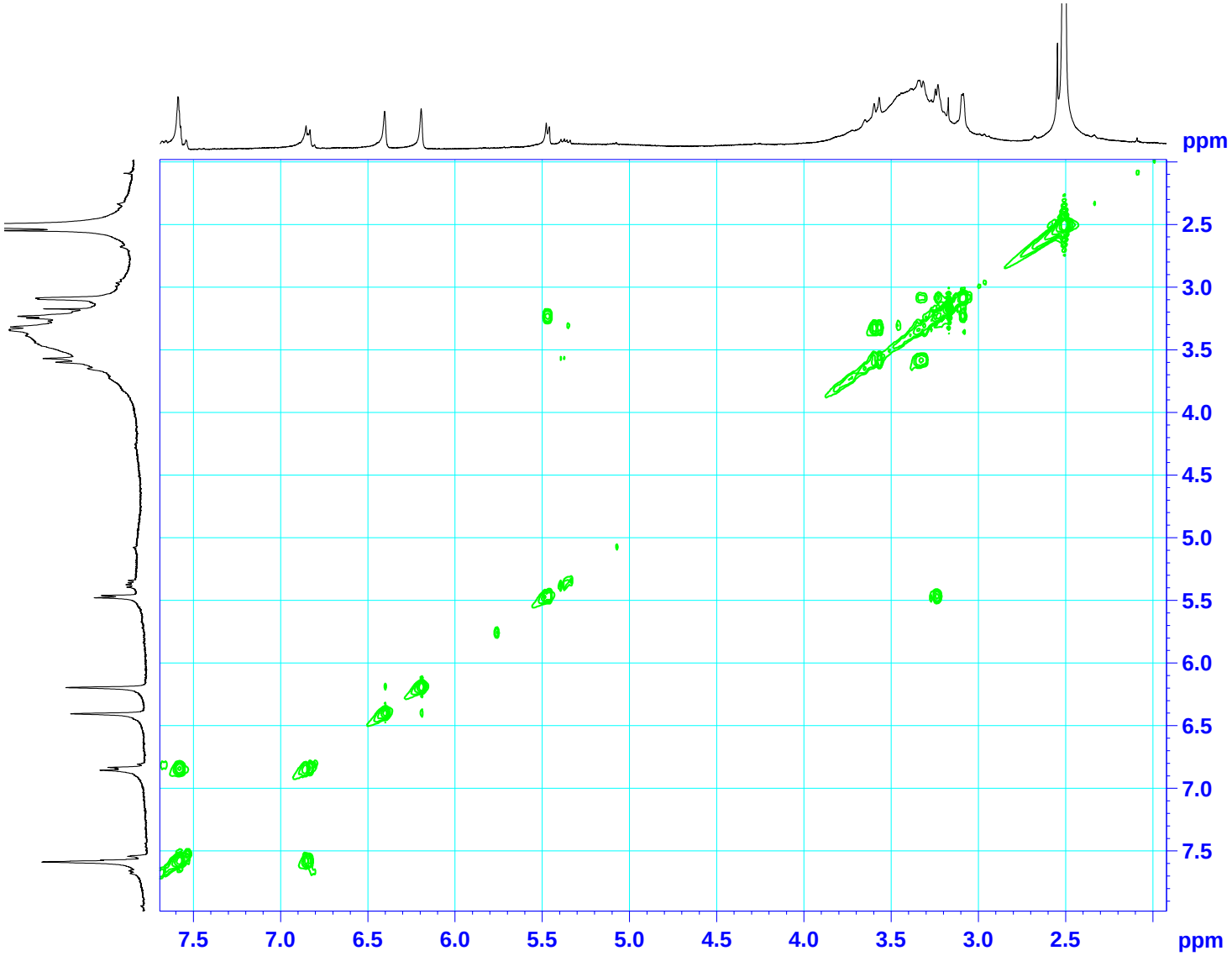
S29: APT, DEPT-135, DEPT-90 NMR (100 MHz, DMSO-*d6*) spectrum of quercetin-3-*O*-*β*-glucoside (**4**)



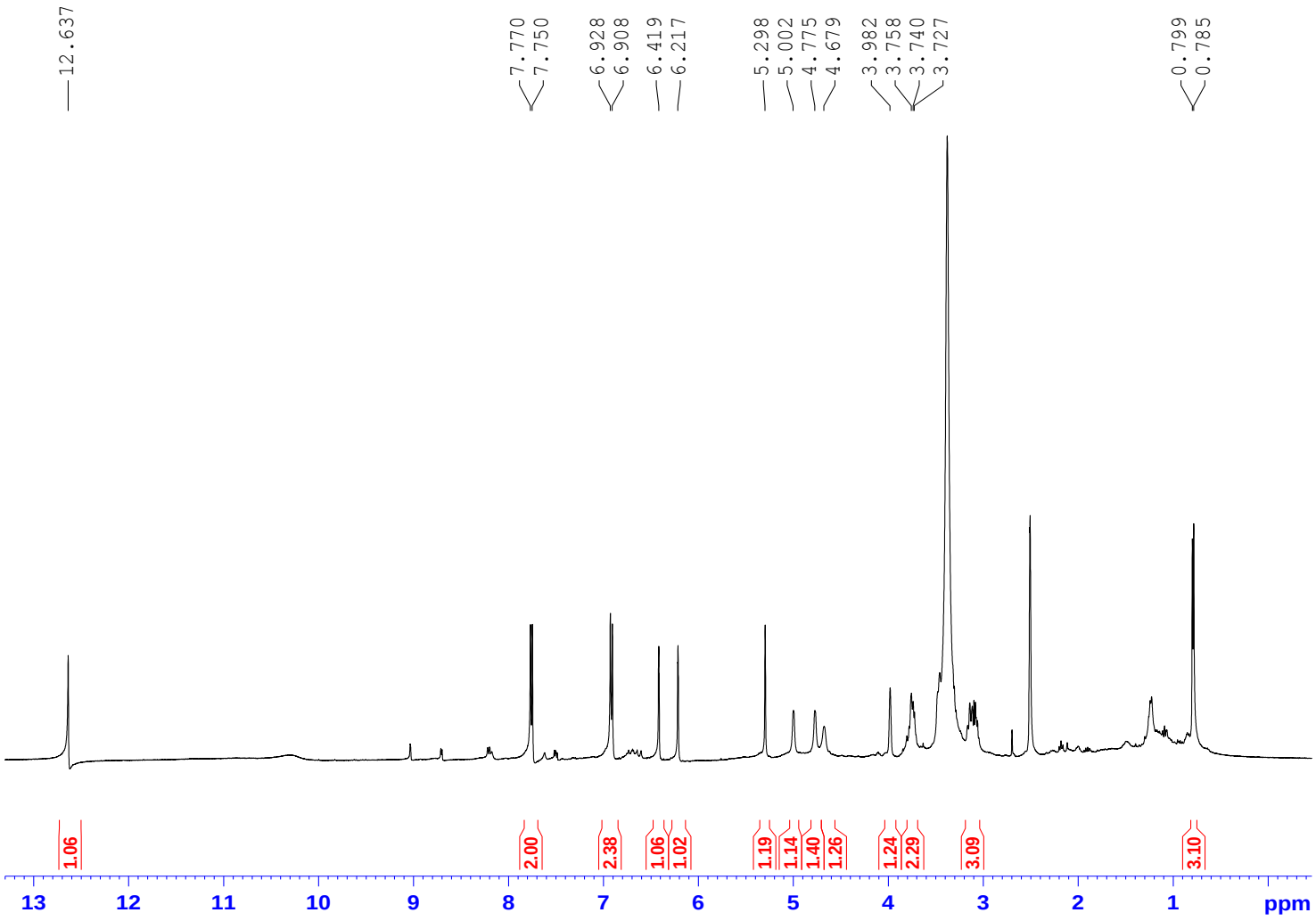
S30: HETCOR (400 MHz, DMSO-*d6*) Spectrum of quercetin-3-*O*-*β*-glucoside (**4**)



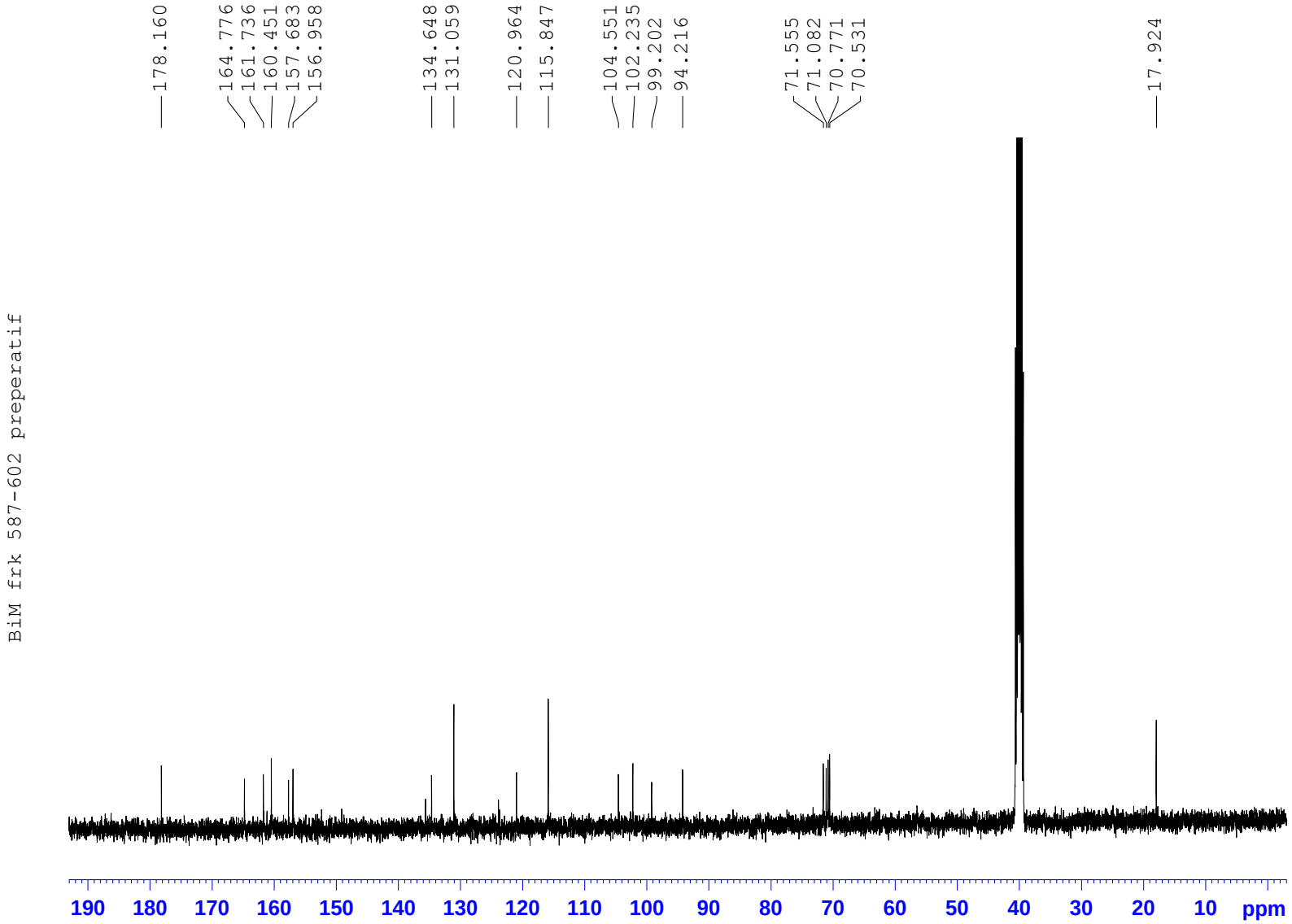
S31: HMBC (400 MHz, DMSO-*d6*) spectrum of quercetin-3-*O*-*β*-glucoside (**4**)



