SUPPLEMENTARY MATERIAL

Two new norlignans from the aerial parts of *Pouzolzia sanguinea* (Blume) Merr.

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ABSTRACT

Two new norlignans, pouzolignan N (**1**) and pouzolignan O (**2**), together with five known norlignans, pouzolignan F (**3**), pouzolignan G (**4**), pouzolignan H (**5**), pouzolignan L (**6**), and gnetifolin F (**7**) were isolated from the aerial parts of *Pouzolzia sanguinea* (Blume) Merr.. Their chemical structures were elucidated via HR-ESI-MS and NMR spectroscopic methods. Absolute configurations at stereocenters were confirmed by comparisons of CD spectra with those of TD-DFT calculations. Compounds **1**–**6** exhibited chemical structures unique to *Pouzolzia* species. At a concentration of 30 µM, compounds **1**–**7** exhibited weak cytotoxic activity toward CAL27 and MDA-MB-231 cell lines (cell viability from 65.3 ± 0.86 to 98.8 ± 1.23%). They also inhibited anoctamin-1 activity with inhibitory rates from 8.1 ± 0.87 to 24.3 ± 1.41%.

Keywords: *Pouzolzia sanguinea* (Blume) Merr., Pouzolignan N, Pouzolignan O, Nuclear magnetic resonance, Cytotoxicity, Anoctamin-1 inhibitor.

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Content

Cytotoxic assay

Cytotoxic effects of the isolated compounds **1-7** towards CAL27 and MDA-MB-231 cell lines were evaluated by MTT assay in a 96 wells plate. Cells were cultured in supplemented medium containing 10% fetal bovine serum, 100 U/mL penicillin, and 100µg/mL streptomycin (DMEM for CAL-27 cells and RPMI 1640 for MDA-MB-231 cells). The exponentially growing cells (1×10^5 cells/mL) were treated for 3 days with the compounds (final concentration 30 µM). After incubation, 50 µL of MTT solution (2 mg/mL) was added to each well and the cells were then incubated at 37°C for 4 h. The media was then carefully aspirated and dimethylsulfoxide (150 µL) was added to each well to dissolve the formazan crystals. Absorbance was read at 540 nm on a microplate reader (TECAN GENIOUS). Experiments were performed in triplicate. The cell viability is expressed as the percentage of absorbance in sample wells compared to the untreated control.

Anoctamin-1 (ANO1) inhibitory activity

Yellow fluorescent protein (YFP) reduction analysis was performed using FLUOstar Omega microplate reader and MARS Data Analysis Software. ANO1 and YFP expressing FRT cells were plated in 96-well black-walled microplates at a density of 1.5×10^4 cells per well. Each well was washed twice in PBS (200 µL/wash) and then the compounds (2 µL) were added to reach 30 µM (final concentration). After incubation at 37°C for 10 min, plates were transferred to a plate reader for fluorescence analysis. Each well was individually measured for ANO1-mediated I^- influx by monitoring YFP fluorescence continuously (400 ms per point) for 2s (baseline). After that, 100 µL of 140 mM I^- solution containing 200 µM ATP was added at 2s and then YFP fluorescence was recorded for 6s. The initial iodide influx rate was determined from the initial slope of fluorescence reduction by nonlinear regression after iodide injection with ATP.

Theoretical calculation of CD spectra for compound 1

Conformational studies were done using Spartan 14 program. Conformer optimization and TDDFT calculation were performed by Gaussian 16 program. The calculated CD spectra were generated based on the Boltzmann distribution of optimized conformers by SpecDis v1.64 software. Two possible enantiomers of **1**, including **1a** (2*R*,3*S*,5*S*) and **1b** (2*S*,3*R*,5*R*) were applied to conformational searches at ground state and semi-empirical AM1 set. The stable conformers (Boltzmann distributions over 1.0%) were optimized by DFT calculations at the B3LYP/6-31G(d,p) basic set and solvent methanol as polarizable continuum model (PCM). After optimization, conformers were subjected to TDDFT calculations at B3LYP/6-31G(d,p) set and methanol as a PCM. The CD spectra at 30 excited states for each conformer were combined to obtain theoretical CD spectra for each enantiomer. Half-band was taken at $\zeta = 0.25$ eV.

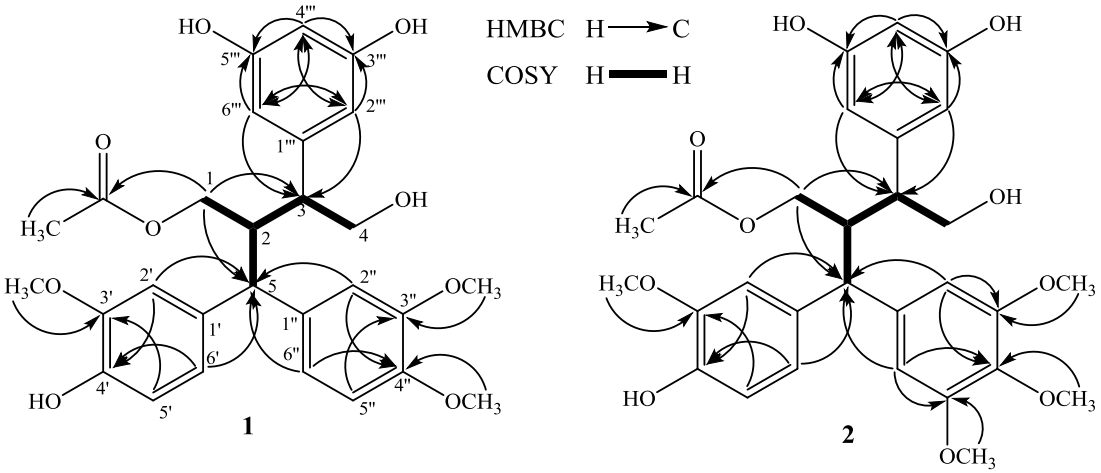

Figure S1. Important HMBC and COSY correlations of compounds **1** and **2**.

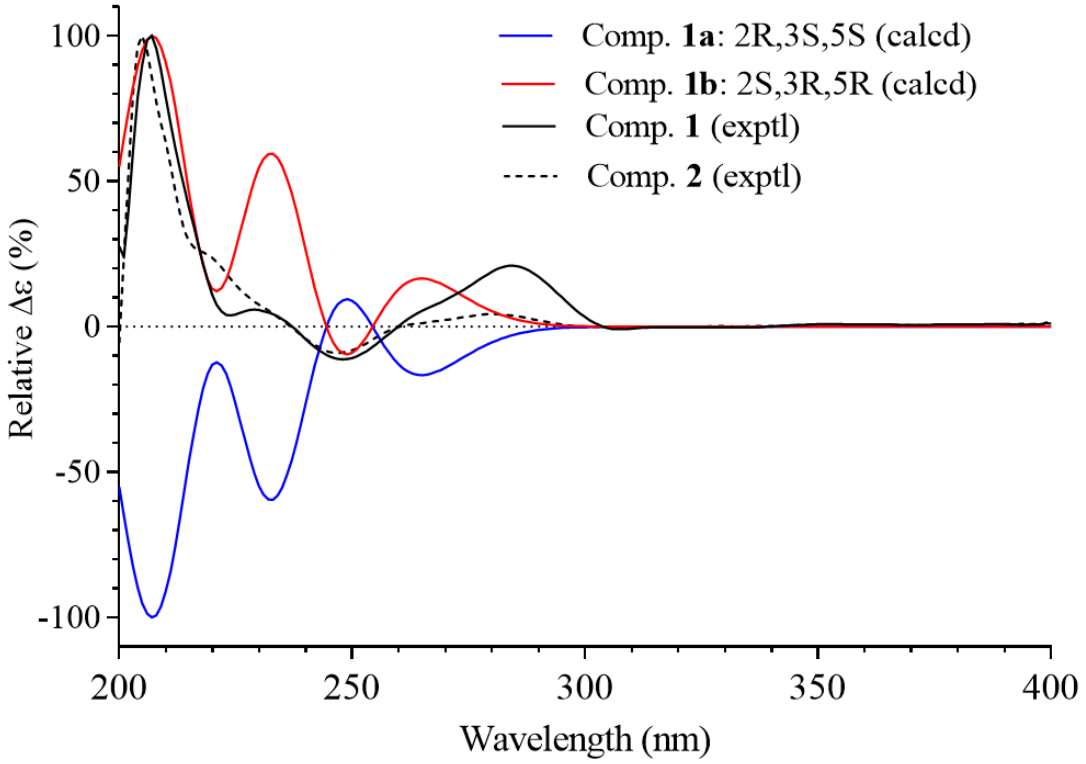

Figure S2. Experimental CD spectral of compounds **1-2** and TD-DFT calculated CD spectra of enantiomers **1a** (2R3S,5S), **1b** (2S,3R,5R).