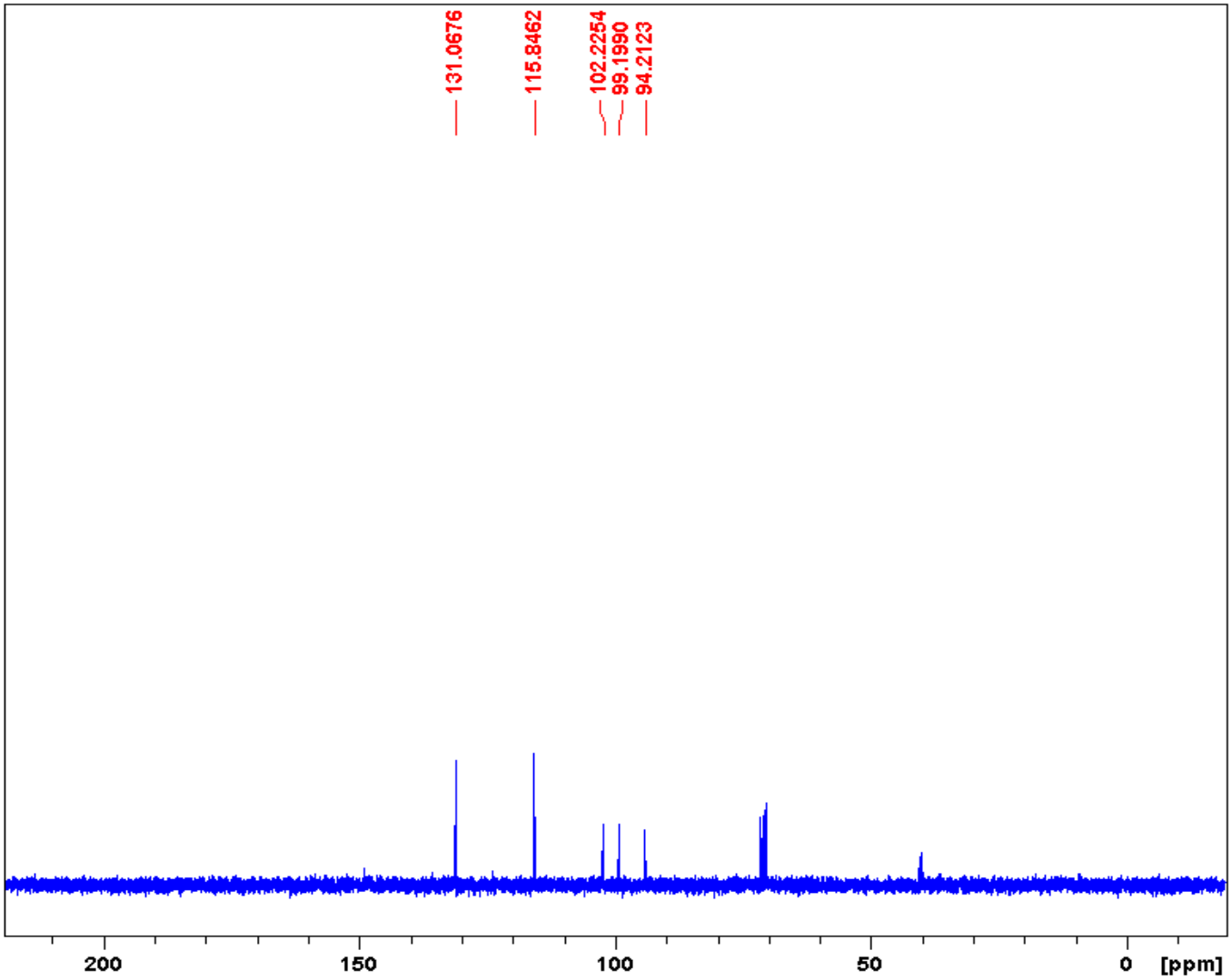
S32: COSY-90 (400 MHz, DMSO-*d6*) spectrum of quercetin-3-*O*-*β*-glucoside (**4**)



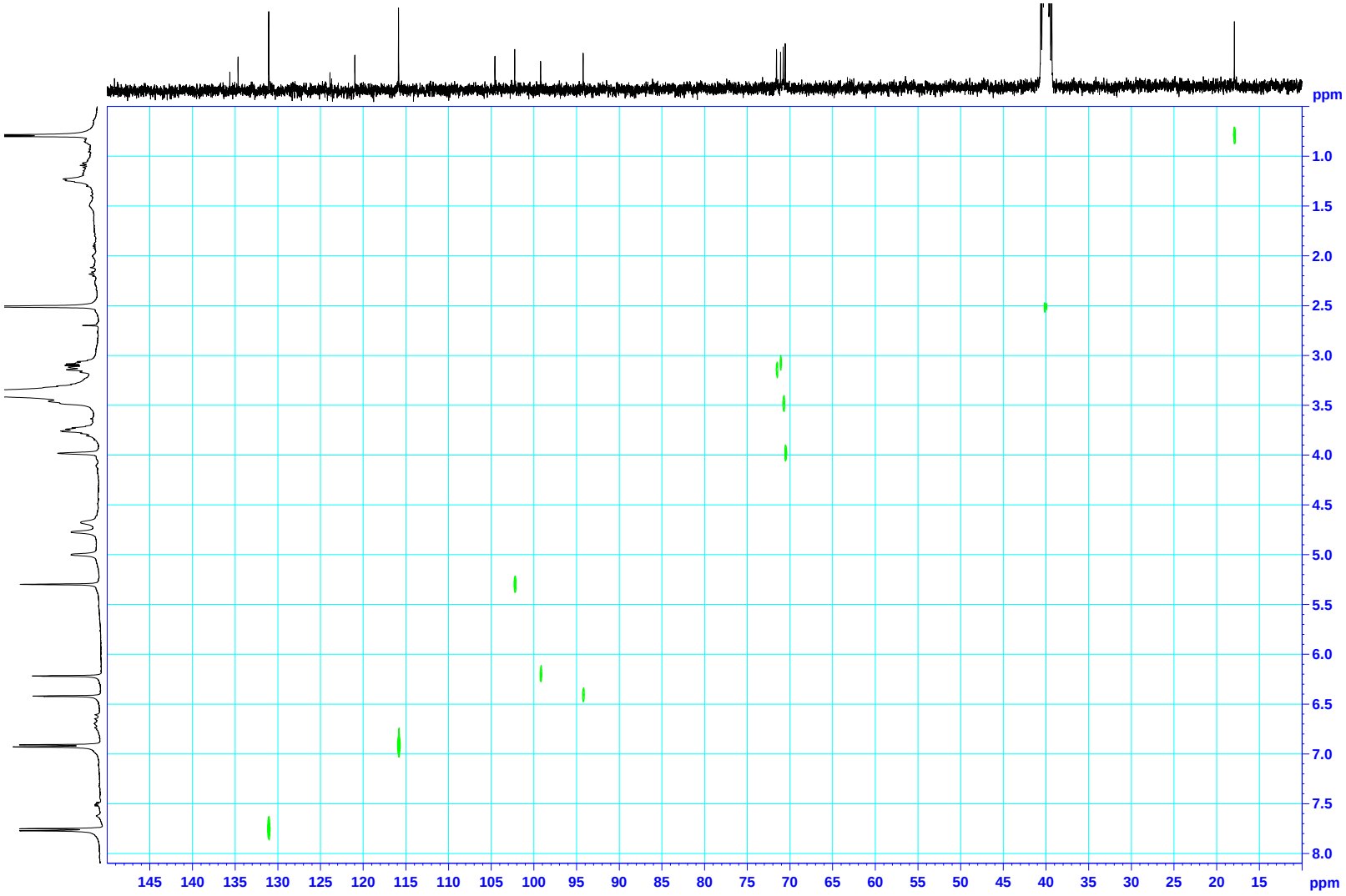
S33: ^{1}H-NMR (400 MHz, DMSO-*d6*) spectrum of kaempferol-3-*O*-*β*-rhamnopyranoside (**5**)



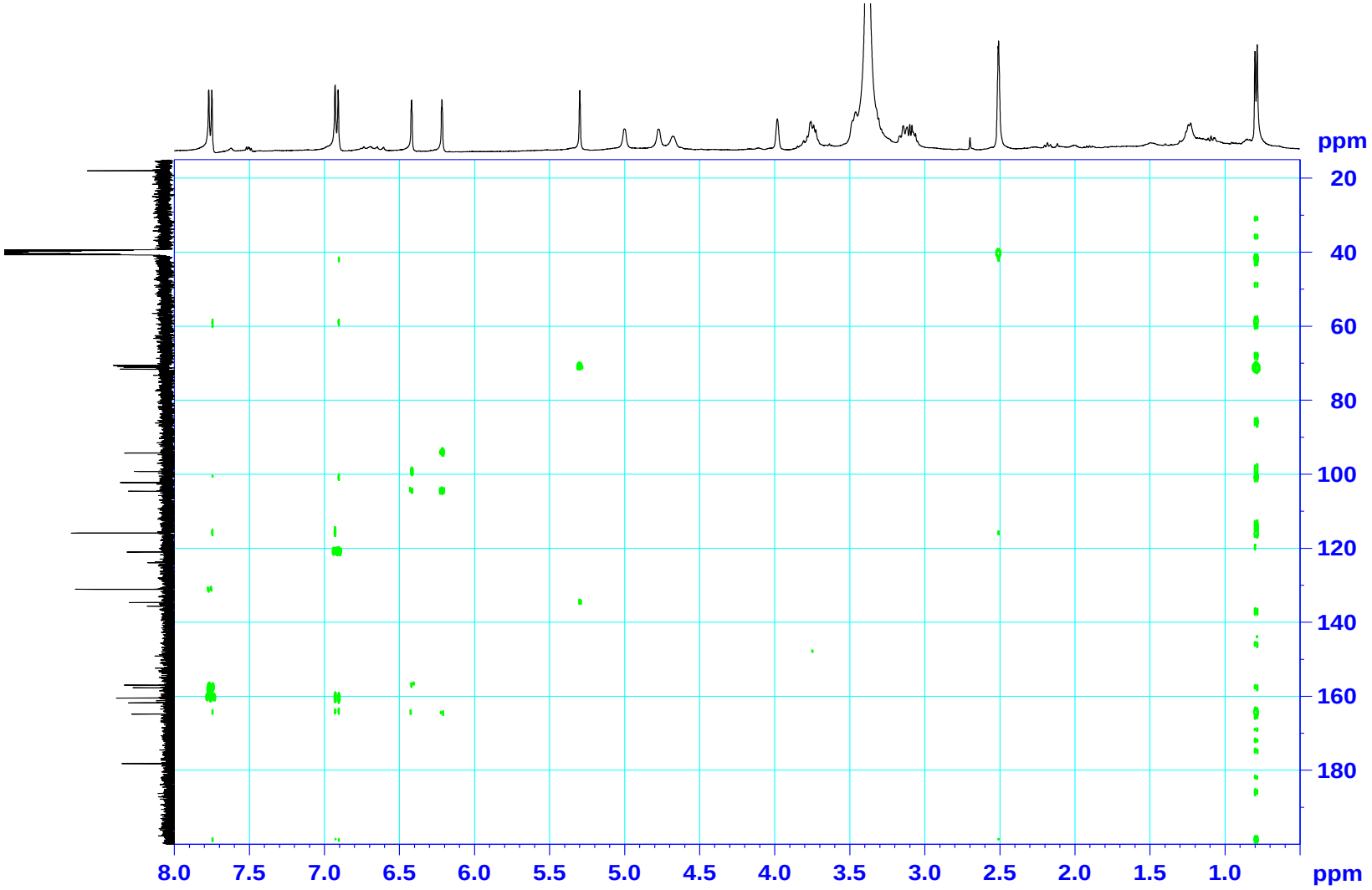
S34: ^{13}C-NMR (100 MHz, DMSO-*d6*) spectrum of kaempferol-3-*O*-*β*-rhamnopyranoside (**5**)



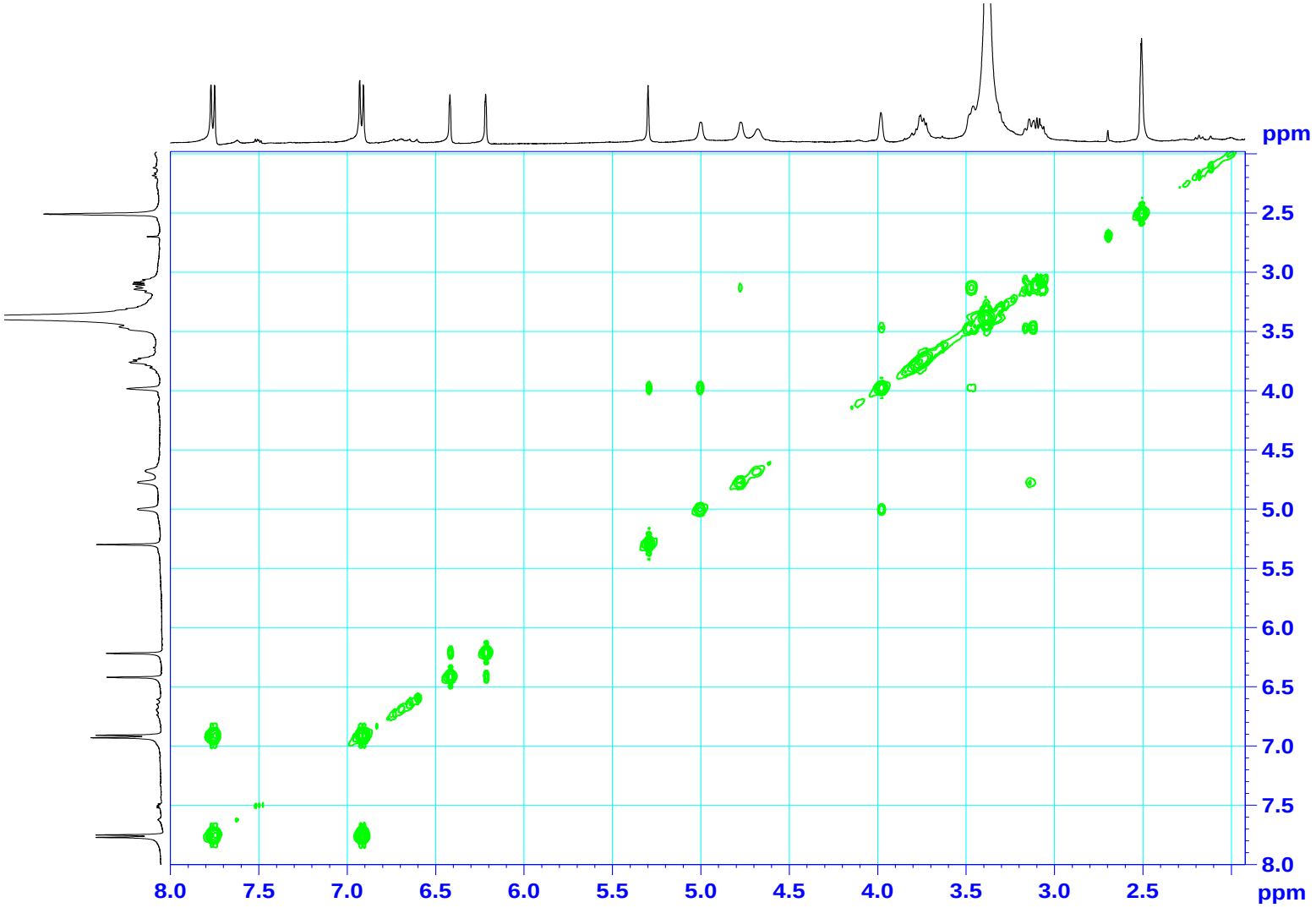
S35: DEPT-90 NMR (100 MHz, DMSO-*d6*) spectrum of kaempferol-3-*O*-*β*-rhamnopyranoside (**5**)



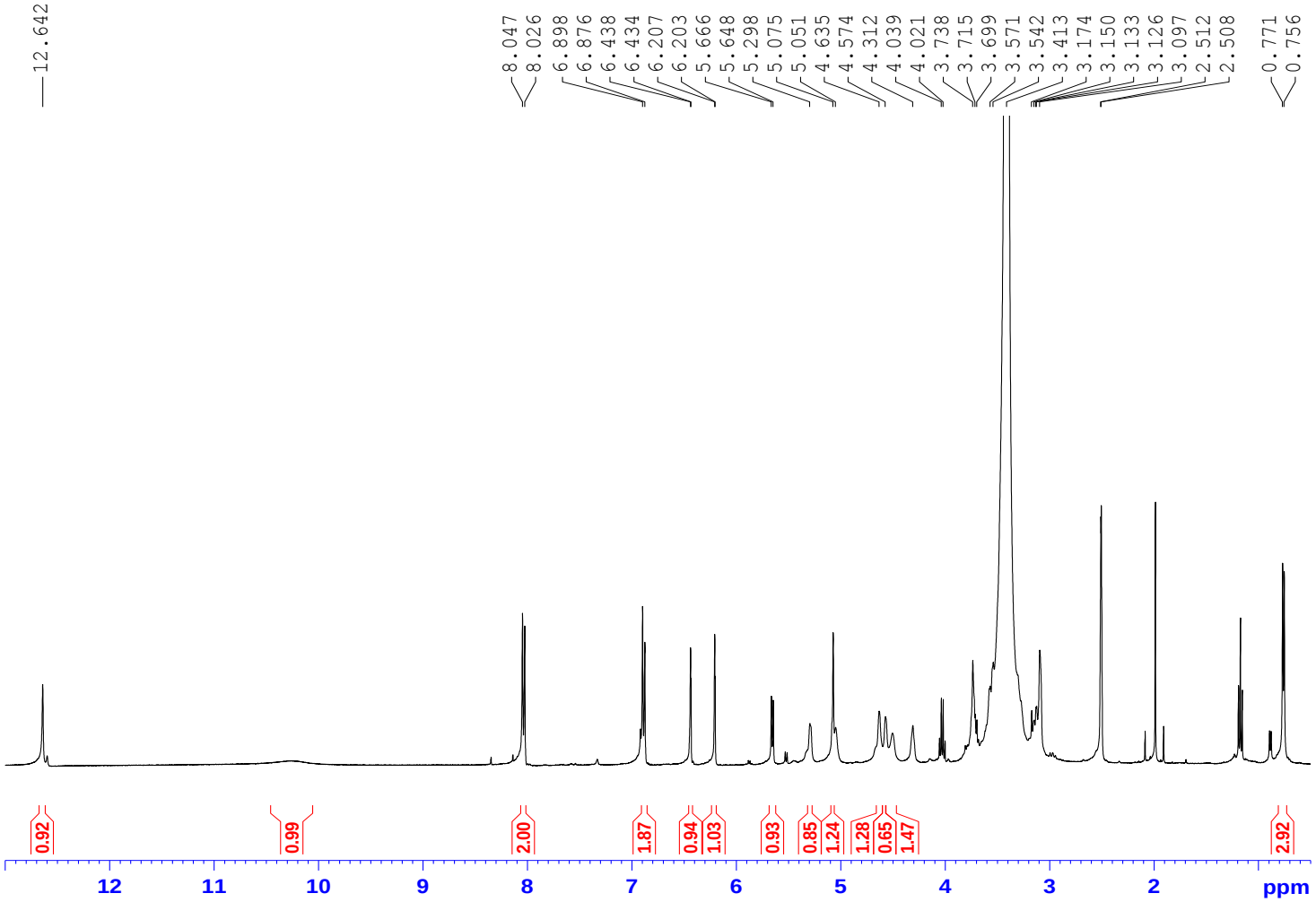
S36: HETCOR (400 MHz, DMSO-*d6*) spectrum of kaempferol-3-*O*-*β*-rhamnopyranoside (**5**)