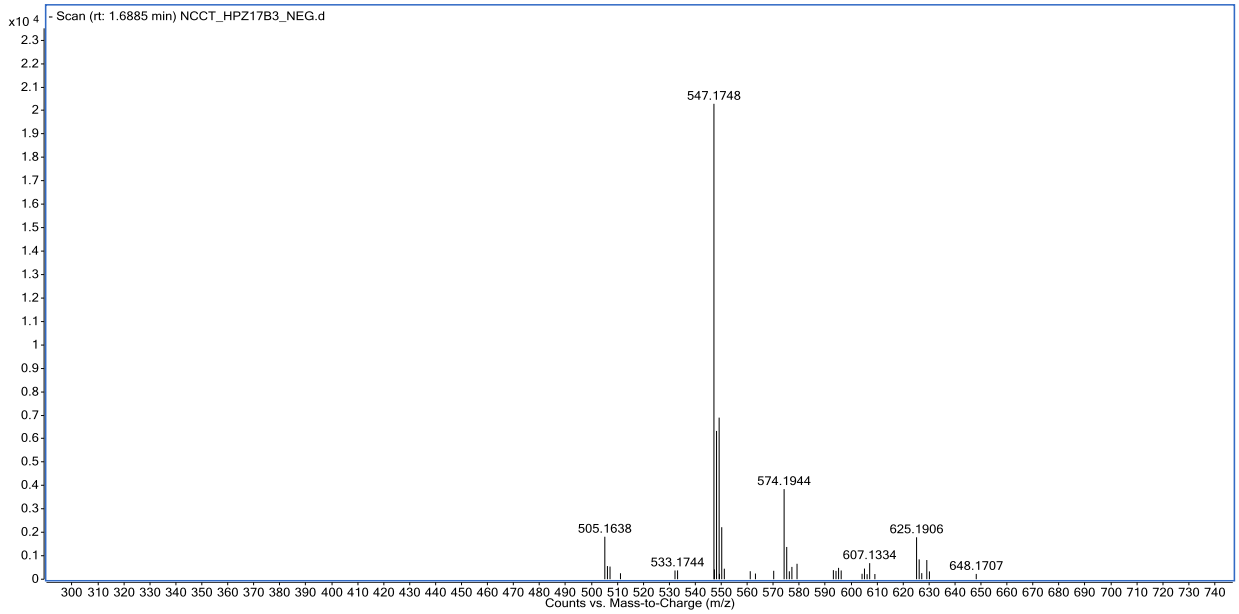

Figure S3. HR-ESI-MS of compound **1**

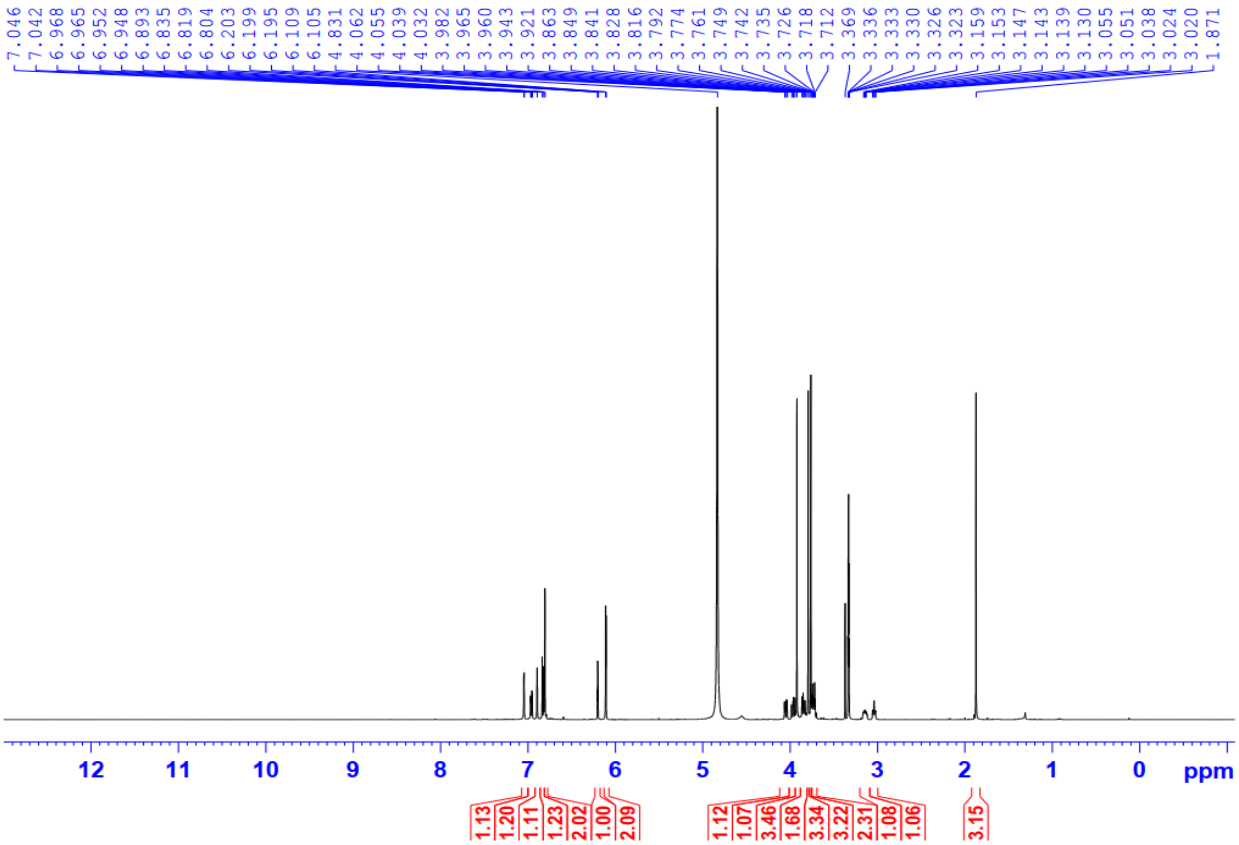

Figure S4. ^{1}H-NMR spectrum of compound **1** in CD_3OD

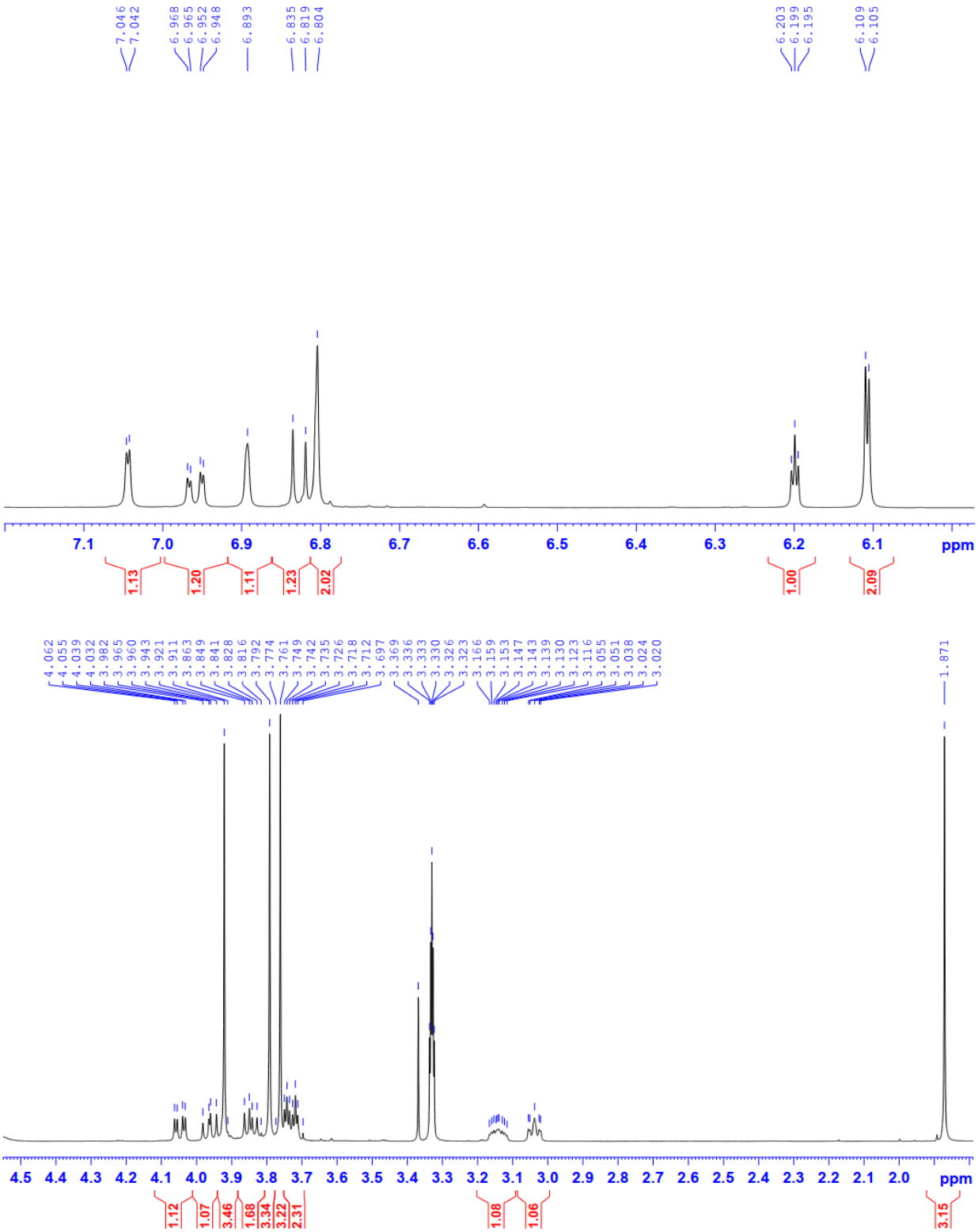

Figure S5. Extended ^{1}H-NMR spectrum of compound **1** in CD_3OD

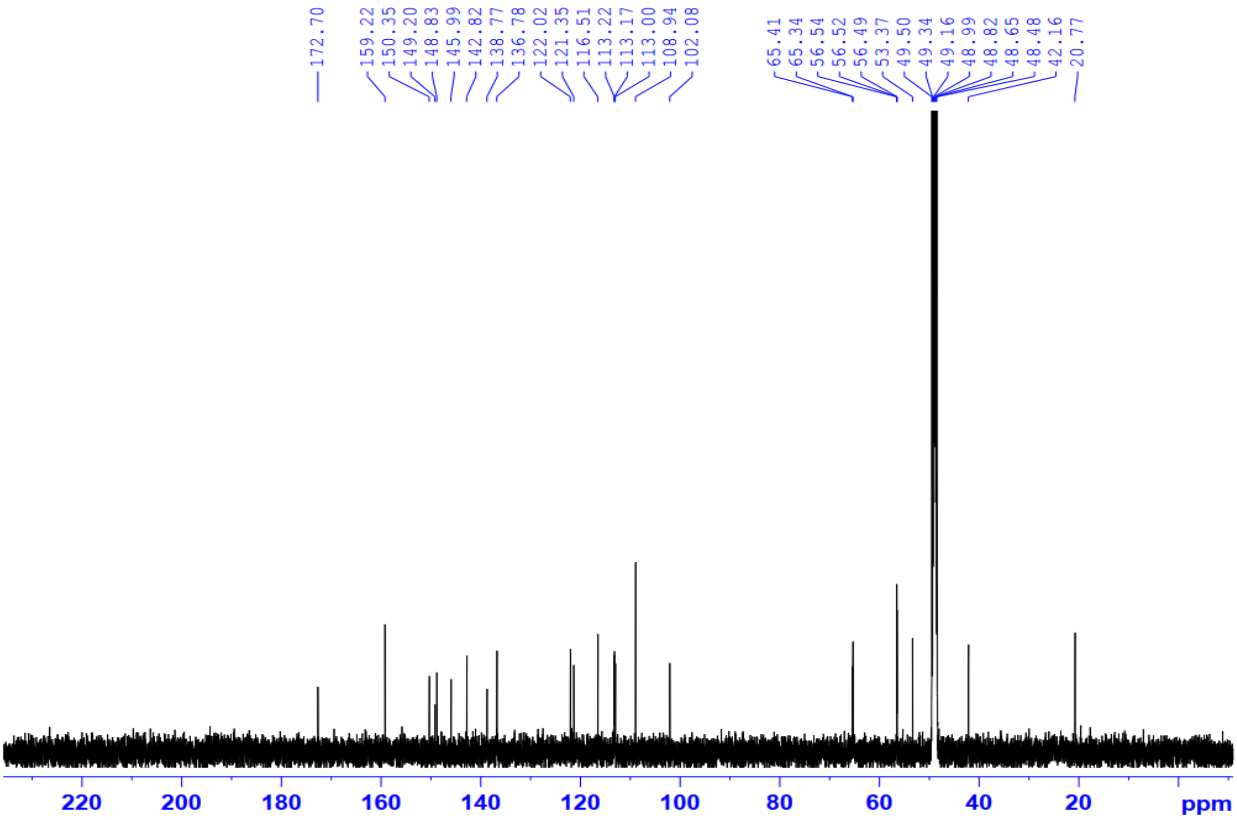

Figure S6. ^{13}C-NMR spectrum of compound **1** in CD_3OD

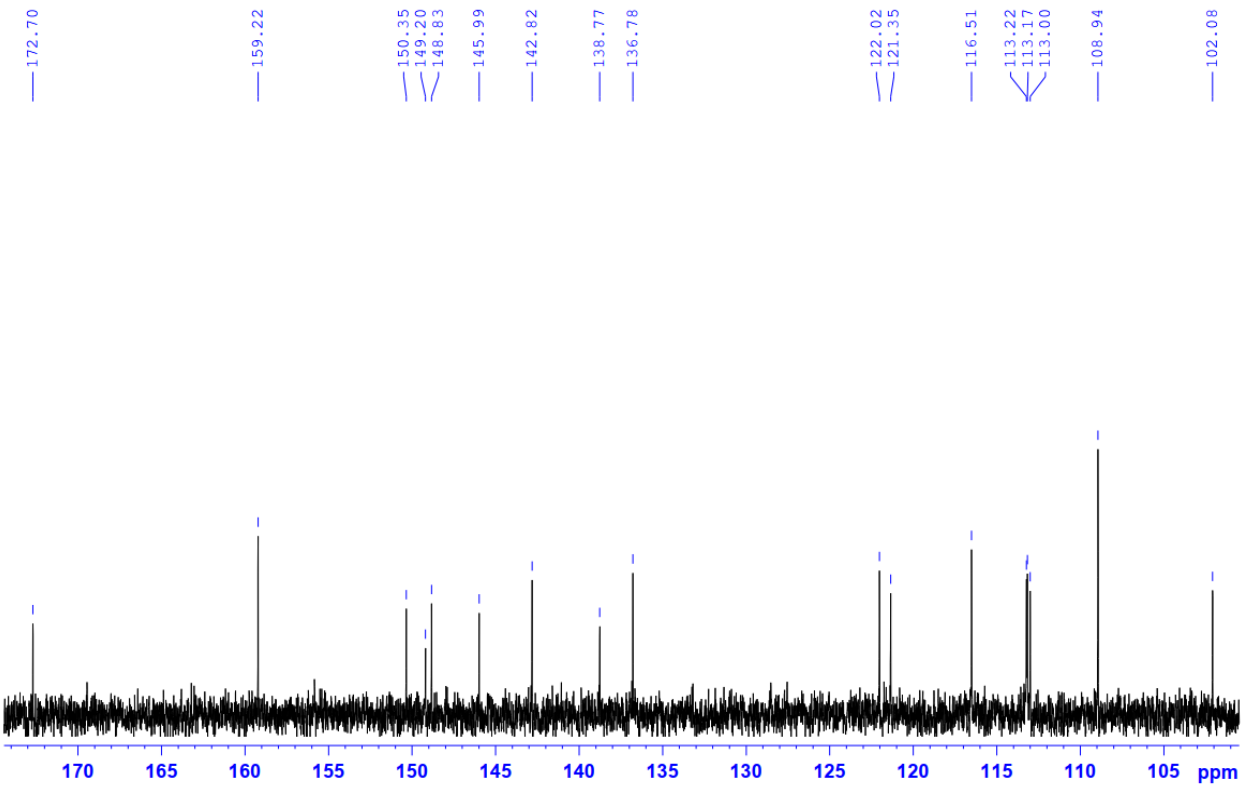

Figure S7. Extended ^{13}C-NMR spectrum of compound **1** in CD_3OD

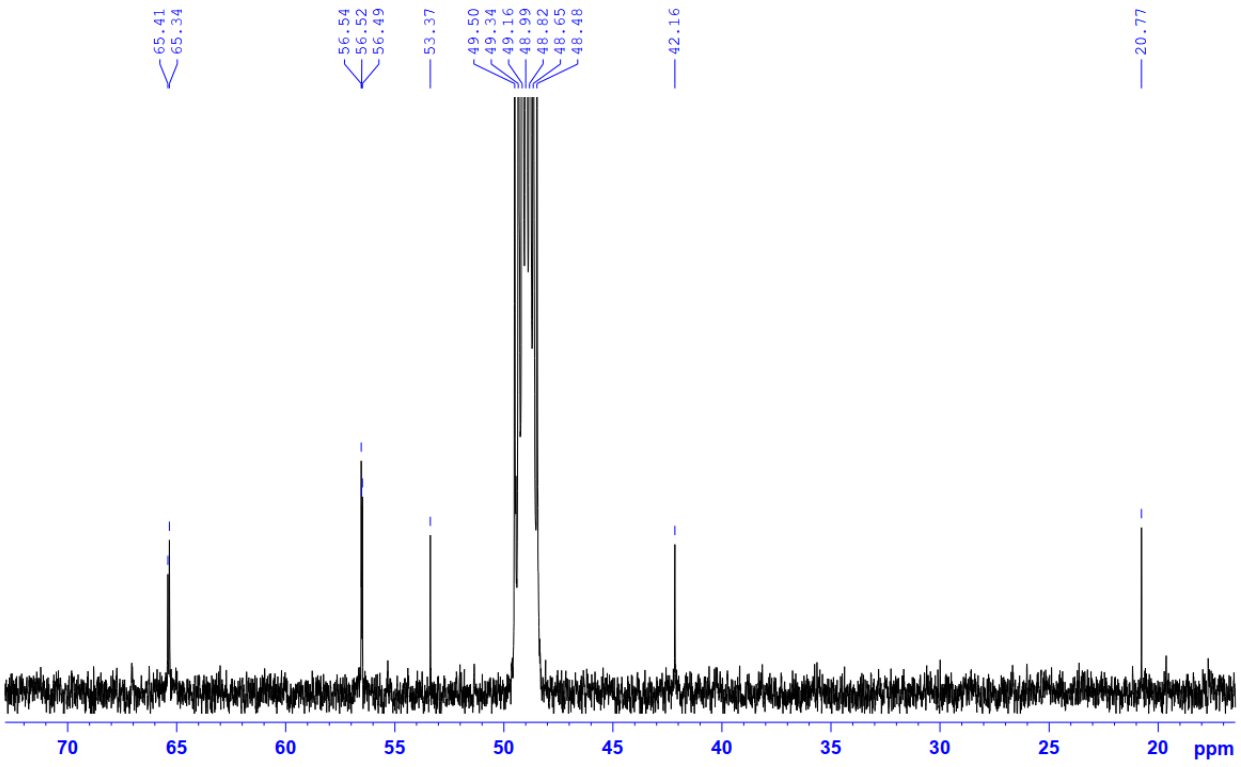

Figure S8. Extended ^{13}C-NMR spectrum of compound **1** in CD_3OD