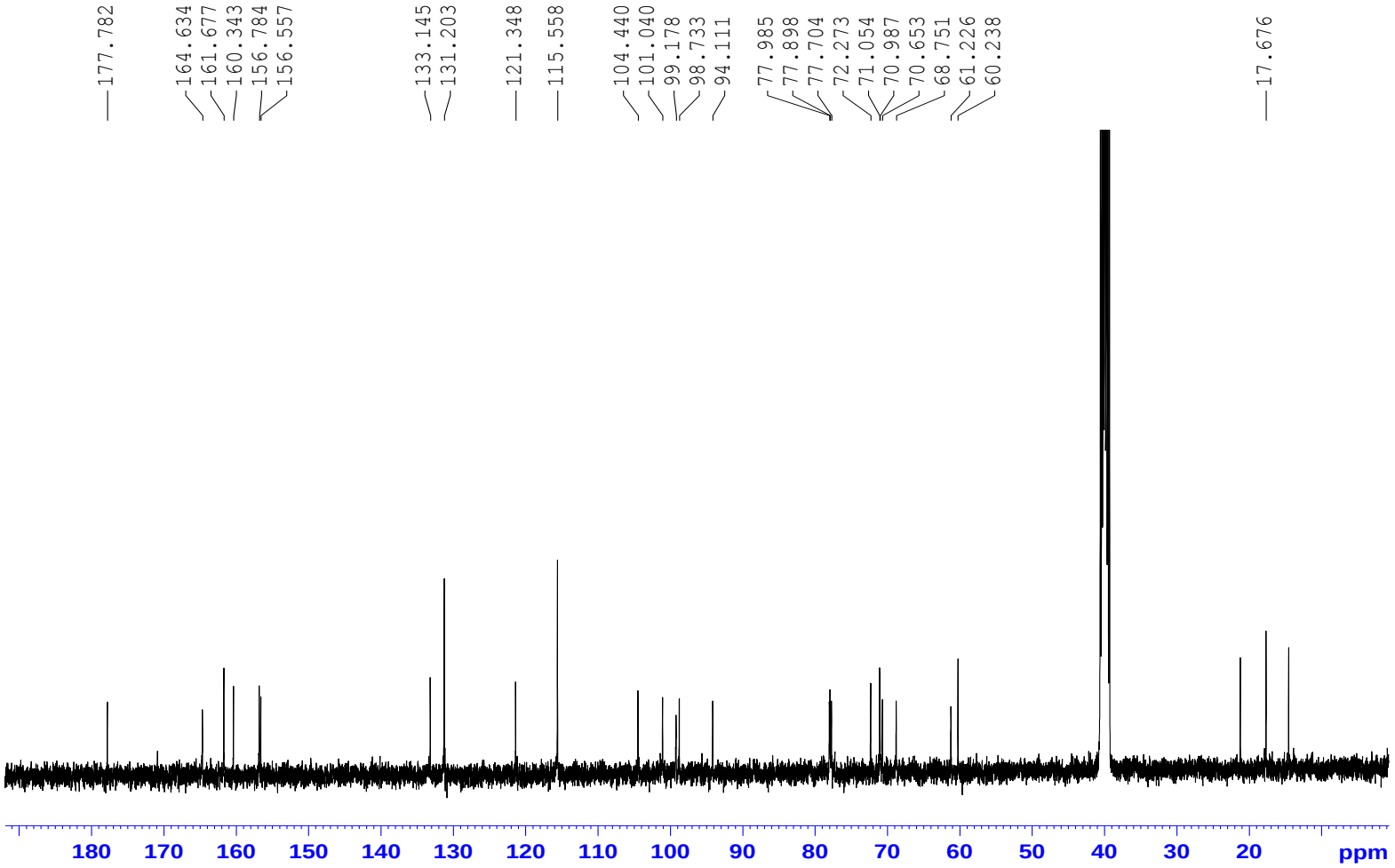
S37: HMBC (400 MHz, DMSO-*d6*) spectrum of kaempferol-3-*O*-*β*-rhamnopyranoside (**5**)



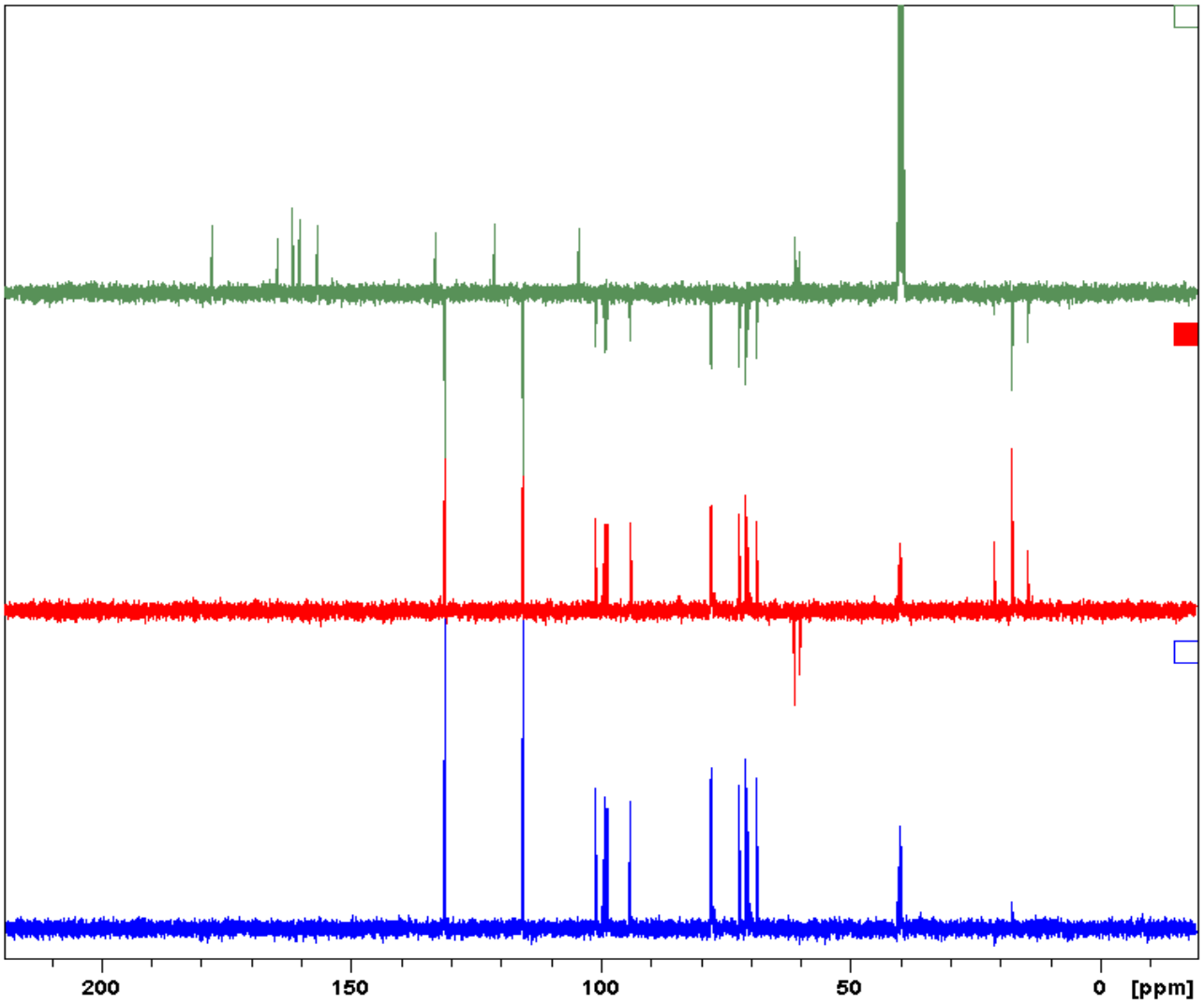
S38: COSY-90 (400 MHz, DMSO-*d6*) spectrum of kaempferol-3-*O*-*β*-rhamnopyranoside (**5**)



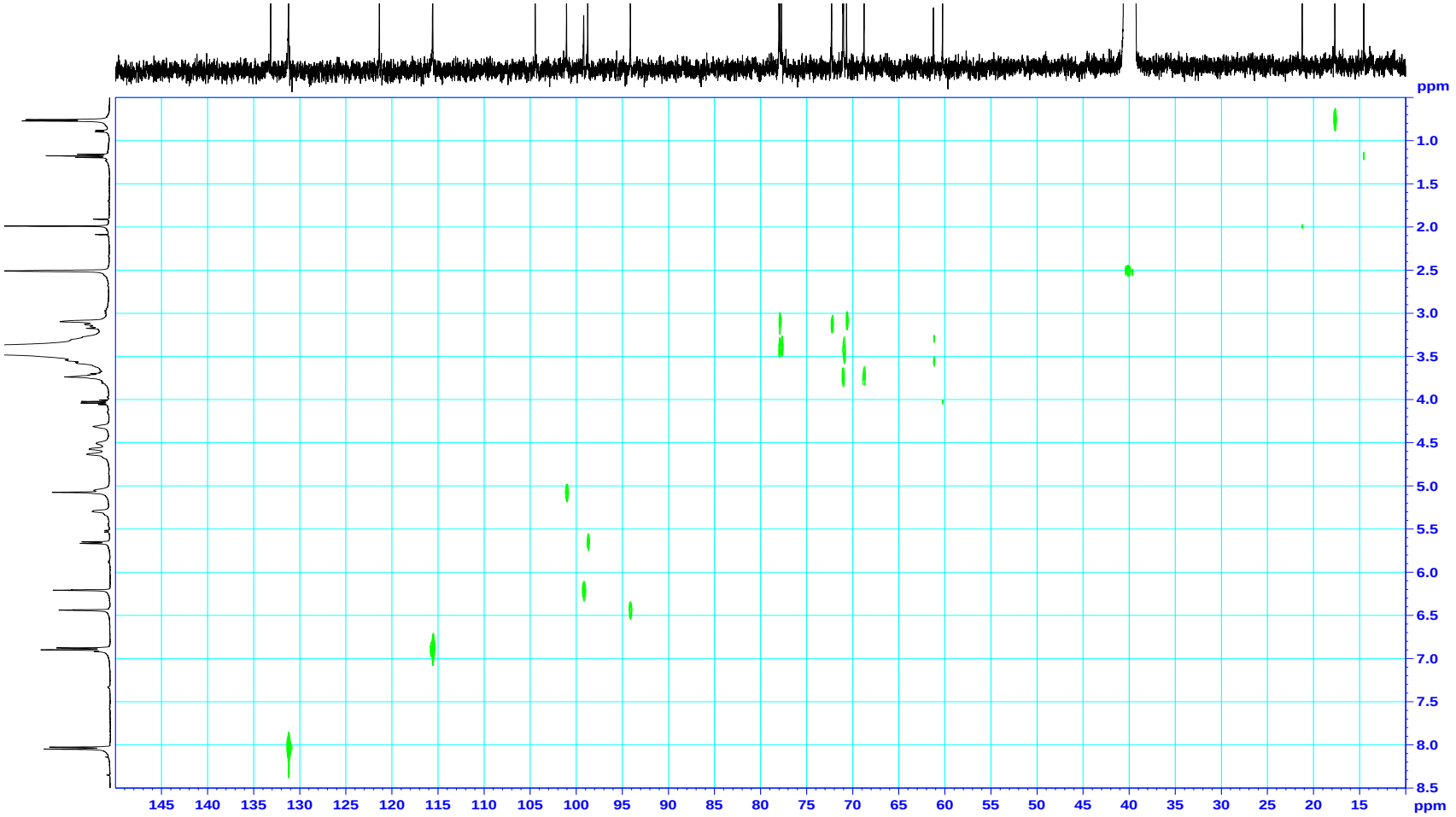
S39: ^{1}H-NMR (400 MHz, DMSO-*d6*) spectrum of kaempferol-3-*O*-*β*-D-neohesperidoside (**6**)