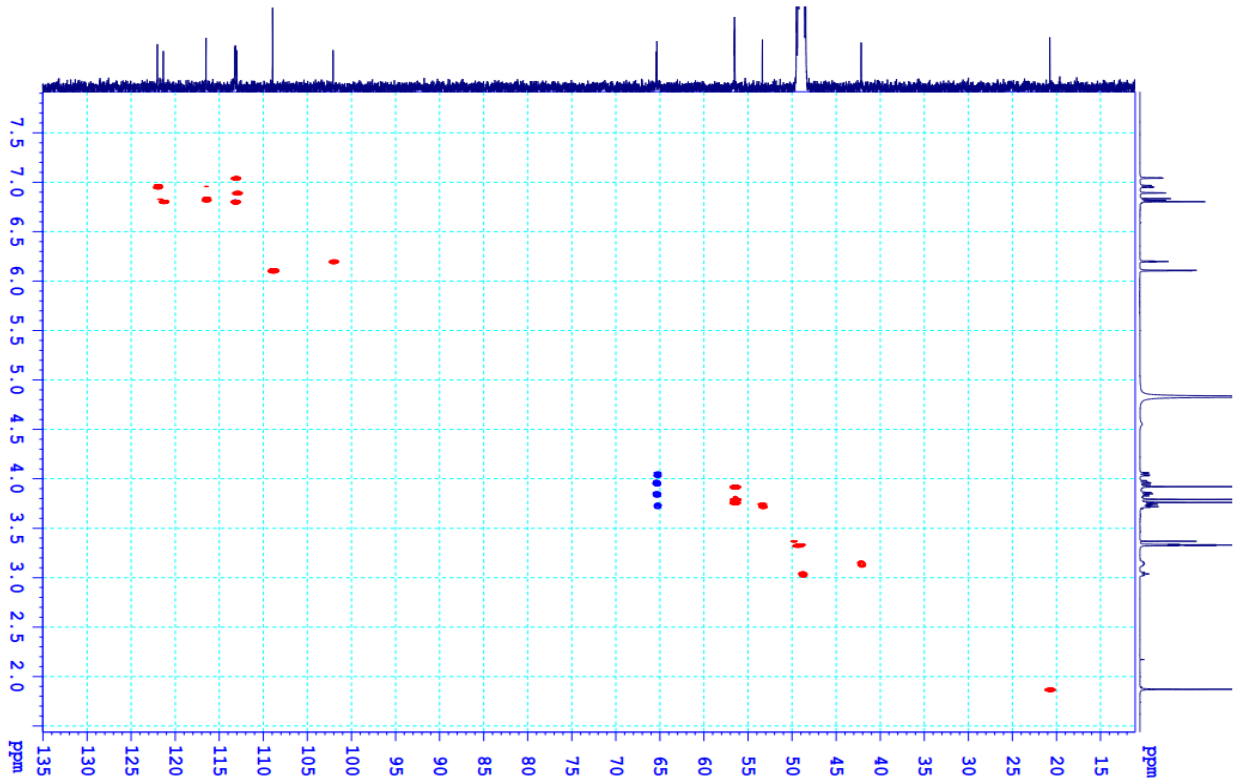

Figure S9. HSQC spectrum of compound **1**

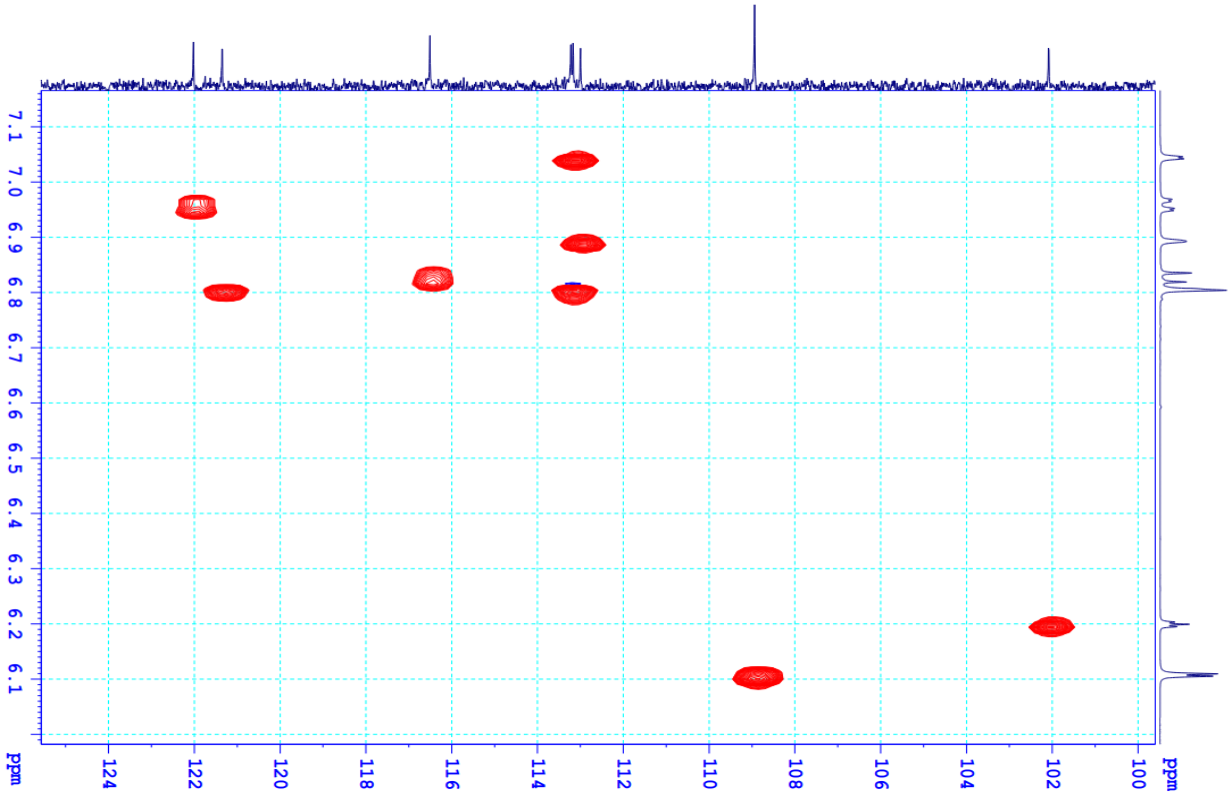

Figure S10. Extended HSQC spectrum of compound **1**

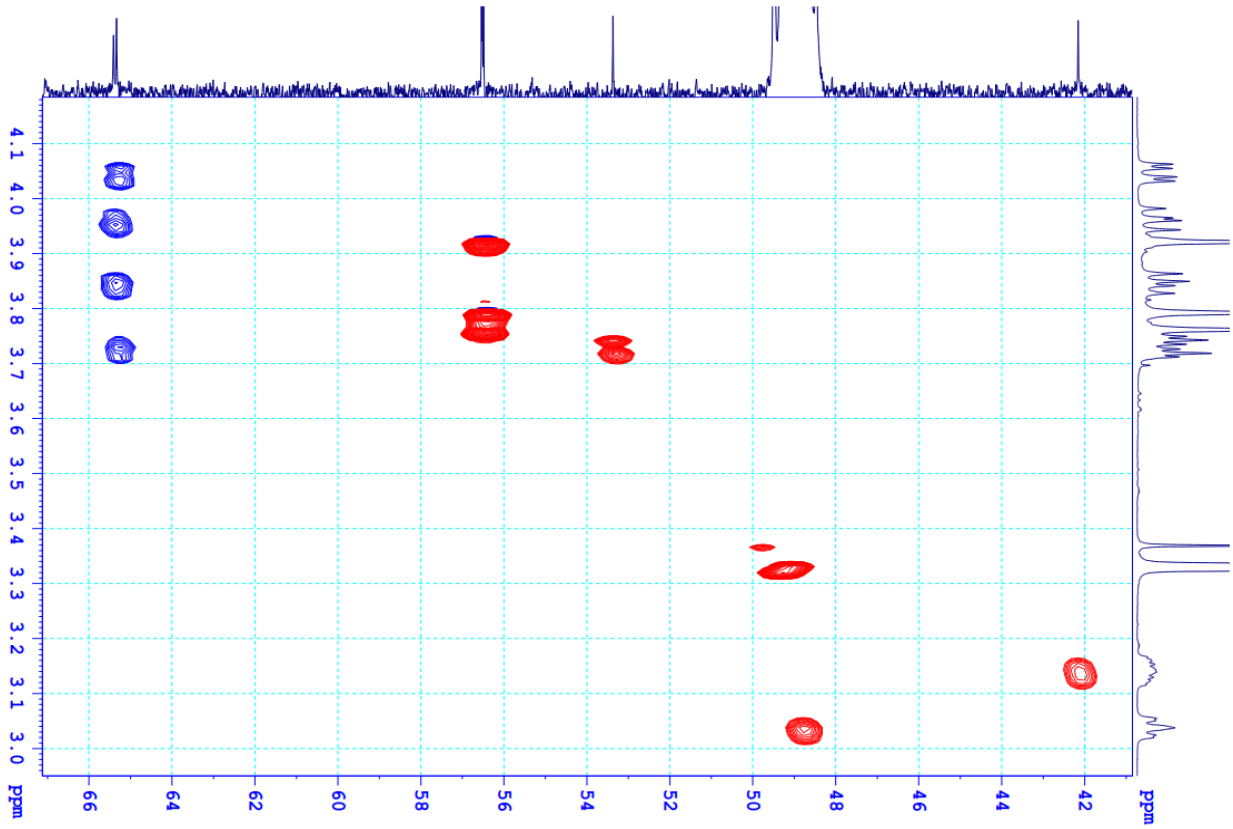

Figure S11. Extended HSQC spectrum of compound **1**

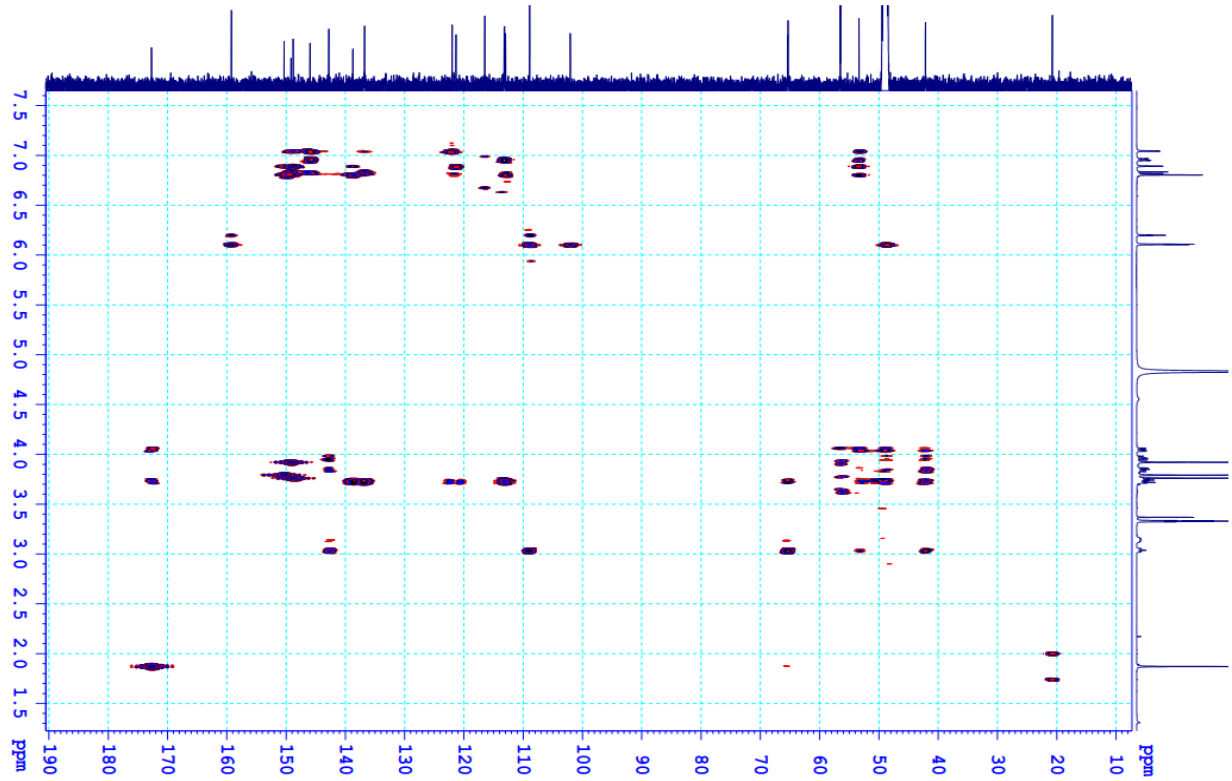

Figure S12. HMBC spectrum of compound **1**

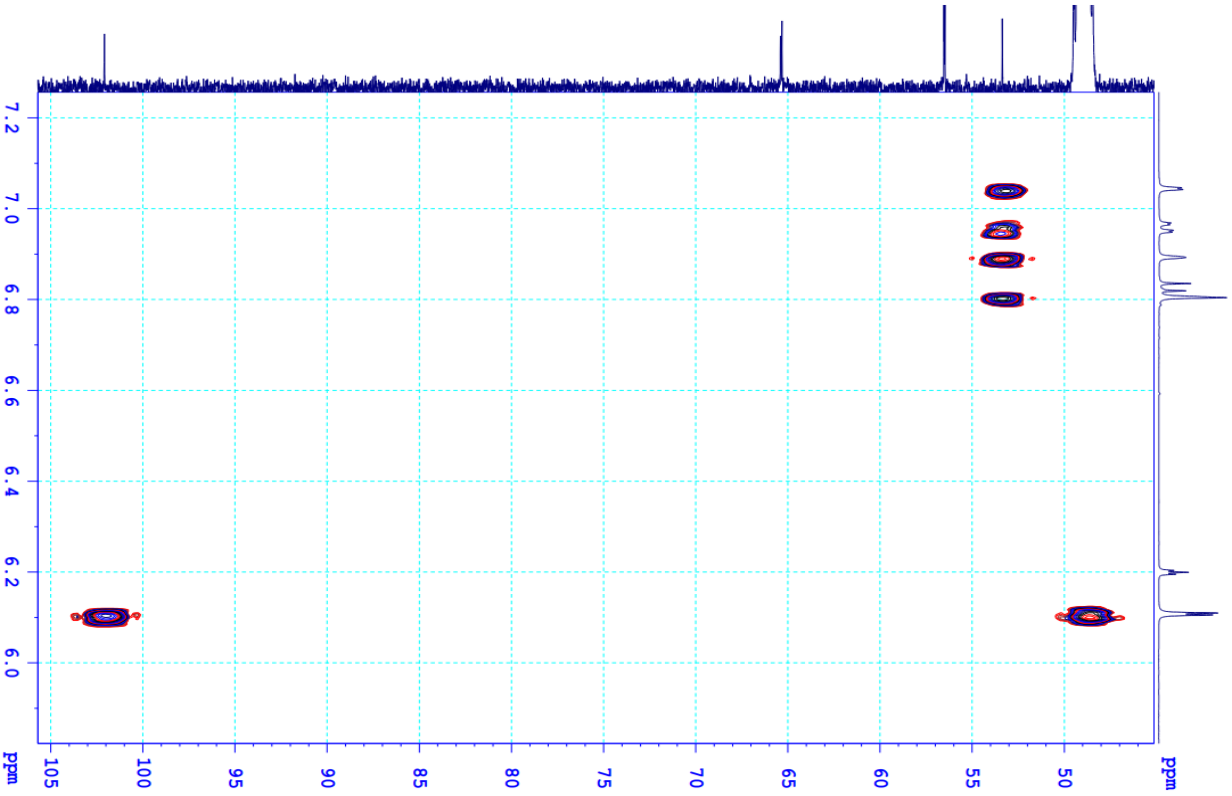

Figure S13. Extended HMBC spectrum of compound **1**

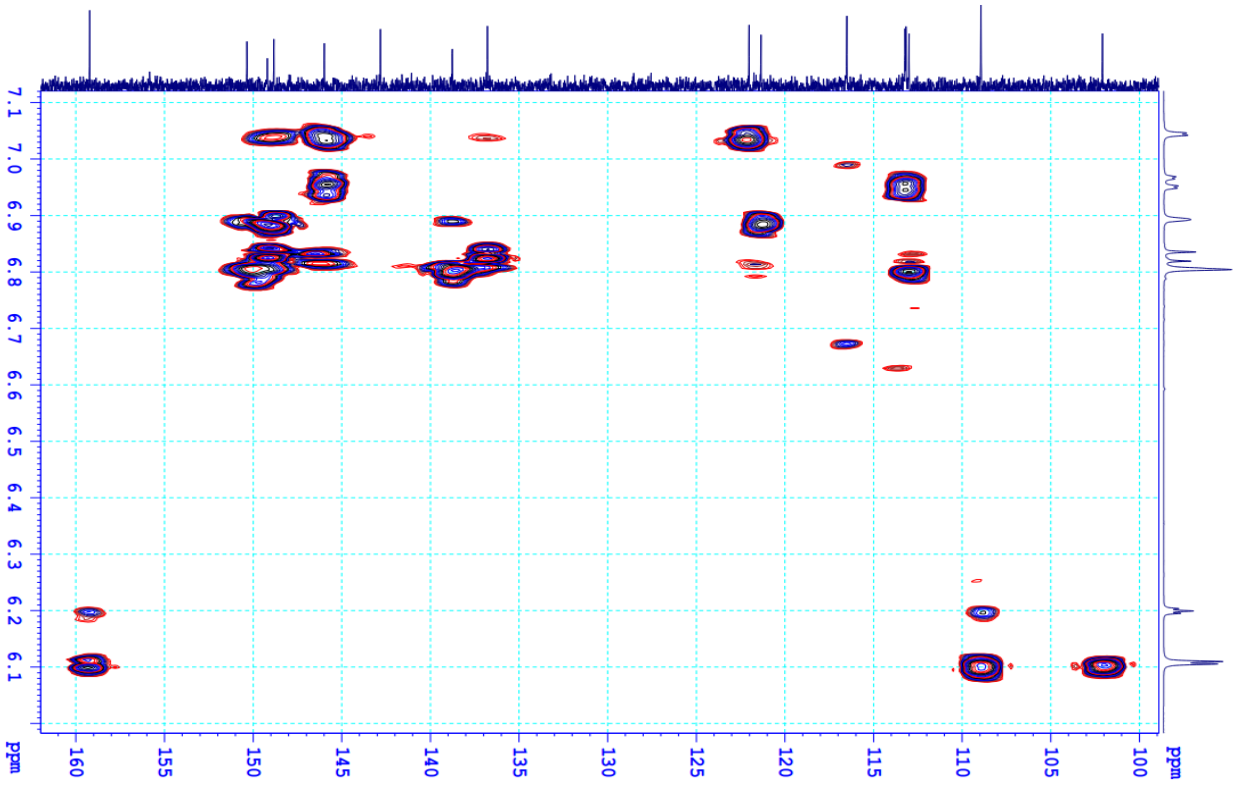

Figure S14. Extended HMBC spectrum of compound **1**

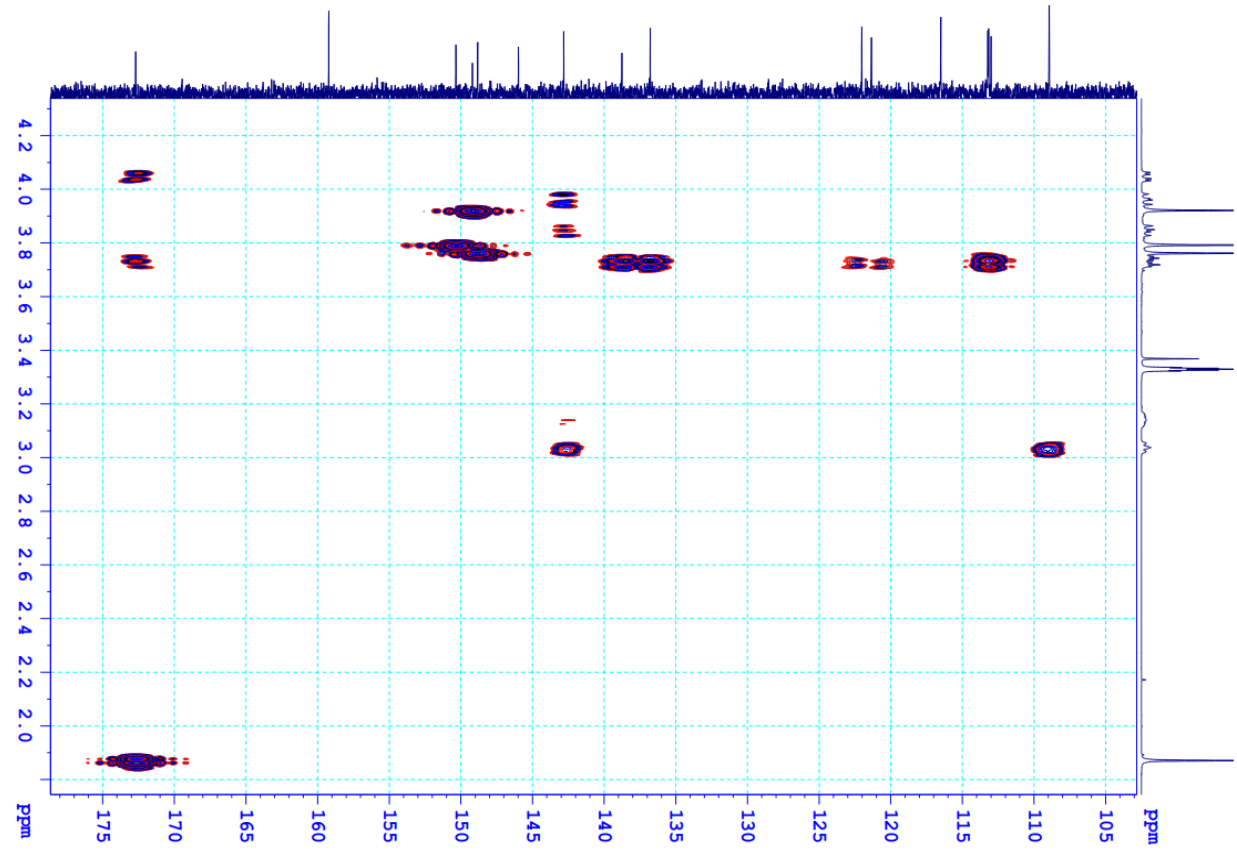

Figure S15. Extended HMBC spectrum of compound **1**

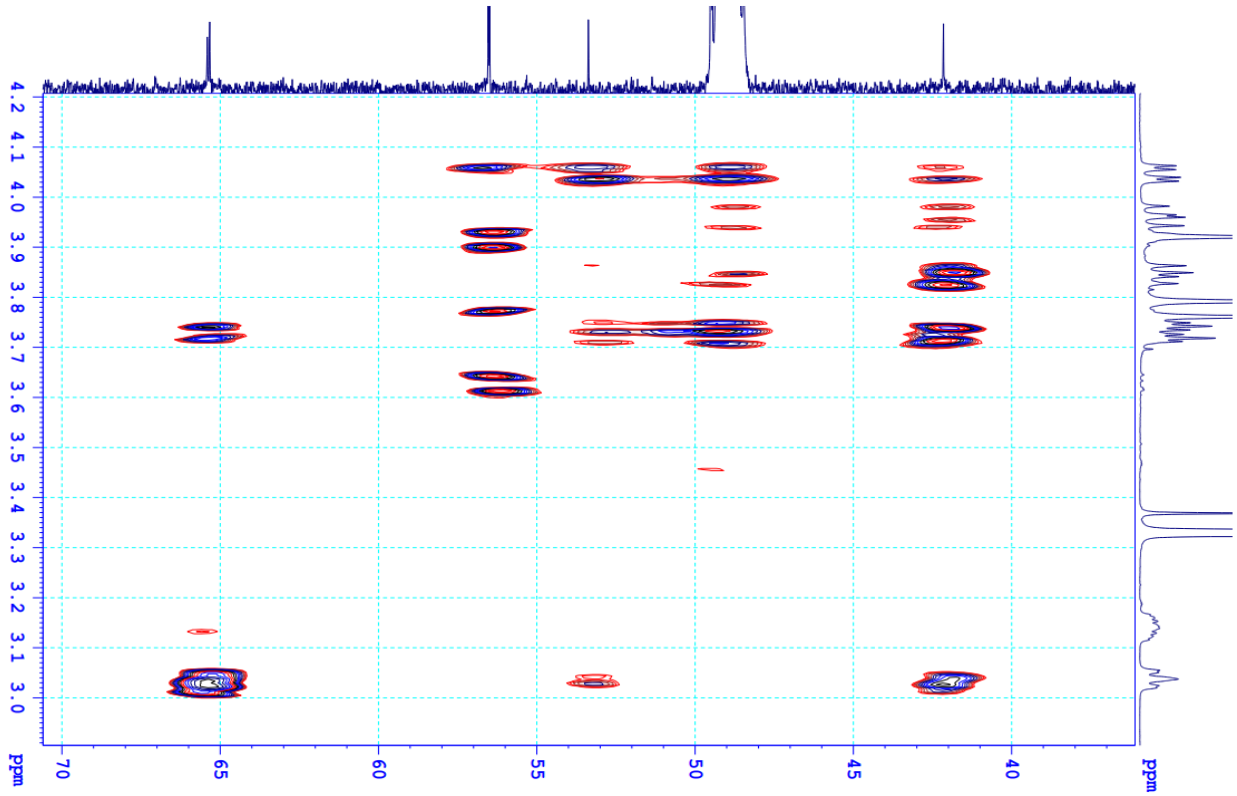

Figure S16. Extended HMBC spectrum of compound **1**

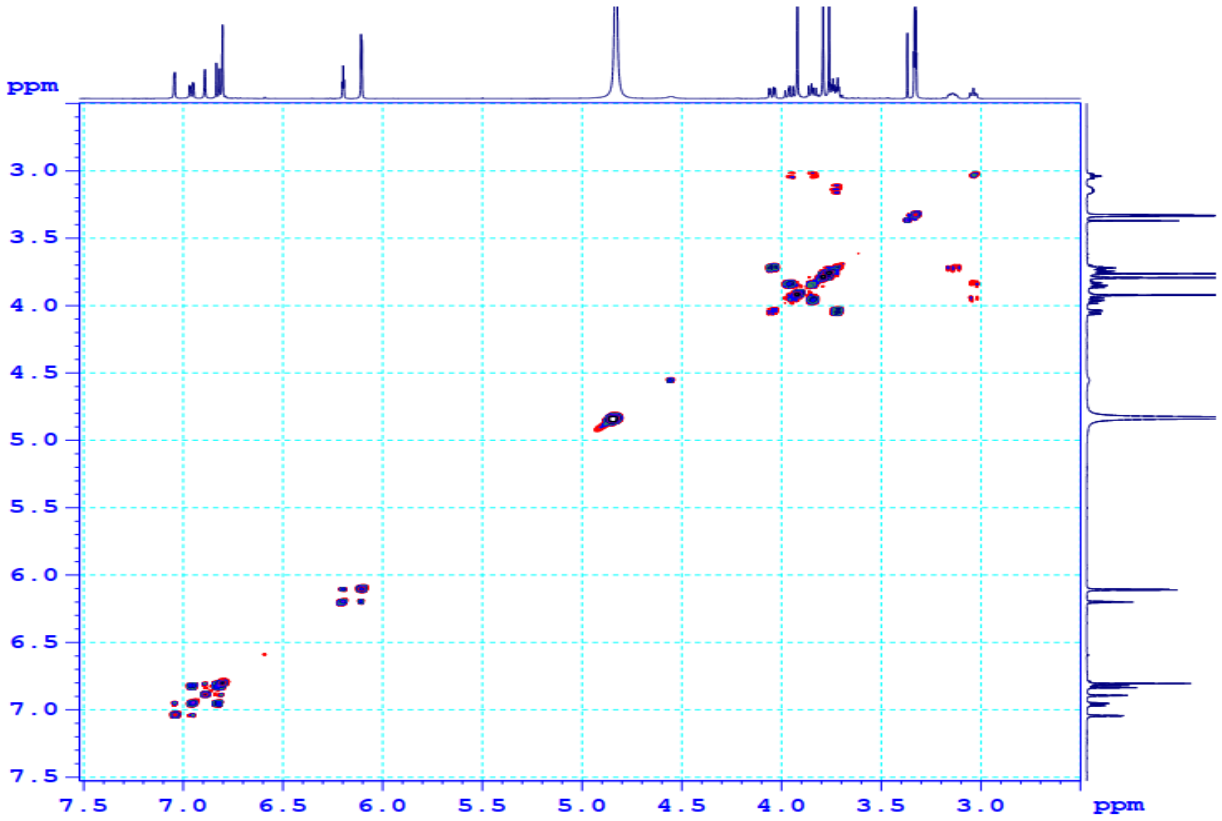

Figure S17. H-H COSY spectrum of compound **1**

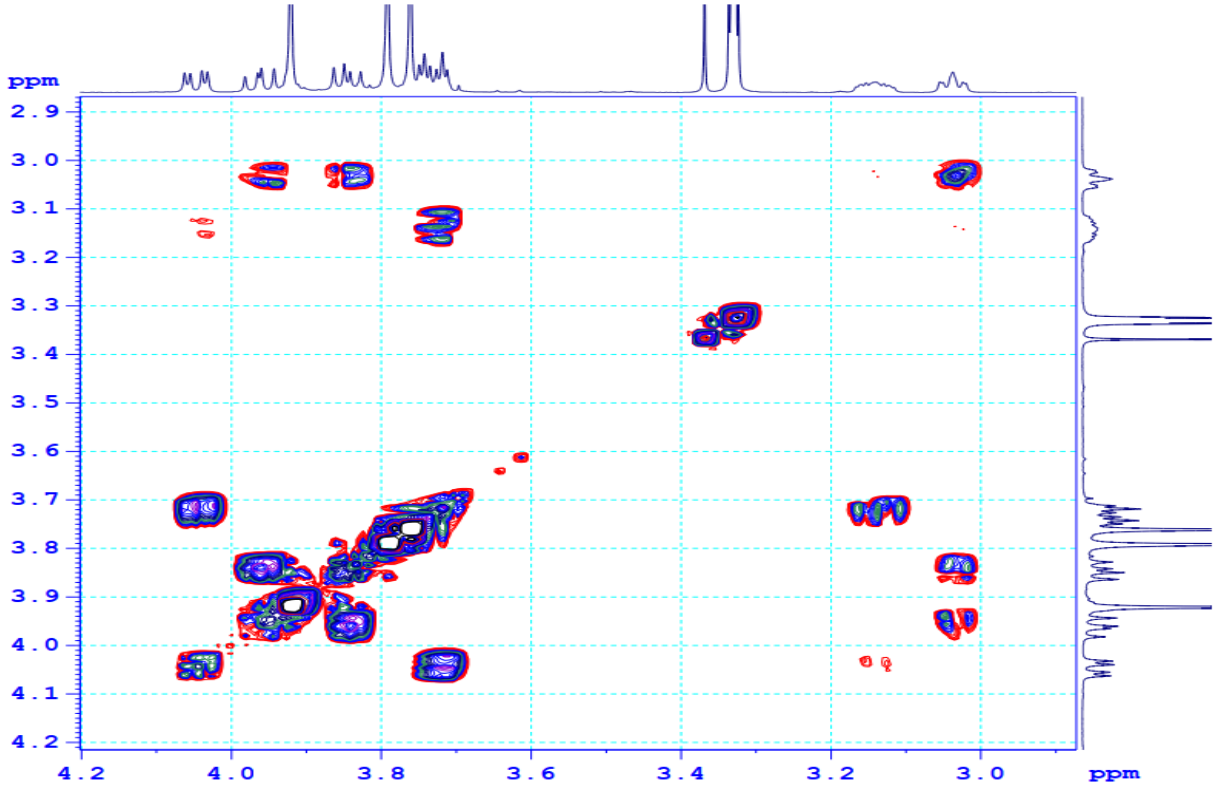

Figure S18. Extended H-H COSY spectrum of compound **1**

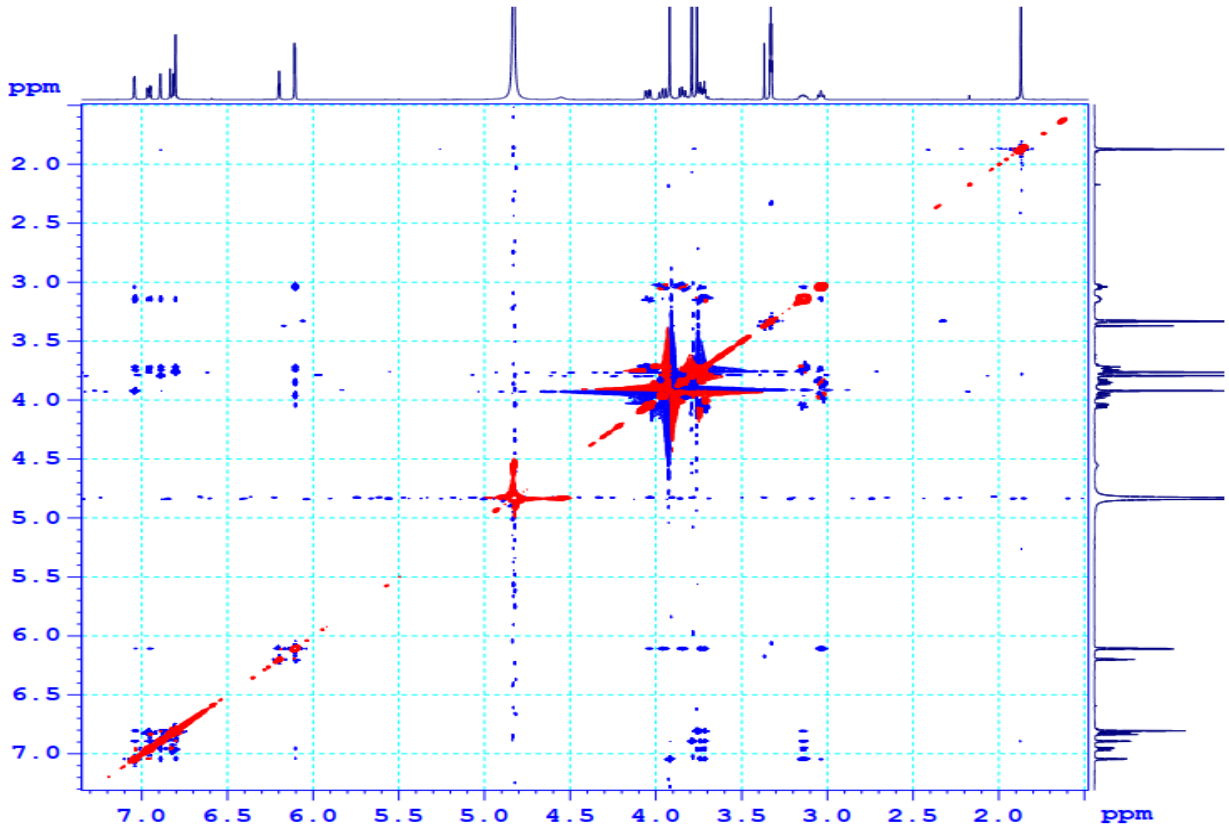

Figure S19. NOESY spectrum of compound **1**

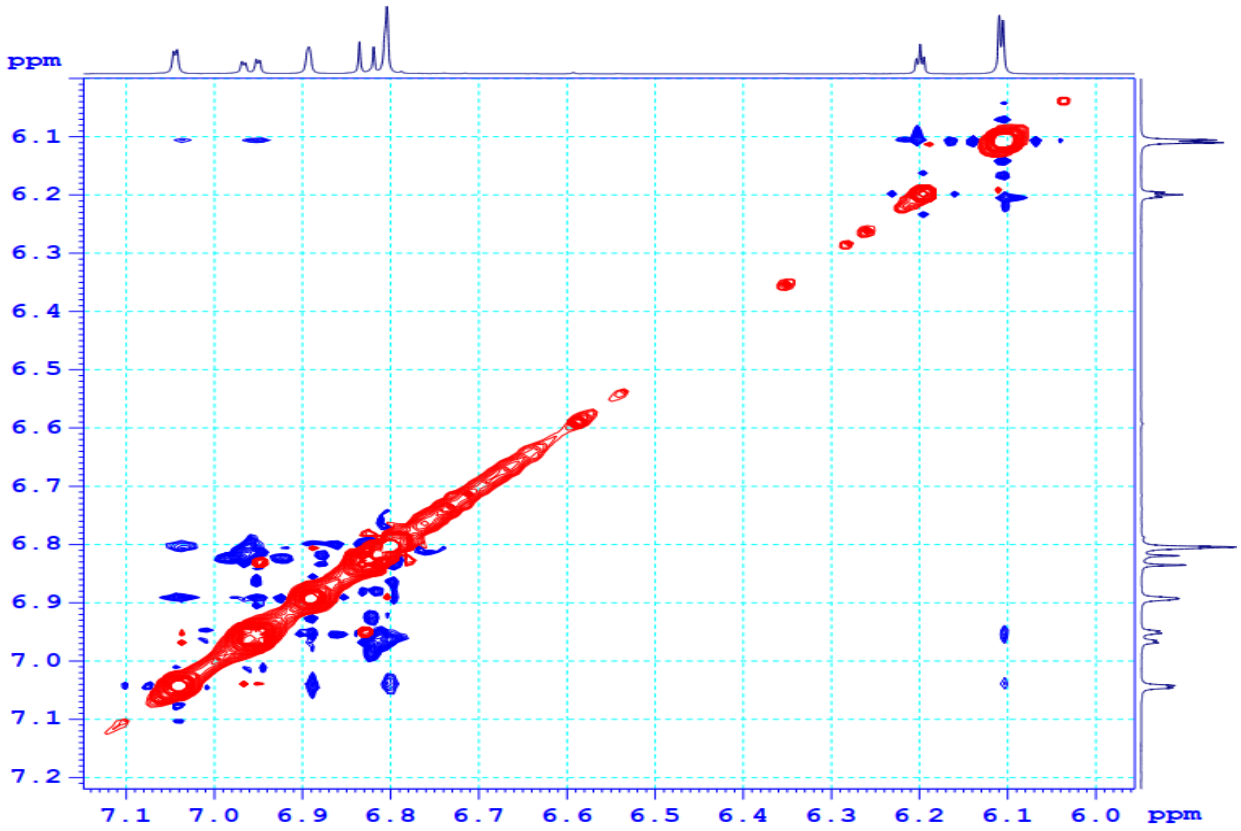

Figure S20. Extended NOESY spectrum of compound **1**

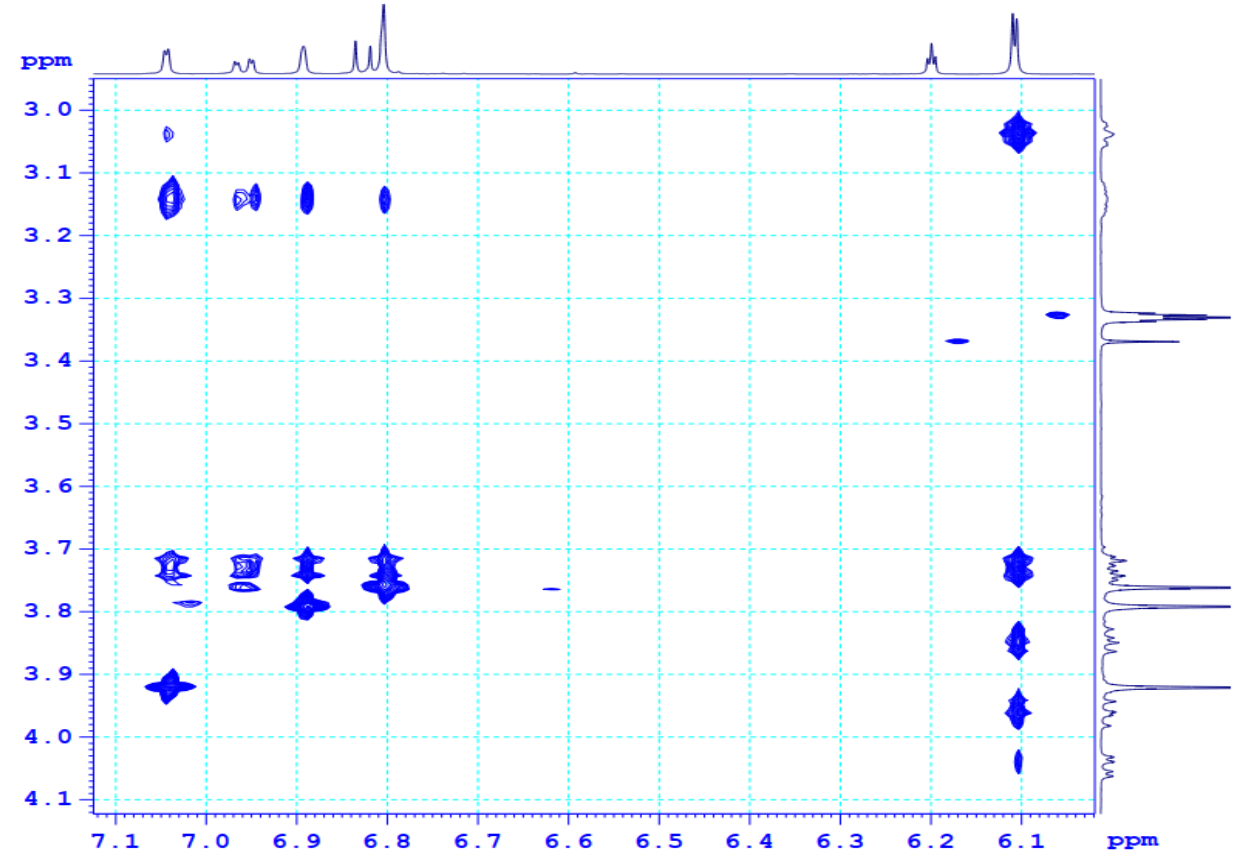

Figure S21. Extended NOESY spectrum of compound **1**

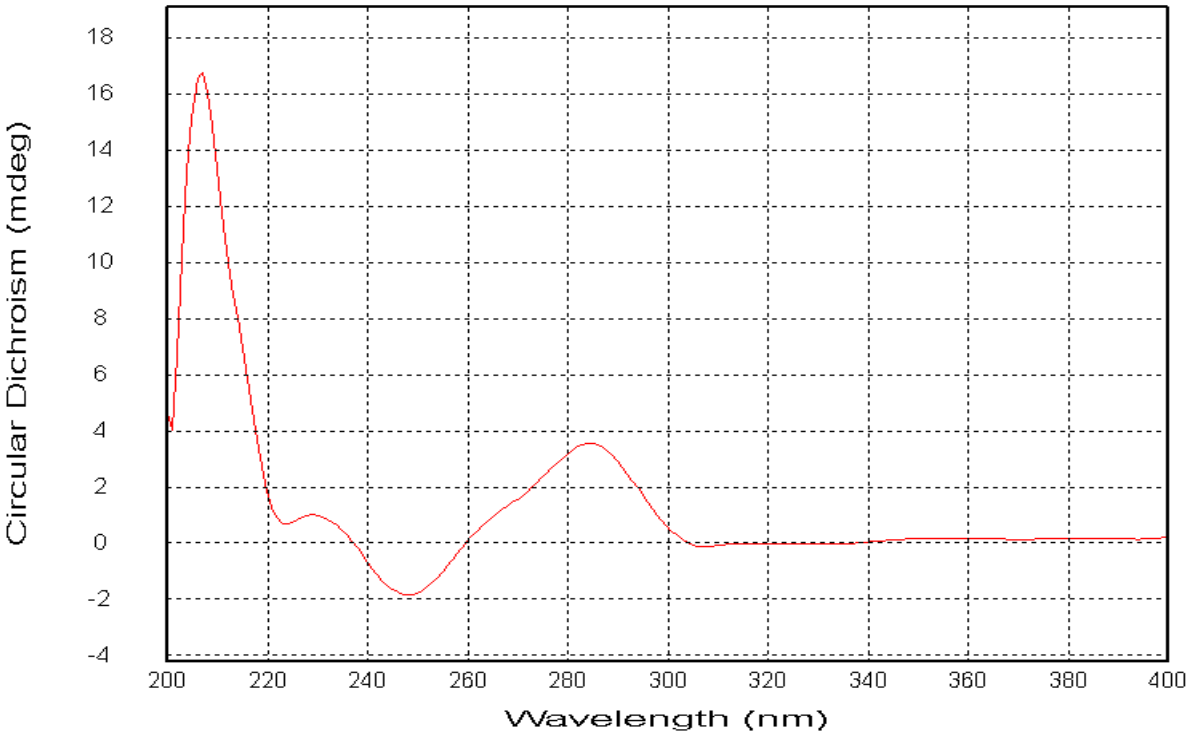