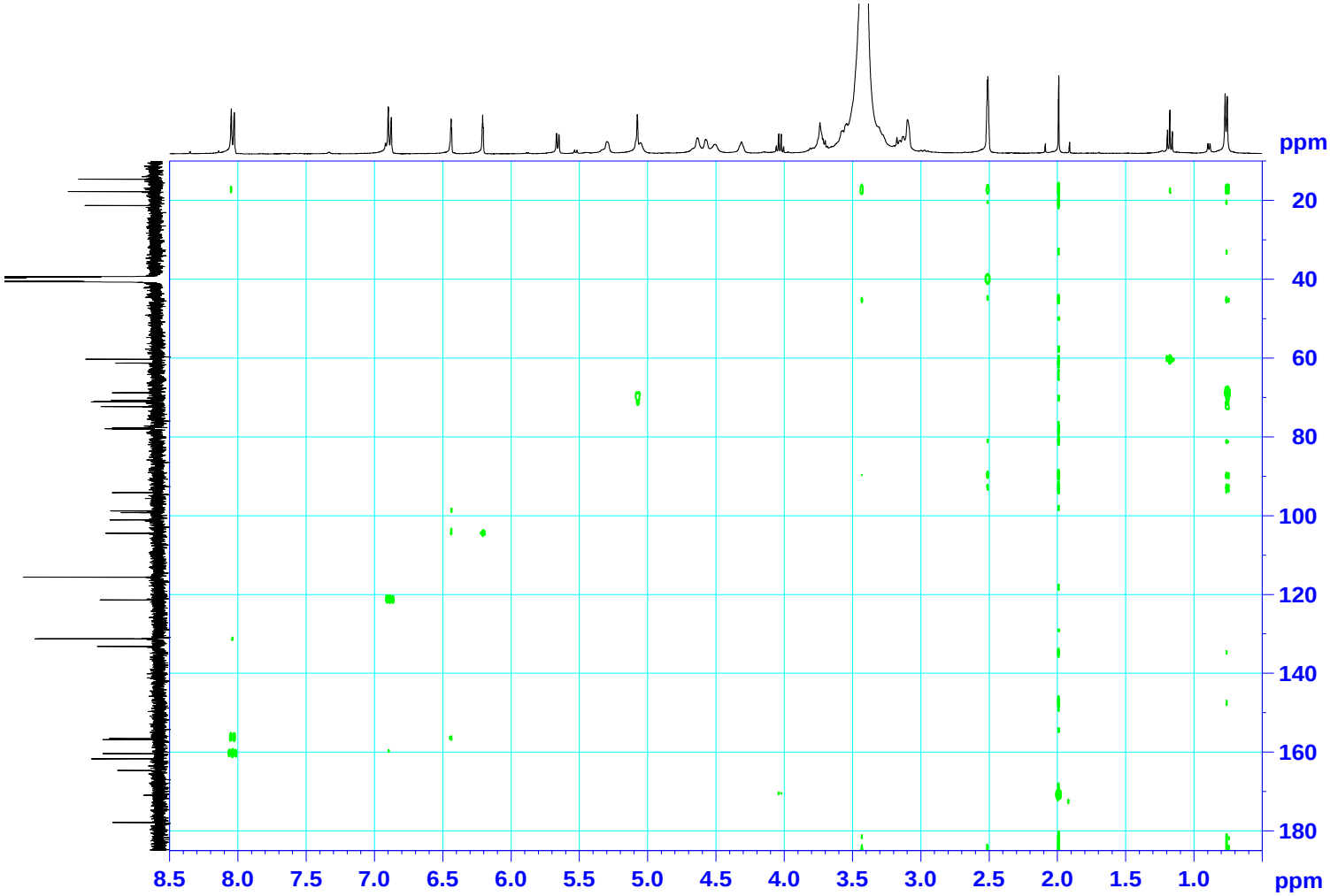
S40: ^{13}C-NMR (100 MHz, DMSO-*d6*) spectrum of kaempferol-3-*O*-*β*-D-neohesperidoside (**6**)



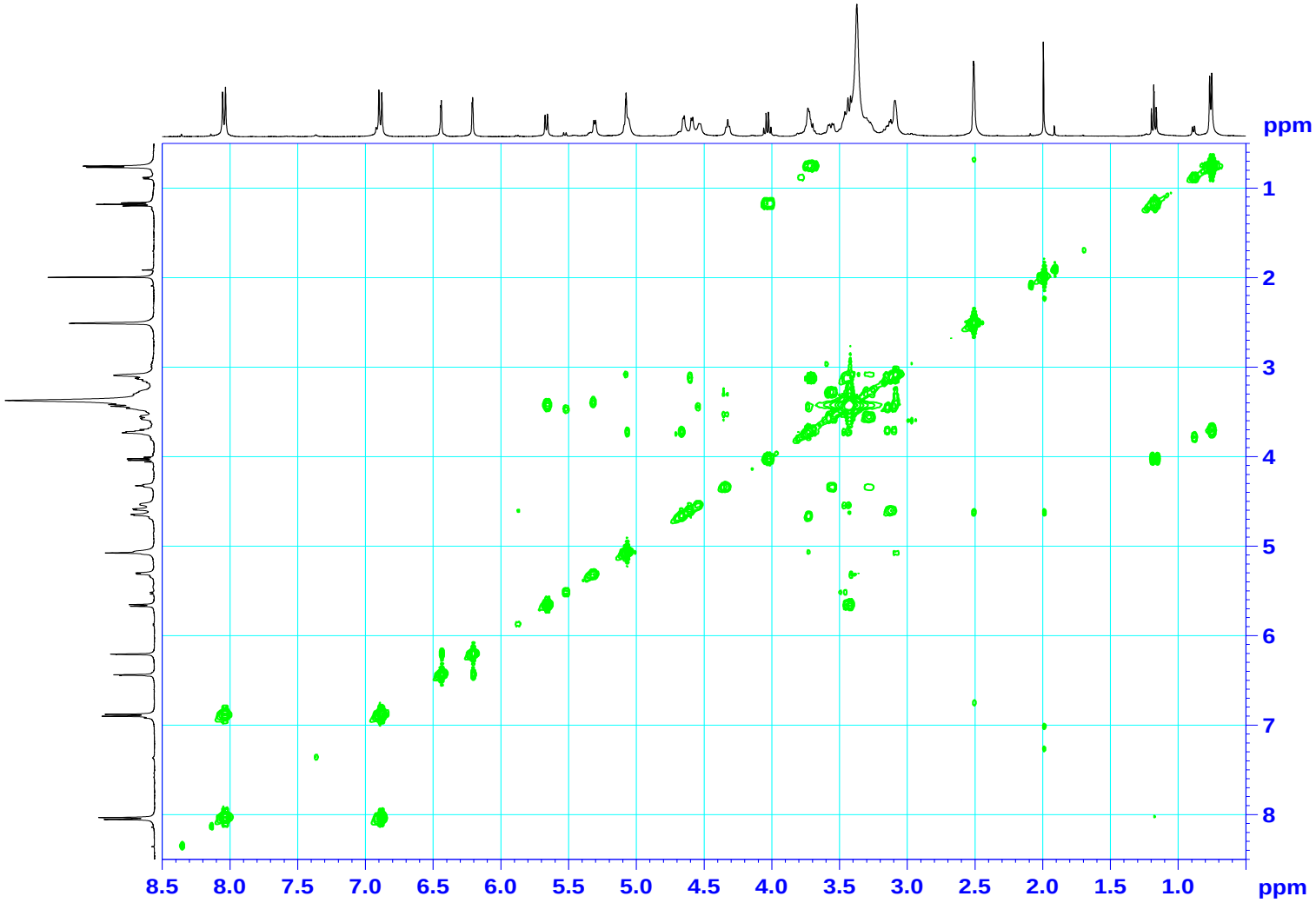
S41: APT, DEPT-135, DEPT-90 NMR (100 MHz, DMSO-*d6*) spectrum of kaempferol-3-*O*-*β*-D-neohesperidoside (**6**)