Figure S22. CD spectrum of compound **1**

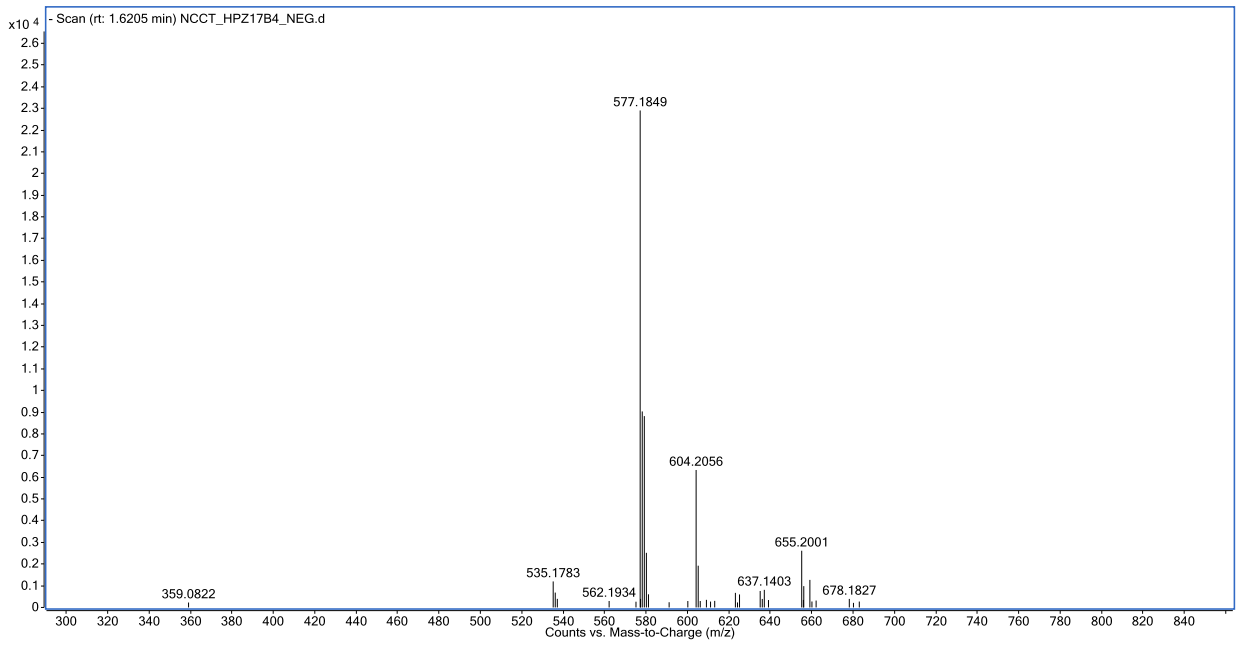

Figure S23. HR-ESI-MS of compound **2**

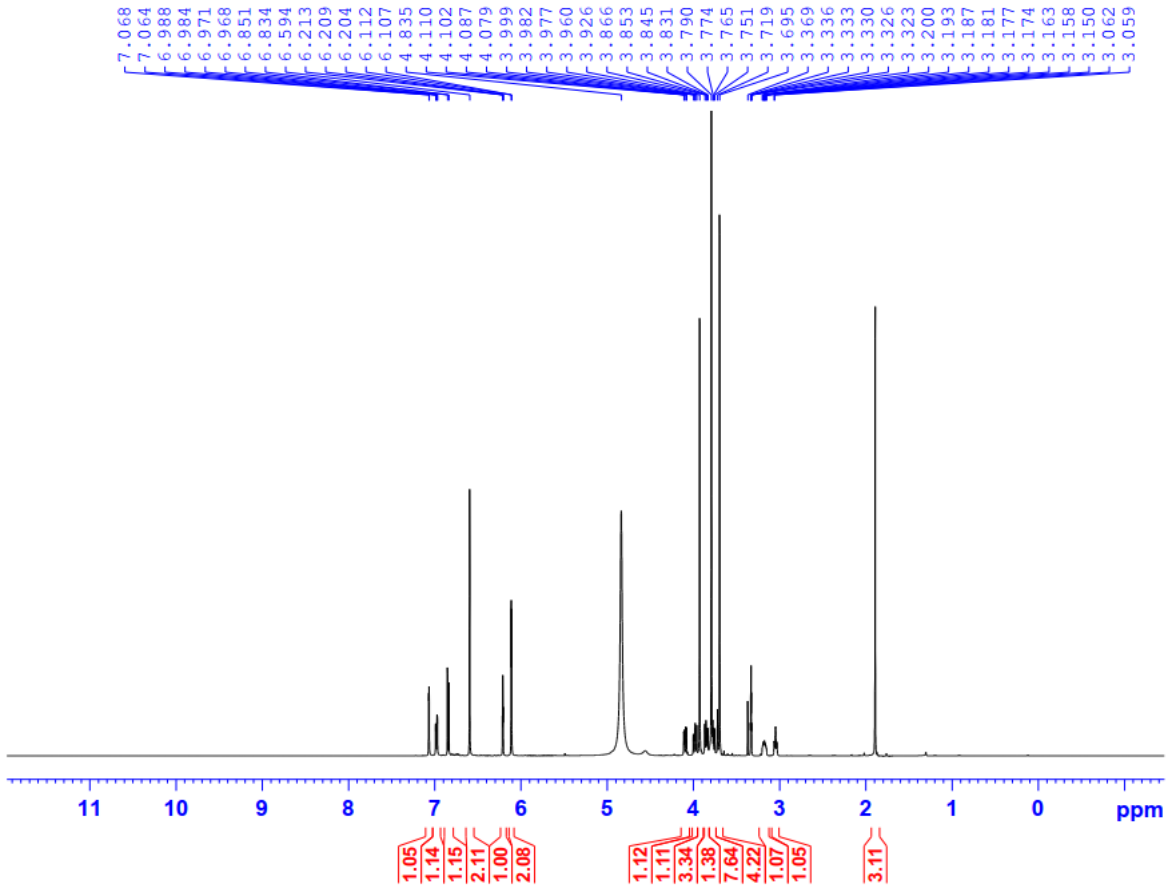

Figure S24. ^{1}H-NMR spectrum of compound **2** in CD_3OD

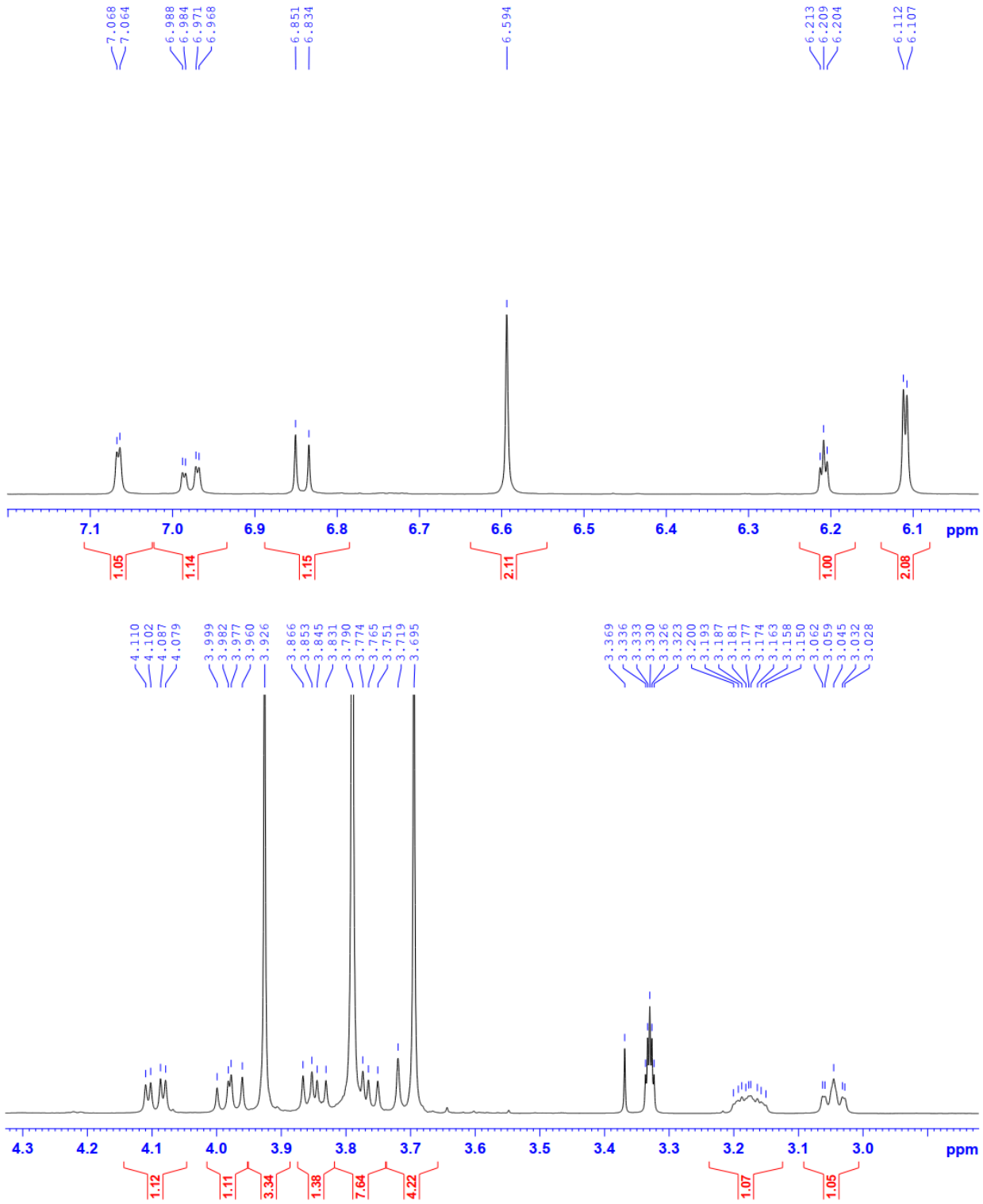

Figure S25. Extended ^{1}H-NMR spectrum of compound **2** in CD_3OD

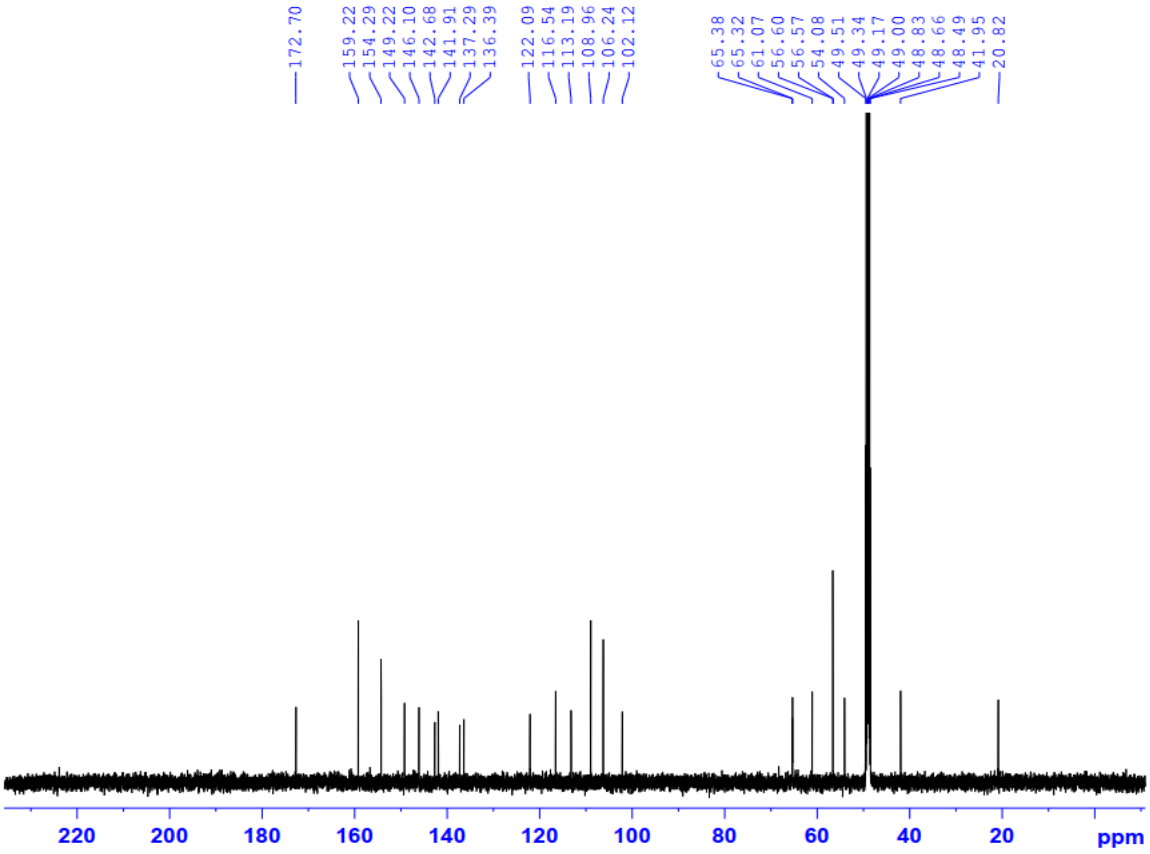

Figure S26. ^{13}C-NMR spectrum of compound **2** in CD_3OD

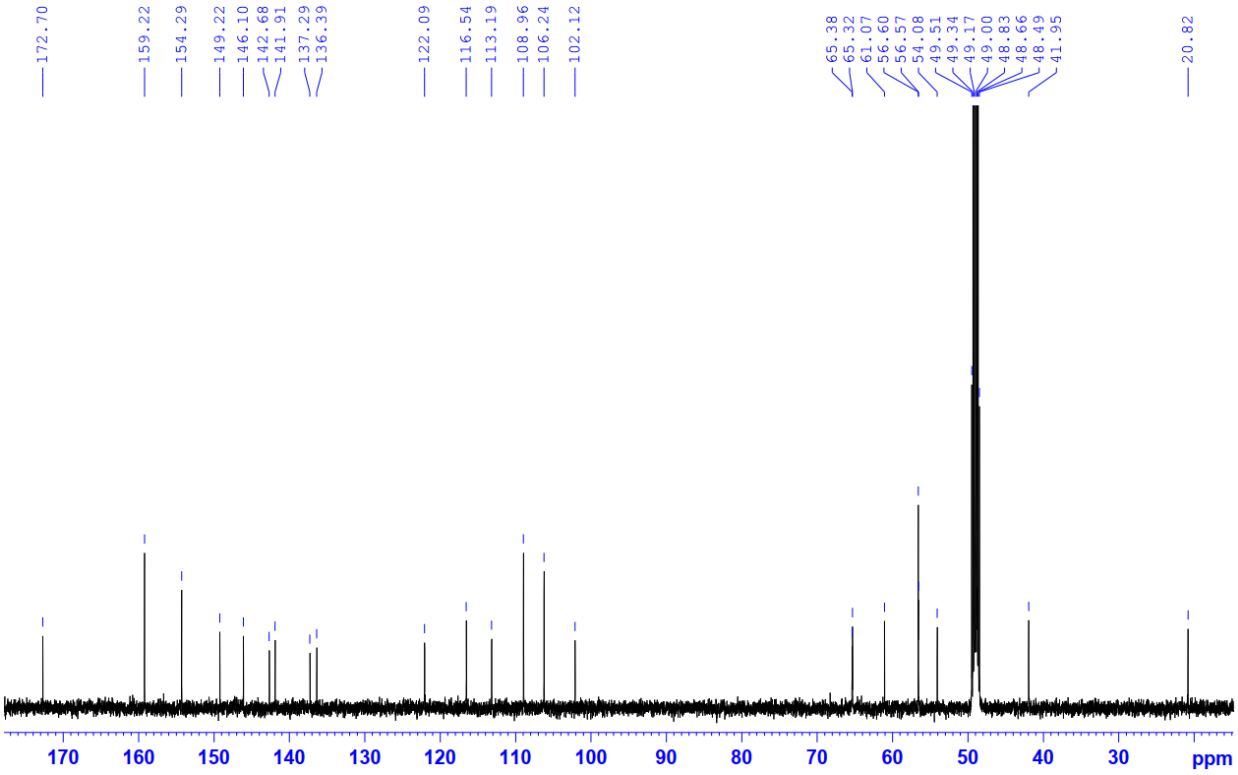

Figure S27. Extended ^{13}C-NMR spectrum of compound **2** in CD_3OD

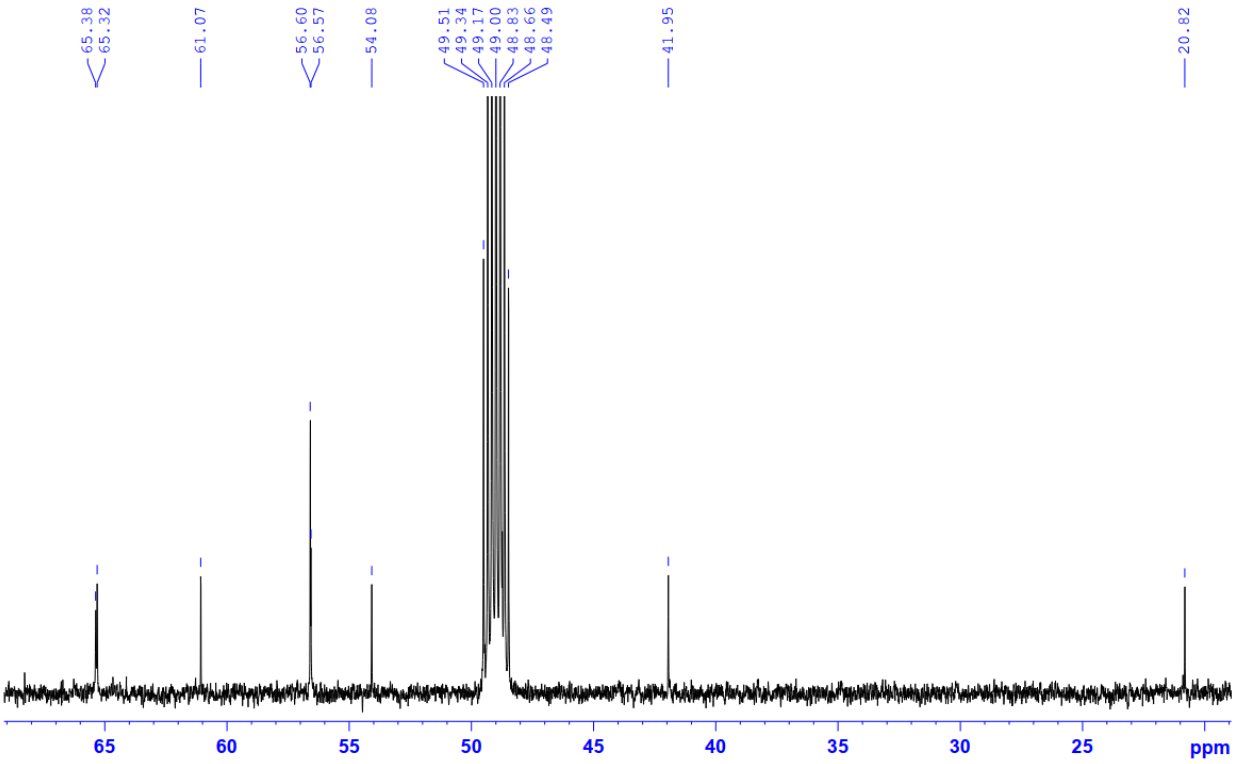

Figure S28. Extended ^{13}C-NMR spectrum of compound **2** in CD_3OD

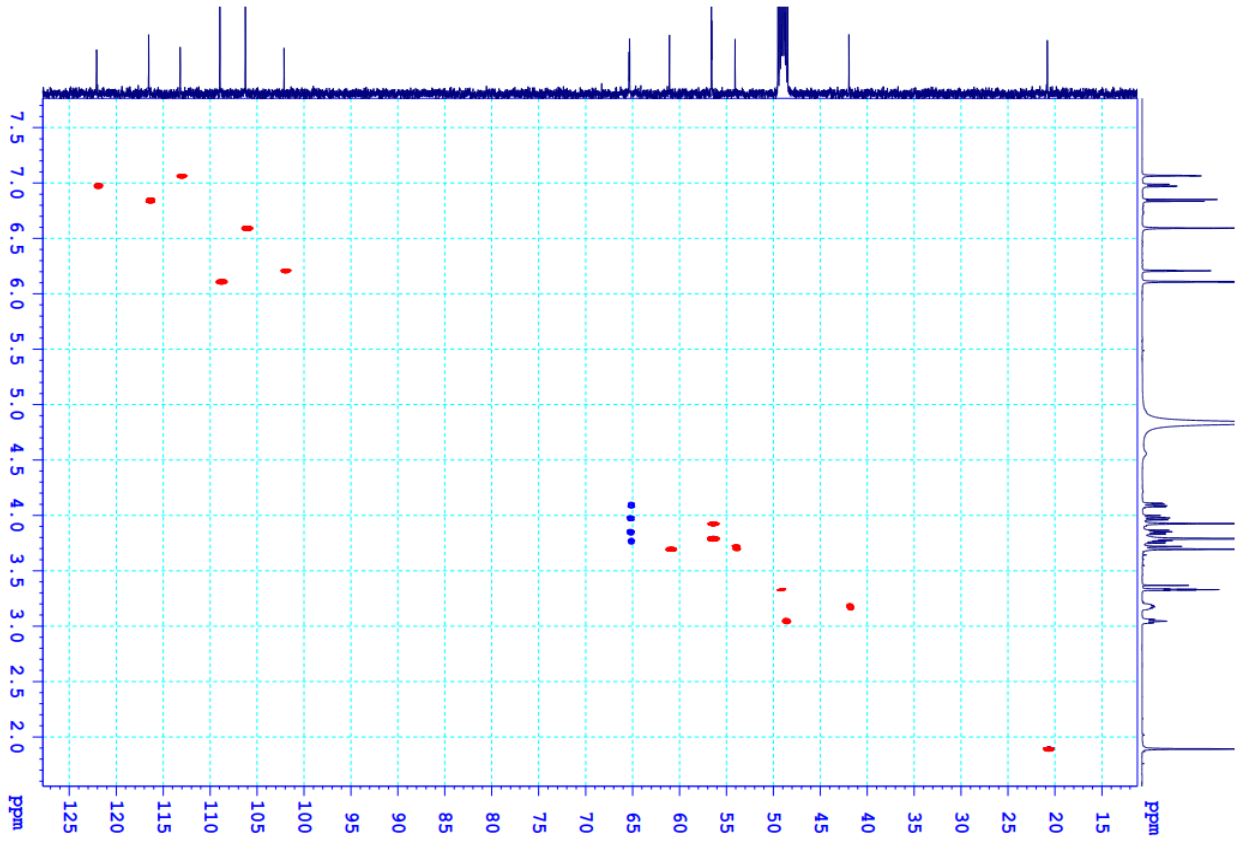

Figure S29. HSQC spectrum of compound **2**

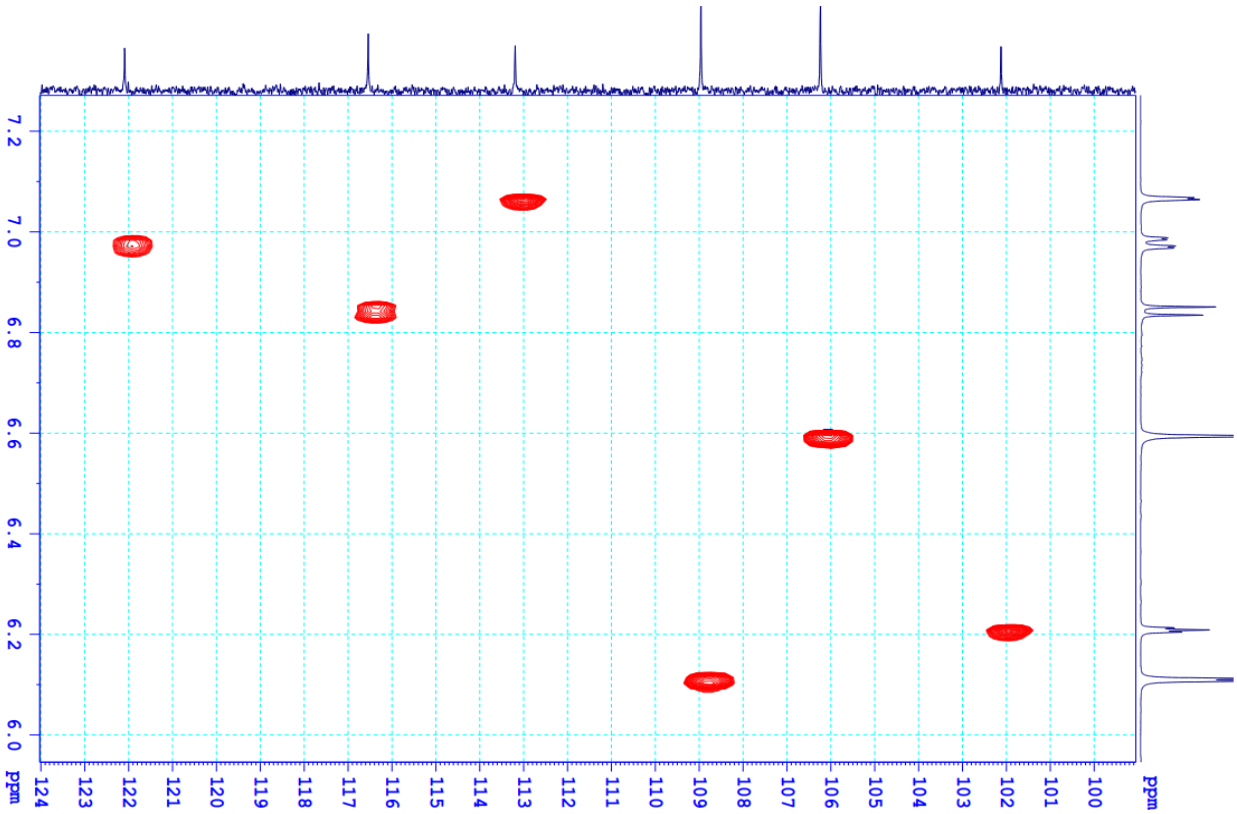

Figure S30. Extended HSQC spectrum of compound **2**

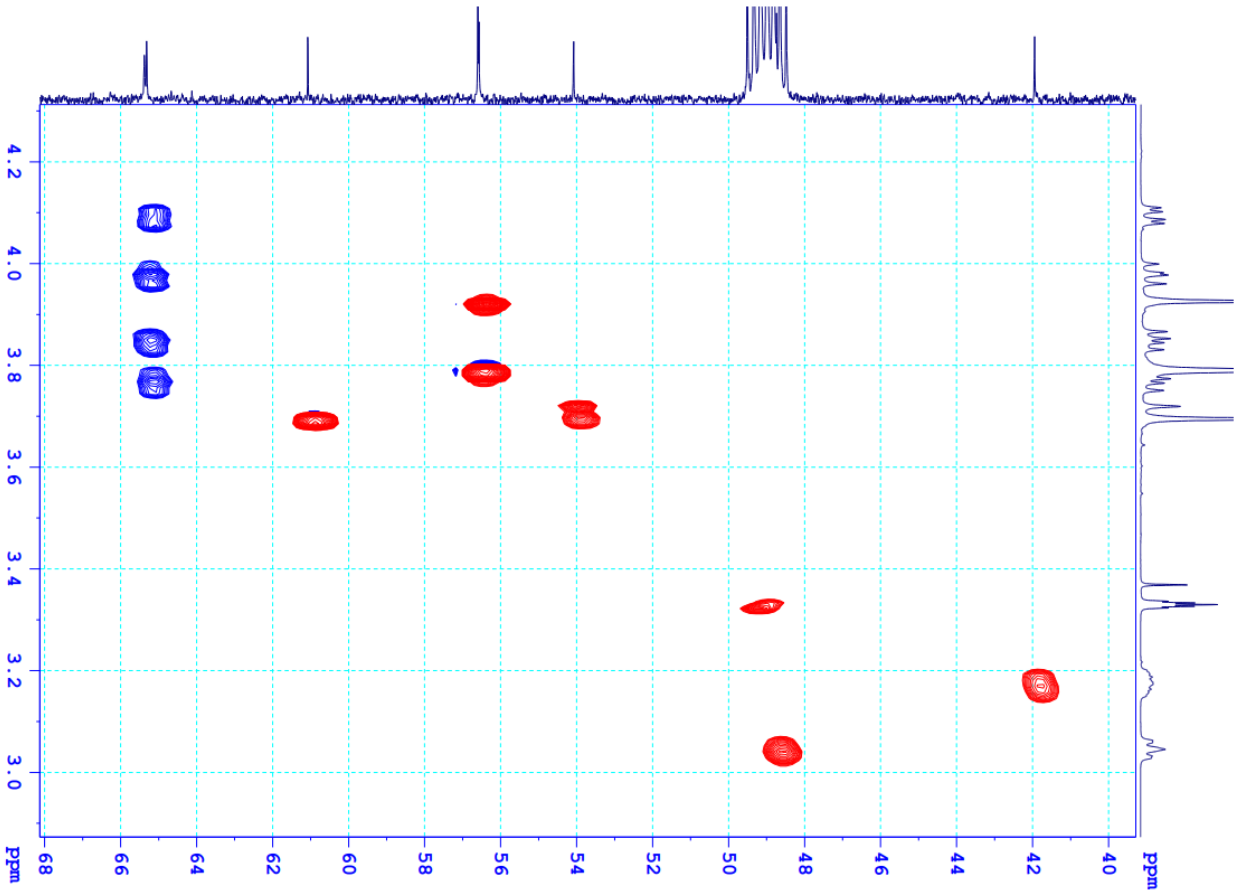

Figure S31. Extended HSQC spectrum of compound **2**

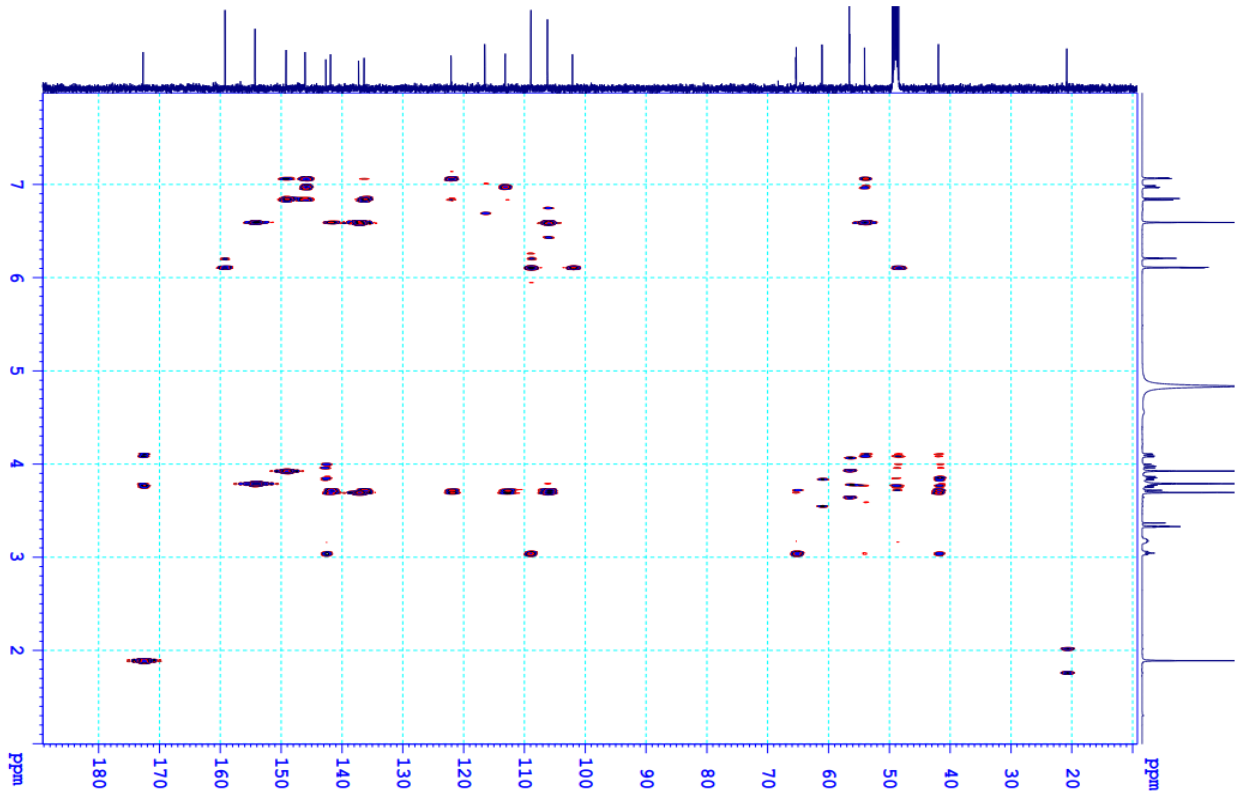

Figure S32. HMBC spectrum of compound **2**

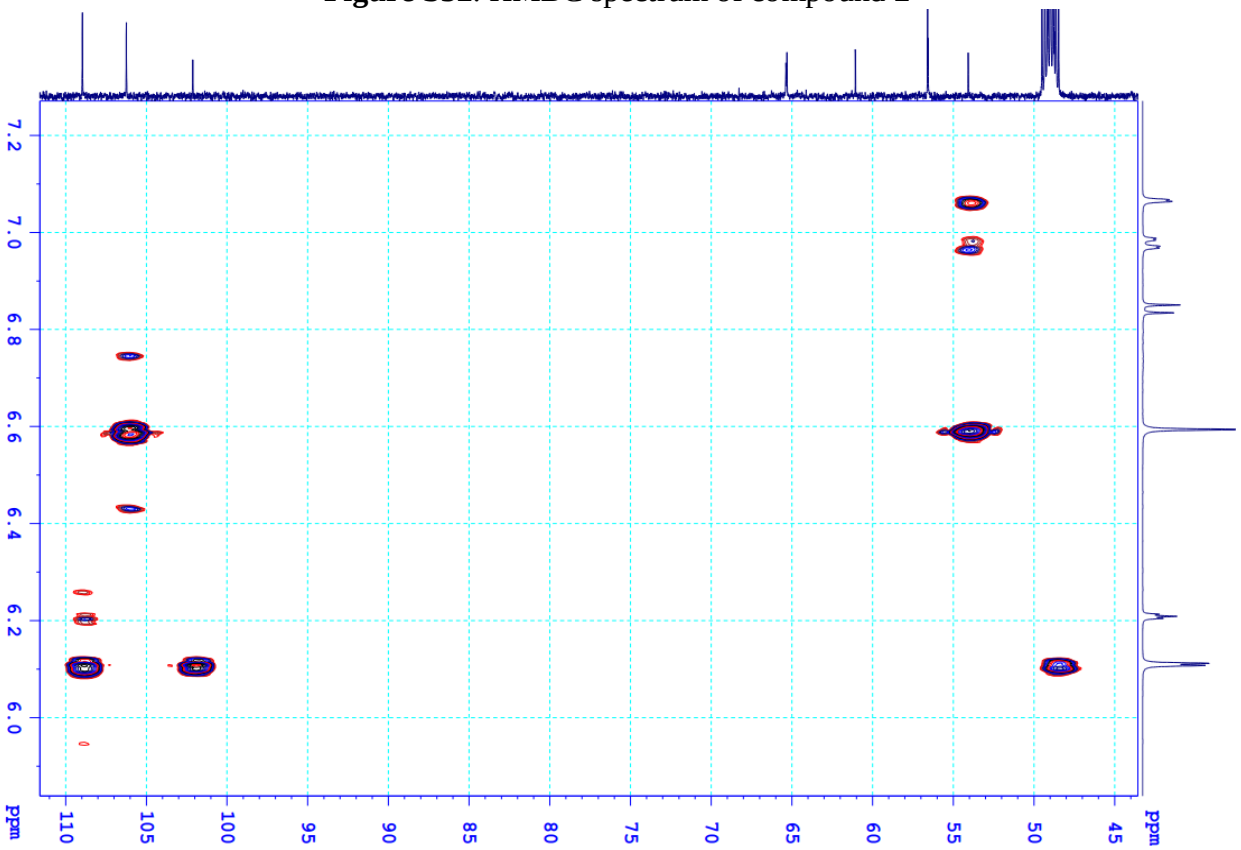

Figure S33. Extended HMBC spectrum of compound **2**

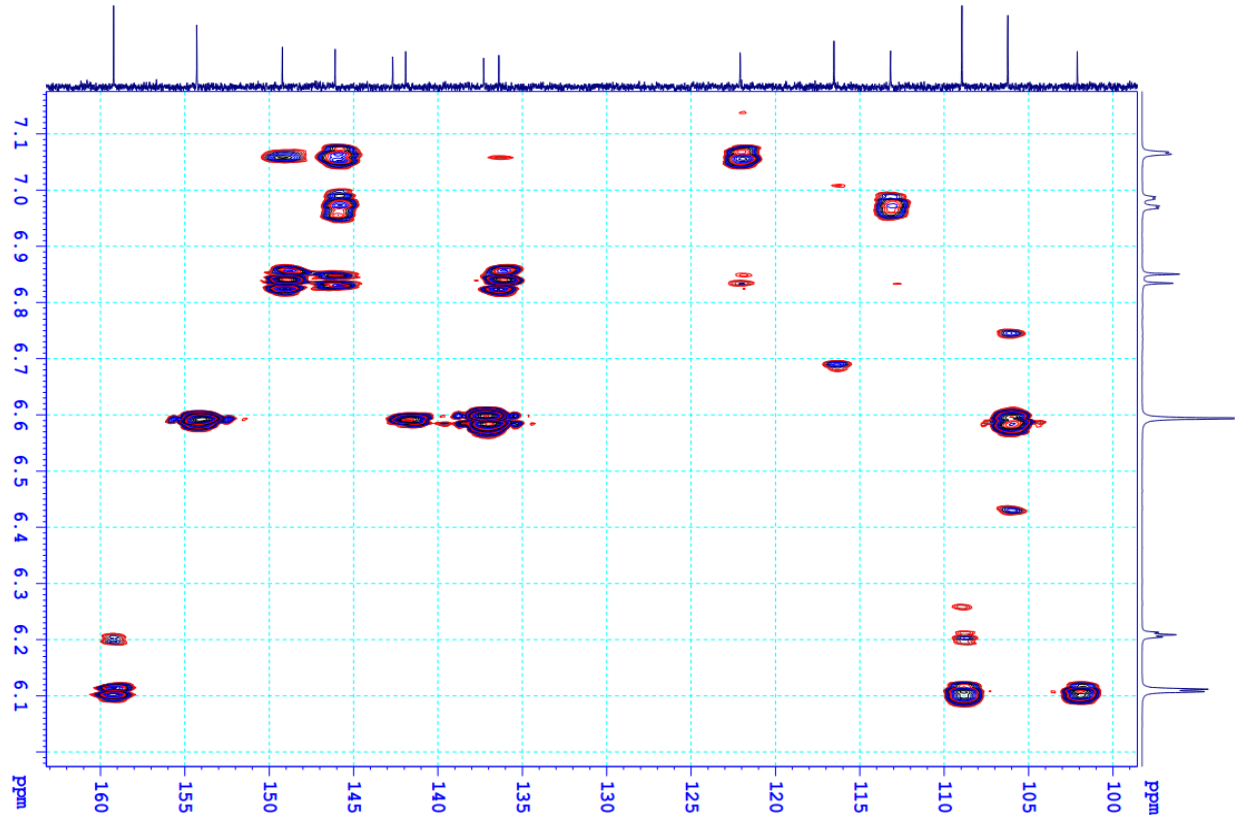

Figure S34. Extended HMBC spectrum of compound **2**

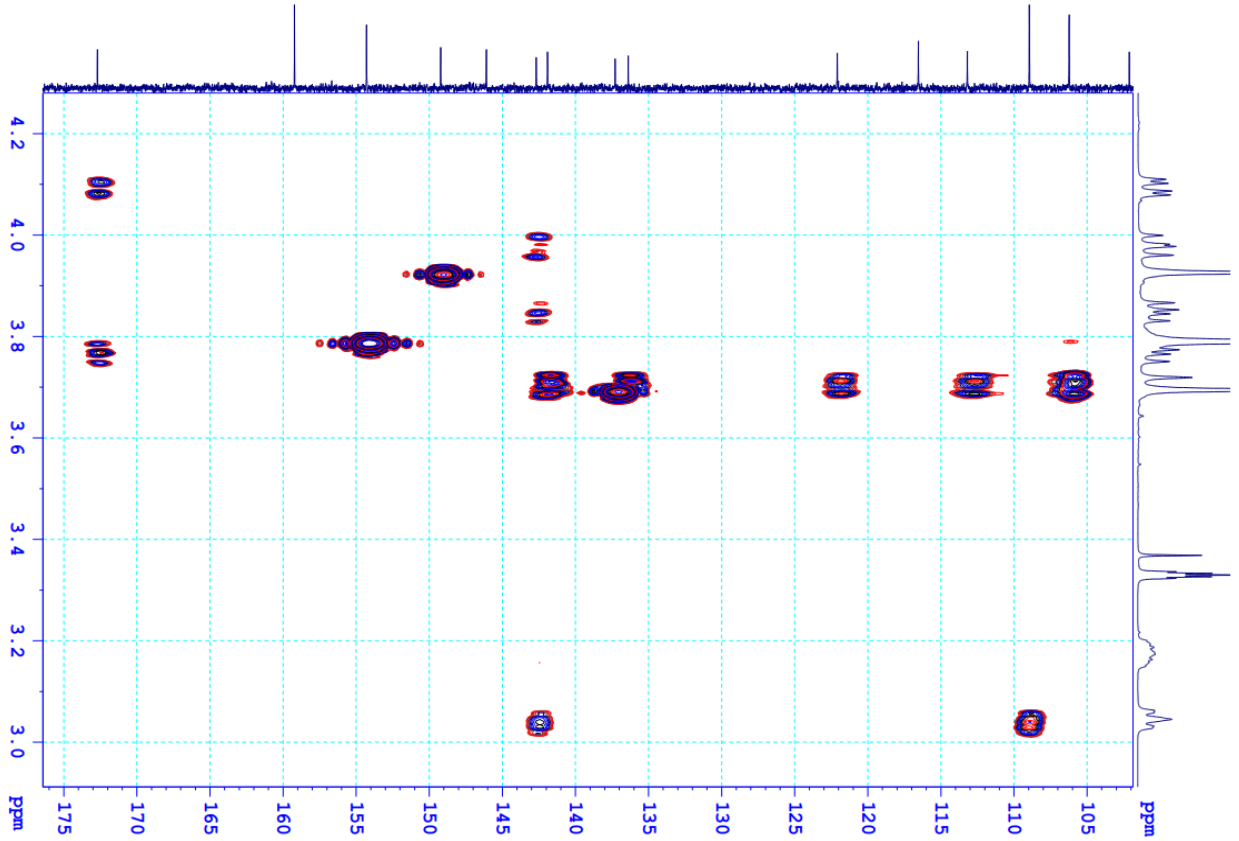