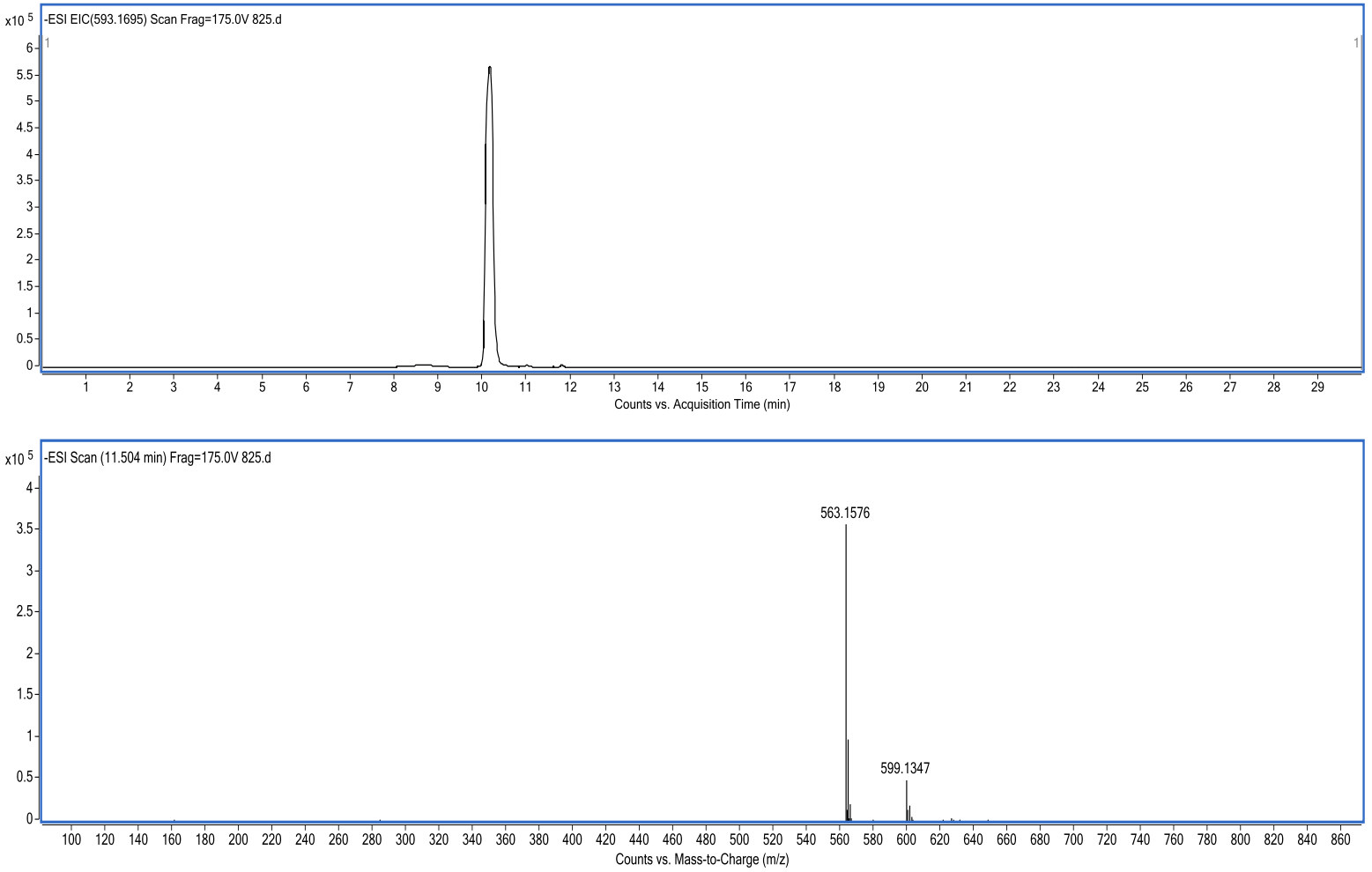
S42: HETCOR (400 MHz, DMSO-*d6*) Spectrum of kaempferol-3-*O*-*β*-D-neohesperidoside (**6**)



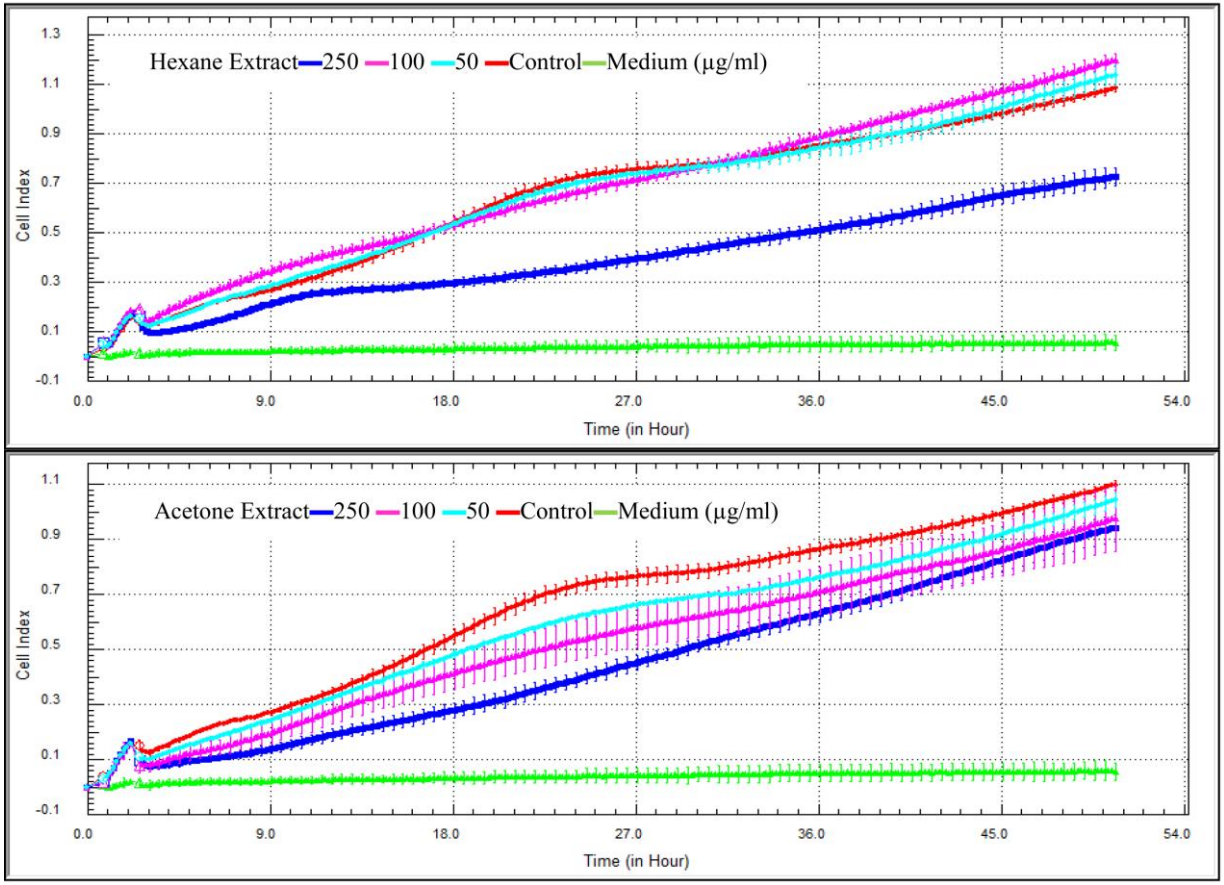
S43: HMBC (400 MHz, DMSO-*d6*) spectrum of kaempferol-3-*O*-*β*-D-neohesperidoside (**6**)



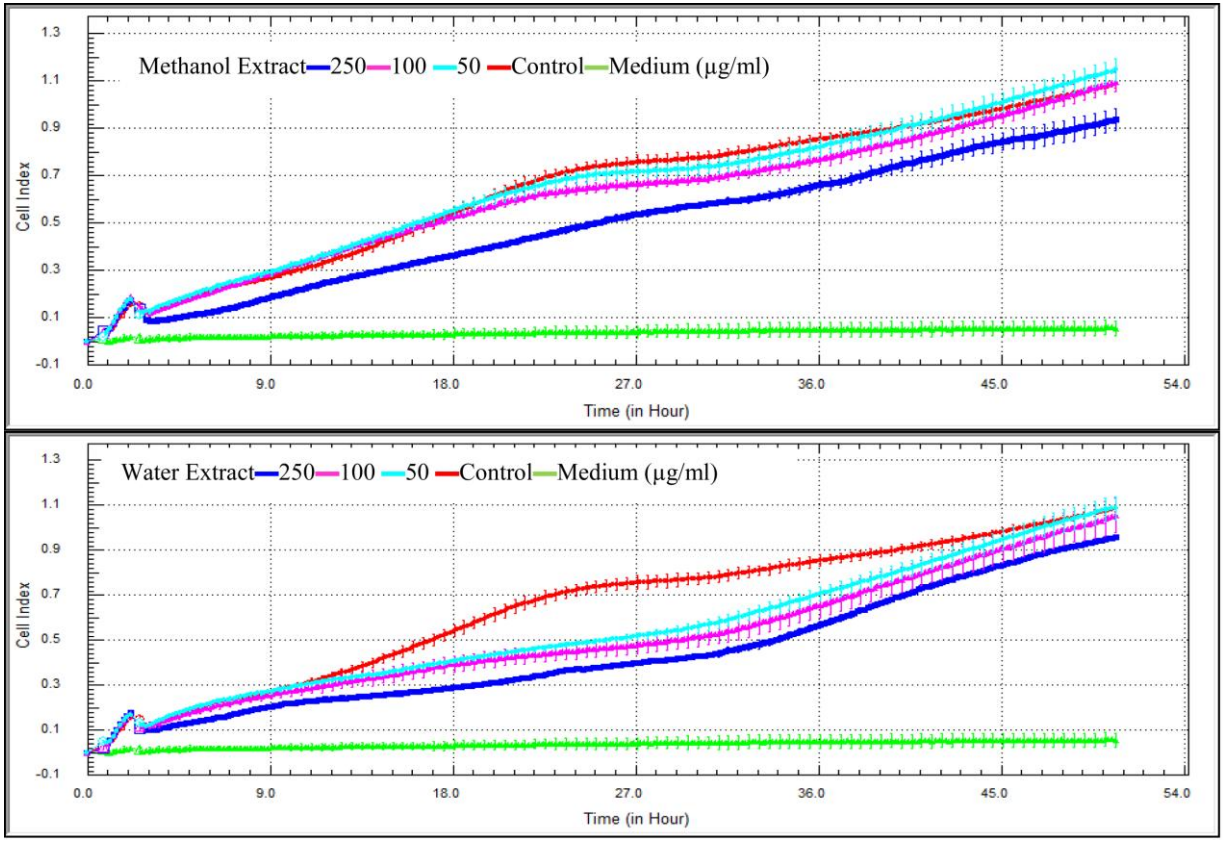
S44: COSY-90 (400 MHz, DMSO-*d6*) Spectrum of kaempferol-3-*O*-*β*-D-neohesperidoside (**6**)



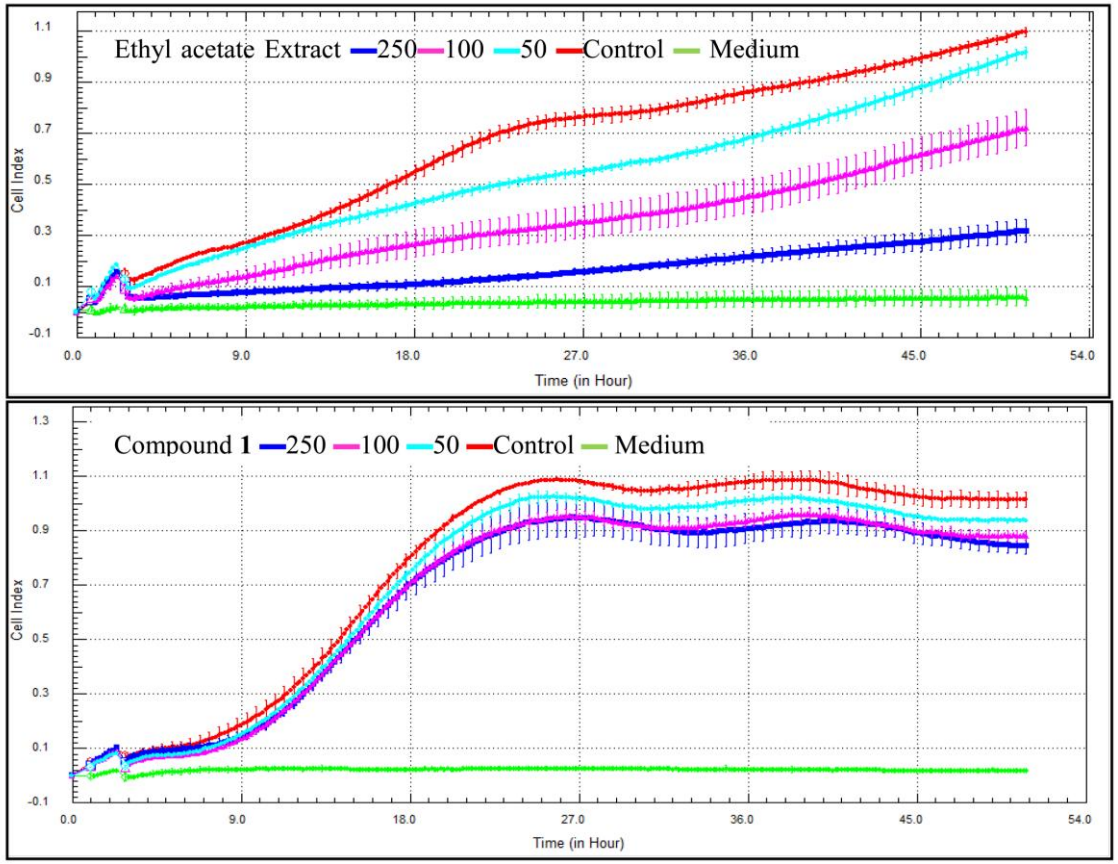
S45: LC-TOF/MS Spectrum of kaempferol-3-*O*-*β*-D-neohesperidoside (**6**)



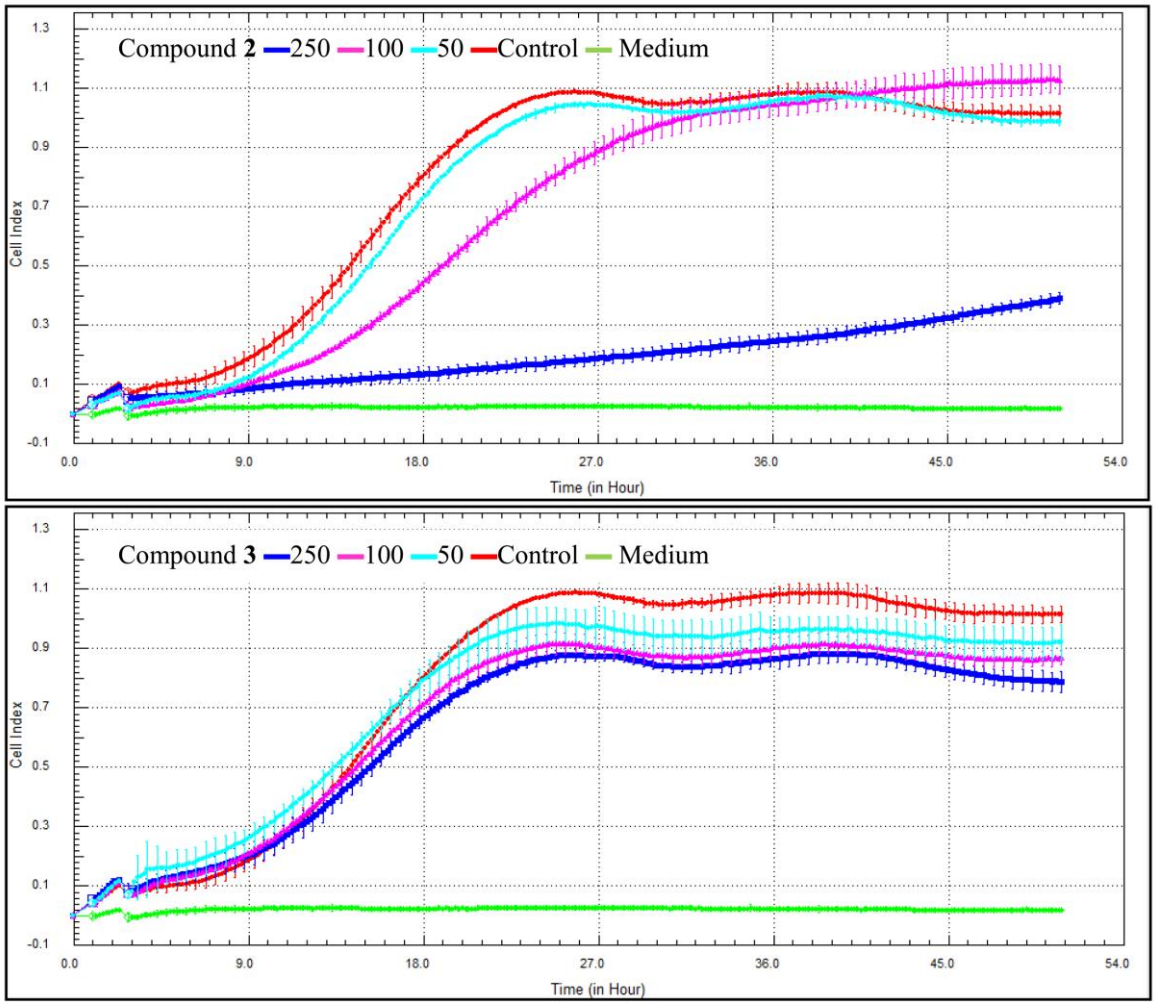
S46: Antiproliferative effects of hexane and acetone extracts



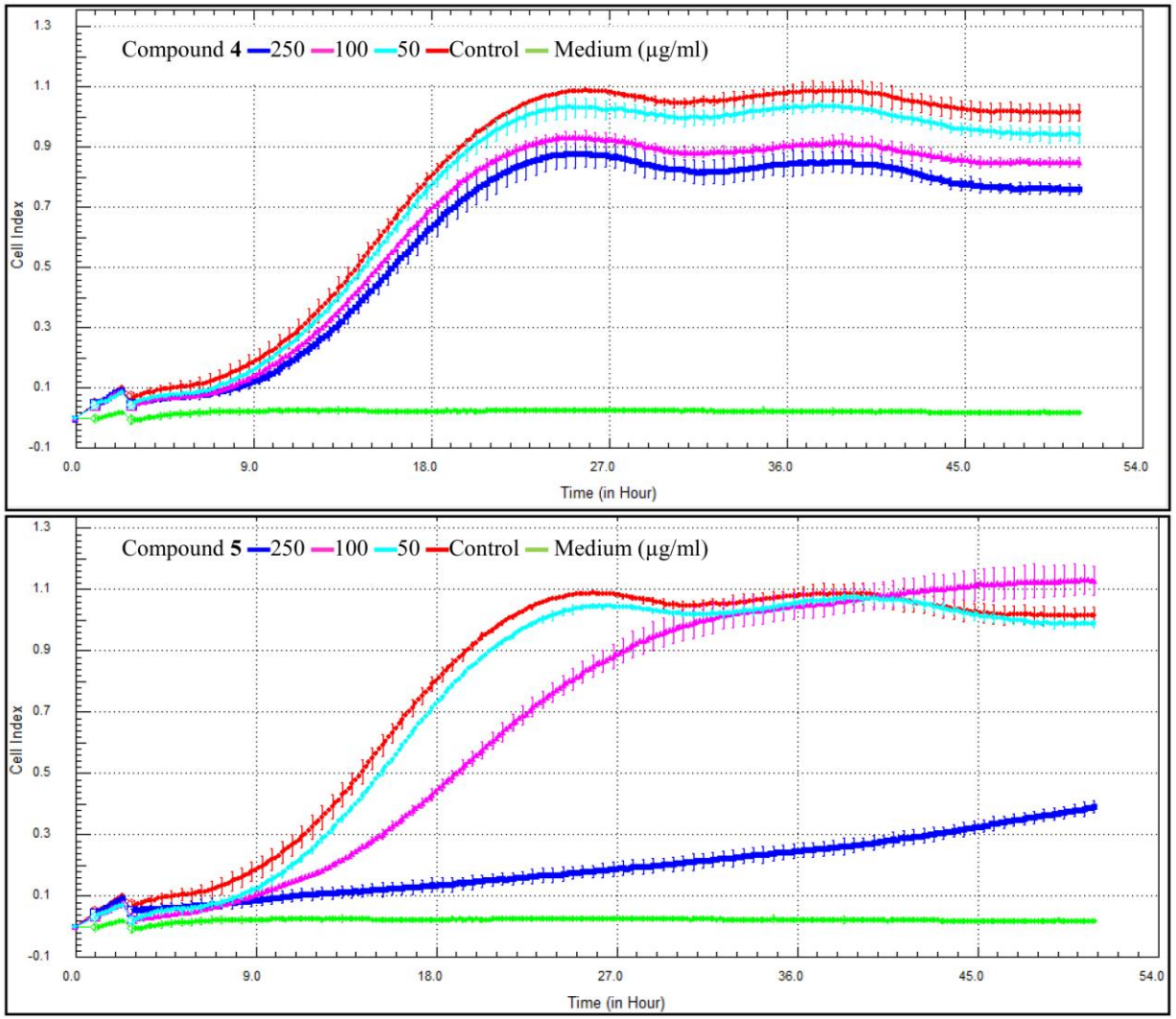
S47: Antiproliferaitve effects of methanol and water extracts