Figure S35. Extended HMBC spectrum of compound **2**

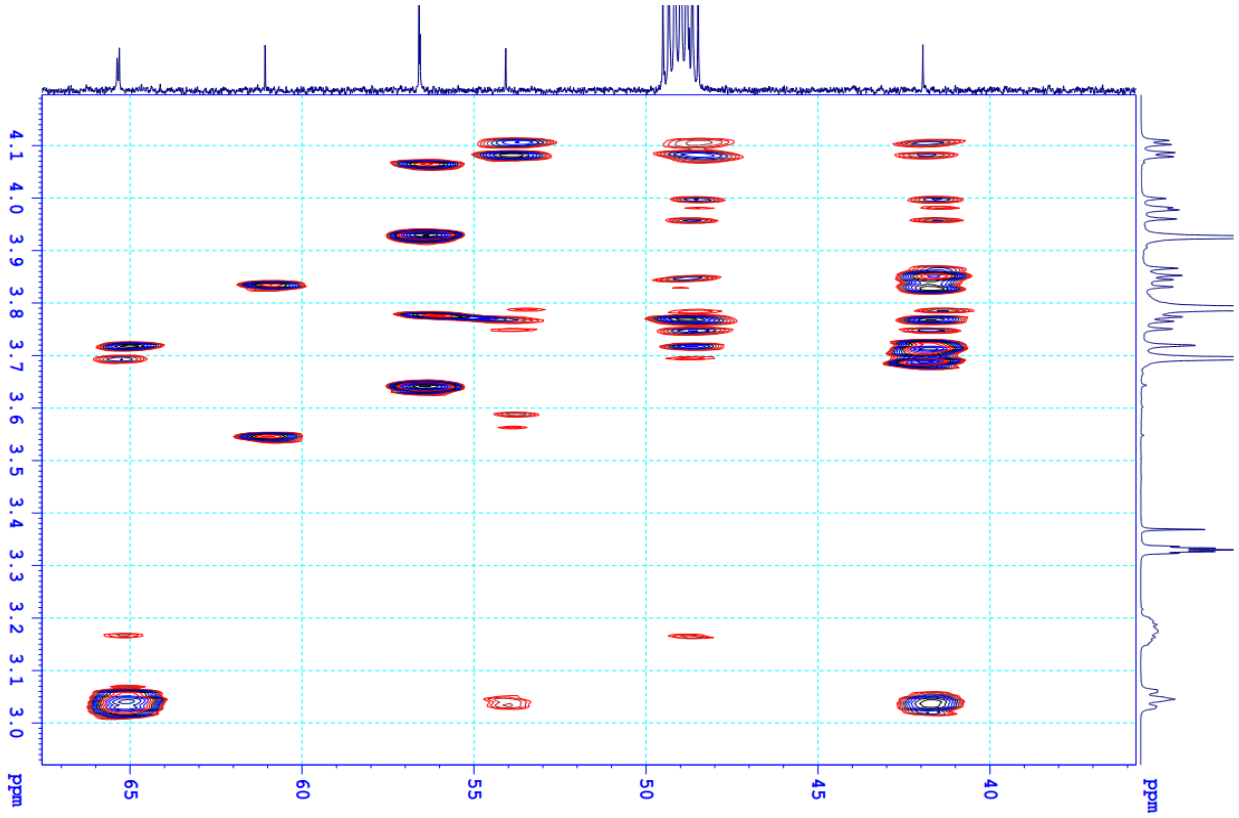

Figure S36. Extended HMBC spectrum of compound **2**

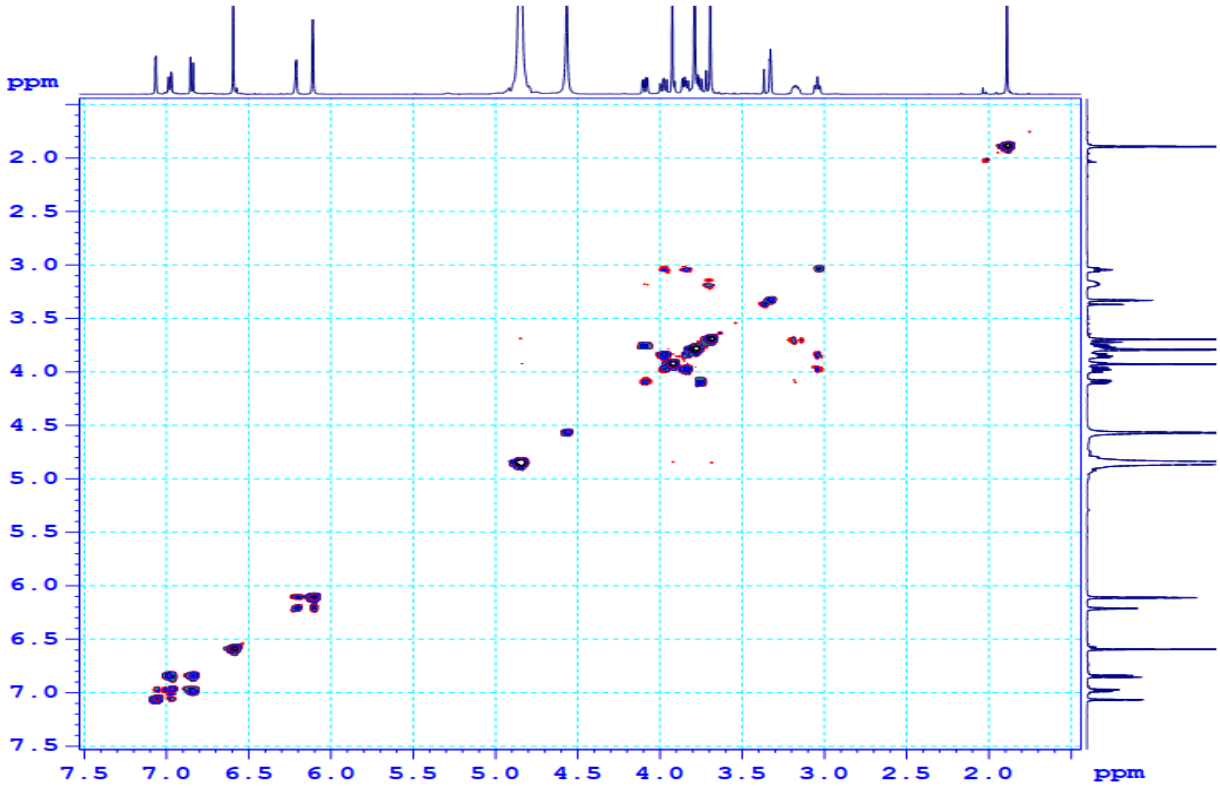

Figure S37. H-H COSY spectrum of compound **2**

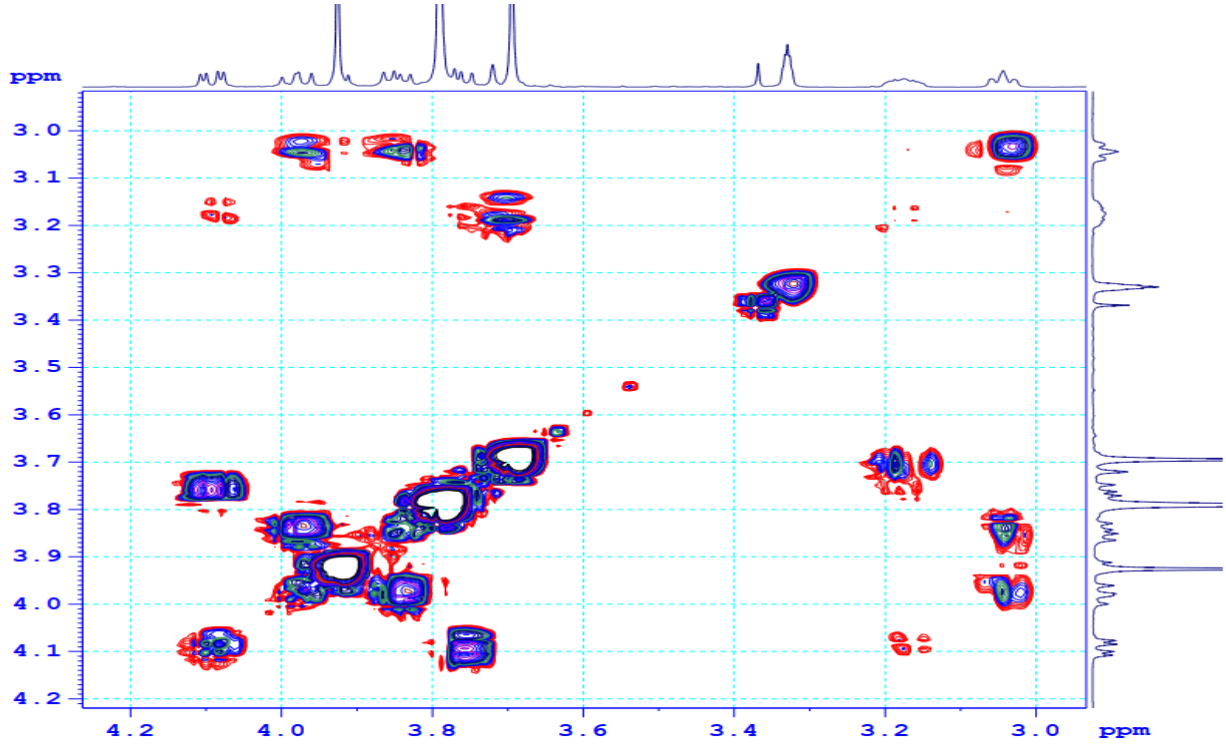

Figure S38. Extended H-H COSY spectrum of compound **2**

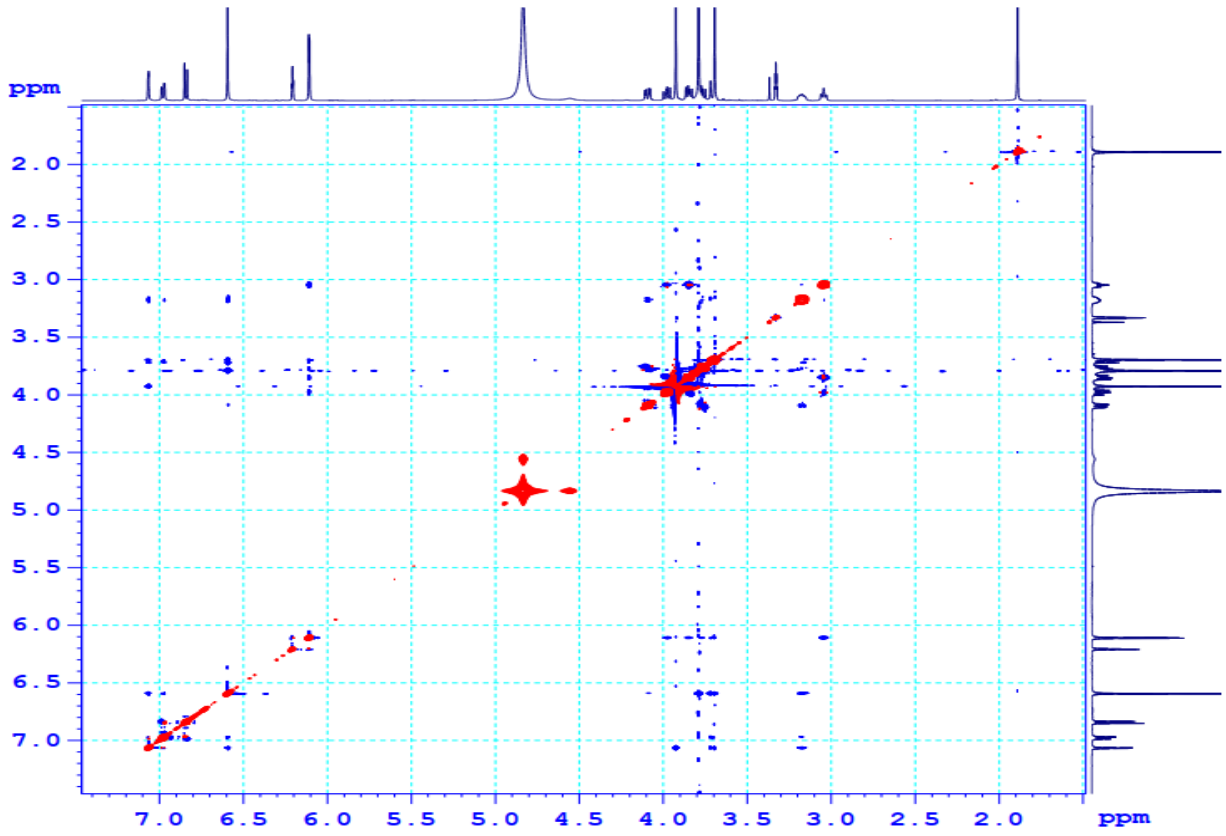

Figure S39. NOESY spectrum of compound **2**

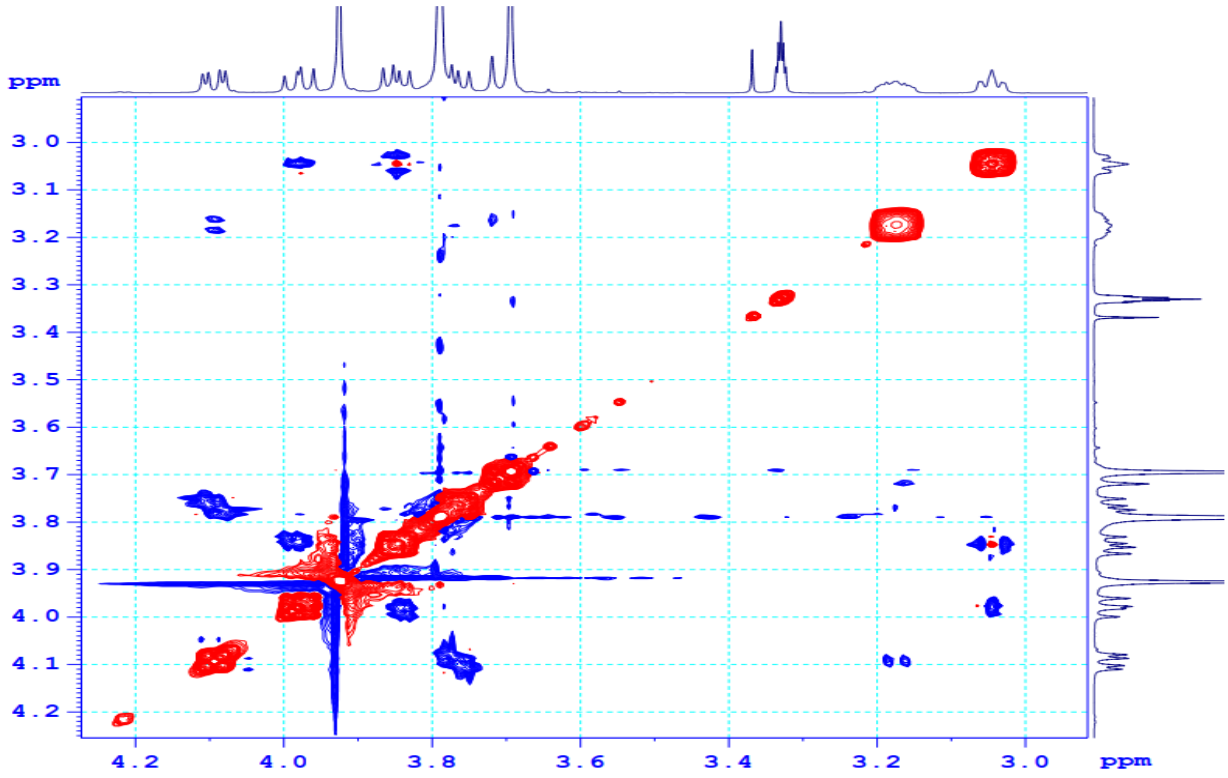

Figure S40. Extended NOESY spectrum of compound **2**

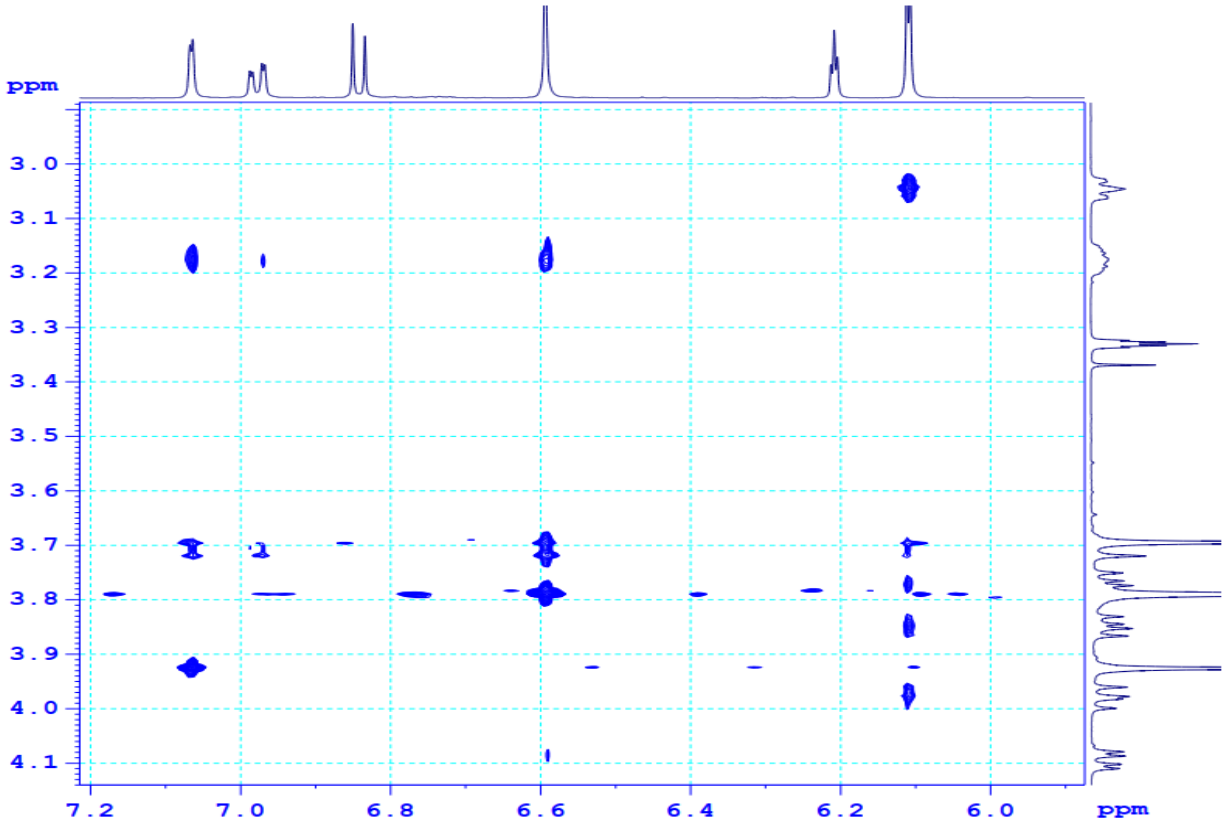

Figure S41. Extended NOESY spectrum of compound **2**

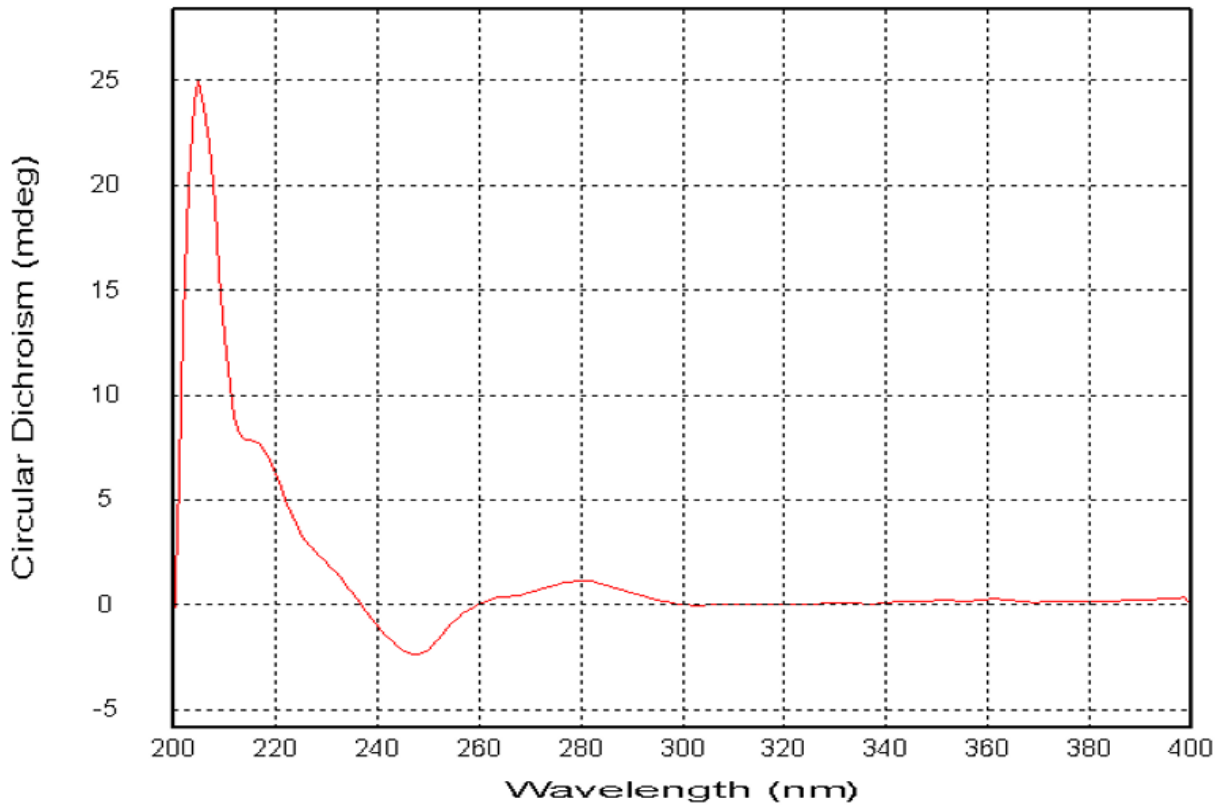

Figure S42. CD spectrum of compound **2**

Table S1. Cytotoxic activity of compounds **1-7** towards CAL and MDA-MB-231 cell lines

Compound	Cell viability (%)	
	CAL27	MDA-MB-231
1	69.4 ± 0.87	89.4 ± 0.62
2	98.8 ± 1.23	90.9 ± 0.65
3	88.1 ± 0.46	86.3 ± 0.67
4	76.3 ± 0.91	84.7 ± 0.66
5	69.5 ± 0.97	85.5 ± 0.74
6	65.3 ± 0.86	89.5 ± 0.91
7	73.0 ± 0.98	78.8 ± 0.85

Compound was tested at concentration of 30 µM

Table S2. ANO1 inhibitory activity of compounds **1-7** at concentration of 30 µM

Compound	Inhibition (%)
1	24.3 ± 1.41
2	17.2 ± 1.63
3	13.8 ± 0.92
4	18.1 ± 1.15
5	11.4 ± 0.73
6	8.1 ± 0.87
7	15.5 ± 1.26