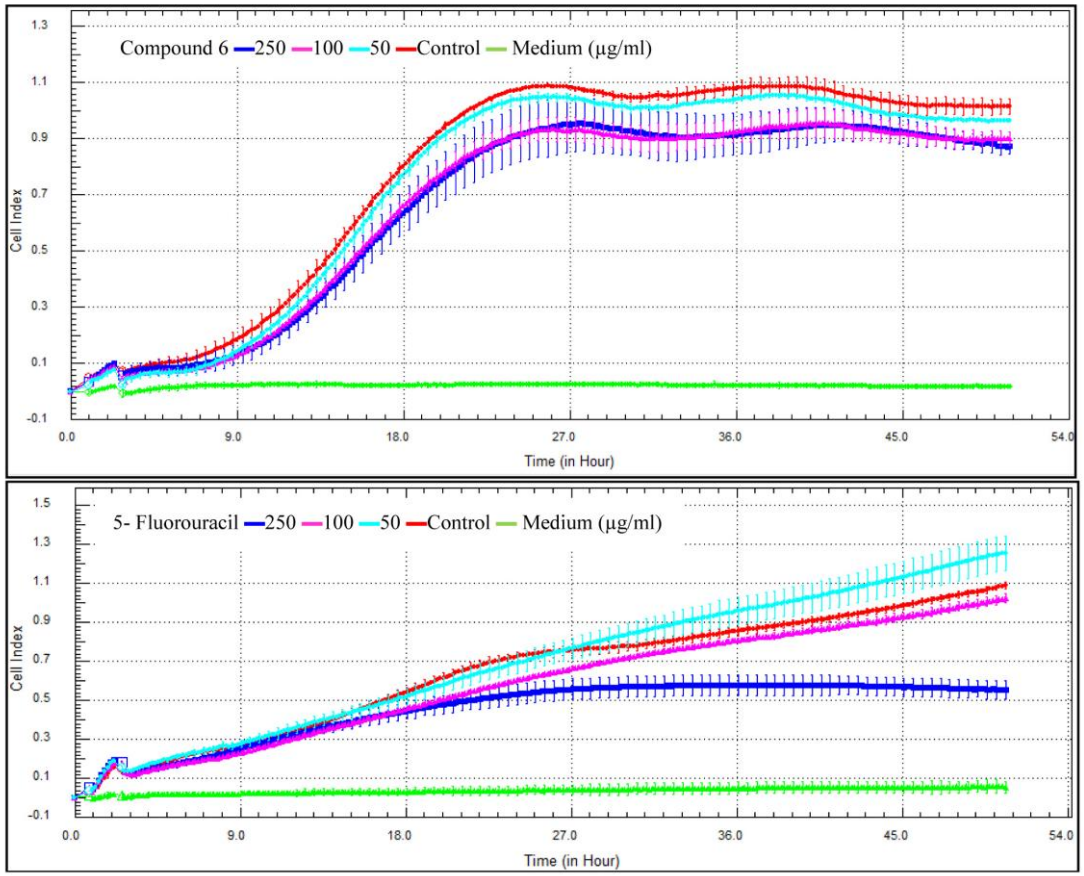
S48: Proliferation assay of ethyl acetate extract and compound **1** against HeLa cell lines



S49: Proliferation assay of compound **2** and compound **3** against HeLa cell lines



S50: Proliferation assay of compound **4** and compound **5** against HeLa cell lines



S51: Proliferation assay of compound **6** and standard, 5-fluorouracil against HeLa cell lines

S52:Table: Antiproliferative activites of the extracts and compounds from *Cynanchum acutum* L. Subsp. *sibiricum* (willd.) Rech. f.

	HeLa IC50 (µg/mL)			
	12 h	24 h	36 h	48 h
Hexane ext.	38,08	23,61	2,910	2,682
Acetone ext.	0,56	0,189	0,224	0,002
Methanol ext.	>100	1,734	0,492	7,023
Water ext.	>100	0,459	0,376	1,614
EtOAc ext.	0,042	0,047	0,064	0,122
Cmp 1	0,20	>100	23,81	>100
Cmp 2	0,20	>100	23,81	>100
Cmp 3	0,09	0,047	0,146	1,723
Cmp 4	0,28	0,055	0,072	0,339
Cmp 5	0,014	0,017	0,054	0,086
Cmp 6	0,028	0,013	0,007	0,041
5-FU	0,036	0,052	0,182	0,368

Table 1. ^{1}H NMR and ^{13}C NMR data of compound **1** (400 MHz for ^{1}H and 100 MHz for ^{13}C in DMSO-*d6*)

Pos.	δ_H	δ_C	Pos.	δ_H	δ_C
1				*β-D*-xsi (C-3_{xsi})	
2		160.6	1"	6.90 (d, *J* = 8.8)	115.7
3		133.1	2"	5.53 (d, *J* = 7.3)	99.8
4		177.5	3"	3.49 (m)	77.6
5		161.2	4"	3.31 (m)	70.1
6	6.18 (d, *J* = 1.4	99.4	5"α	3.34 (m)	77.3
7		165.5	5"β	2.97 (m)	66.4
8	6.41 (d, *J* = 1.4	94.2		*β-D*-xsi (C-2"$_{rha}$)	
9		156.3	1"'	5.09 (d, *J* = 1.2)	101.2
10		104.1	2"'	3.76 (m)	71.0
1'		121.1	3"'	3.49 (m)	70.9
2'	8.04 (d, *J* = 8.8)	131.1	4"'	3.18 (m)	72.3
3'	6.90 (d, *J* = 8.8)	115.7	5"'	3.81 (m)	68.9
4'		156.8	6"'	0.89 (d, *J* = 6.1)	17.7
5'	8.04 (d, *J* = 8.8)	131.1			
6'	6.90 (d, *J* = 8.8)	115.7			

Table 2. ^{1}H NMR and ^{13}C NMR data of compound **2, 3** and **4** (400 MHz for ^{1}H and 100 MHz for ^{13}C in DMSO-*d6*)

Pos.	Quercetin-3-*O*-*β*-xyloside (**2**)		kaempferol-3-*O*-*β*-glucoside (**3**)		Quercetin-3-*O*-*β*-glucoside (**4**)	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
2		156.8		157.0		156.8
3		133.6		133.6		133.7
4		177.8		177.7		177.8
5		161.6		161.6		161.6
6	6.19 (s)	99.3	6.19 (s)	99.7	6.19 (s)	99.3
7		166.1		166.1		165.1
8	6.41 (s)	94.1	6.41 (s)	94.4	6.40 (s)	94.0
9		156.6		156.5		156.5
10		106.9		103.7		104.3
1'		121.3		121.3	-	121.57
2'	7.58 (d, j=1.92)	116.5	8.06 (d, *J* = 8.3)	131.4	7.58 (d, *J* = 1.9)	116.63

3'		145.5	6.86 (d, *J* = 8.3)	115.53	-	145.32
4'		149.2		160.5	-	149.01
5'	6.85 (d, j=8.32)	116.8	6.86 (d, *J* = 8.3)	115.5	6.85 (d, *J* =7.0)	115.69
6'	7.54 (dd, j=8.32, 1.92)	121.9	8.06 (d, *J* = 8.3)	131.4	7.54 (dd, *J* =7.5, 1.9)	122.03
1"	5.34 (d, j=7.36)	102.3	5.39 (d, *J* = 5.6)	102.3	5.34 (d, *J* =6.9)	101.34
2"	3.34 (m)	74.0	3.54 (m)	71.7	3.24 (m)	74.55
3"	3.18 (m)	76.4	3.39 (m)	73.6	3.23 (m)	76.96
4"	3.32 (m)	69.9	3.66 (m)	68.3	3.08 (m)	70.38
5"α	3.54 (m)	66.5	3.10 (m)	76.2	3.09 (m)	78.02
5"β	3.74 (m)					
6"α			3.33 (m)	60.6	3.35 (m)	61.41
6"β			3.53 (m)		3.58 (m)	

Table 3. ^{1}H NMR and ^{13}C NMR data of compound **5** and **6** (400 MHz for ^{1}H and 100 MHz for ^{13}C in DMSO-*d6*)

	kaempferol-3-*O*-*β*-rhamnopyranoside **(5)**		kaempferol-3-*O*-*β*-D-neohesperidoside **(6)**	
Pos.				
	δ_H	δ_C	δ_H	δ_C
2		157.7		160.3
3		133.6		133.1
4		178.1		177.7
5		161.7		161.6
6	6.21 (d, *J* = 1.6)	99.3	6.21 (s)	99.1
7		164.1		164.6
8	6.41 (d, J = 1.6)	94.1	6.44 (s)	94.1
9		154.9		156.5
10		104.5		104.4
1'	-	121.2		121.3
2'	7.75 (dd, *J* =7.9, 1.6)	131.4	8.04 (d, *J* = 8.6)	131.2
3'	6.92 (dd, *J* =7.9, 1.6)	116.2	6.90 (d, *J* = 8.6)	115.5
4'	-	160.4	-	156.7

5'	6.92 (dd, *J* =7.92, 1.6)	116.2	8.04 (d, *J* =8.6)	131.2
6'	7.75 (dd, *J* =7.92, 1.6)	131.4	6.90 (d, *J* =8.6)	115.5
1"	5.29 (d, *J* =5.72)	102.6	5.66 (d, *J* =7.1)	98.7
2"	3.98 (m)	70.5	3.39 (m)	77.9
3"	3.48 (m)	70.7	3.35 (m)	77.7
4"	3.15 (m)	71.5	3.09 (m)	70.6
5"α	3.08 (m)	71.1	3.42 (m)	77.8
5"β				
6"α	0.82 (d, *J* =6.1)	17.9	3.30 (m)	61.2
6"β			3.54 (m)	
1"'			5.09 (d, *J* =5.0)	101.0
2"'			3.14 (m)	70.0
3"'			3.74 (m)	71.0
4"'			3.42 (m)	72.3
5"'			3.73 (m)	68.7
6"'			0.76 (d, J =6.1)	17.67

S 53: References

Okten S, Cakmak O, Erenler R, Yuce O, Tekin S. 2013. Simple and convenient preparation of novel 6,8-disubstituted quinoline derivatives and their promising anticancer activities. Turk J Chem. 37:896-908.