Supporting Information (SI)

Design and Synthesis of Novel Multi-Target Tetrabromophthalimides as CBS, Topo-II Inhibitors, and DNA Intercalators

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Table of Contents

Table S1. Tetrabromophthalimide derivatives (**2a-2k**) GI% utilizing fifteen cancer and two normal cell lines.

Comp no./ Cell line name	2a	2b	2c	2d	2f	2g	2h	2i	2j	2k	Dox
HNO97	82.81	75.13	51.02	55.77	73.49	65.46	76.78	61.38	65.34	50.52	70.50
HN-9	55.08	57.89	78.77	83.04	60.77	76.35	75.30	78.65	66.84	56.42	82.03
MCF7	54.15	63.46	54.59	65.15	58.64	43.55	45.47	45.15	29.80	39.38	0.80
CaCo2	44.34	54.42	30.13	35.72	44.66	24.13	20.52	42.77	27.74	30.23	2.81
HEP2	33.64	60.85	27.15	54.41	47.51	42.58	28.13	52.93	44.10	29.71	62.10
HEPG2	56.06	62.68	38.41	70.12	63.70	58.15	50.90	40.20	40.73	55.91	73.32
A431	43.80	59.45	70.97	75.99	62.86	80.01	73.23	76.69	69.40	46.96	68.30
A735	47.83	54.48	47.88	71.00	60.32	58.17	66.99	62.50	41.23	35.64	69.38
H1299	37.71	52.08	26.03	52.60	42.58	42.54	35.98	35.58	33.43	37.47	94.96
A549	44.79	72.62	61.61	63.06	39.08	76.29	68.19	60.83	59.07	75.79	86.43
HCT_{116}	27.29	63.29	41.71	5.16	23.84	54.71	15.32	62.53	43.30	58.13	71.01
PC3	26.87	86.33	24.40	78.56	87.40	21.77	65.04	79.44	61.06	88.79	89.95
FaDu	78.91	85.37	76.16	26.17	78.77	83.49	93.60	91.57	84.90	75.73	92.94
MDA-MB-468	90.80	87.81	77.21	41.49	90.42	91.97	95.37	95.67	91.91	88.06	96.02
HeLa	49.85	77.02	34.54	43.39	73.07	59.46	72.48	76.21	54.88	92.15	92.71
OEC	24.35	50.65	20.34	31.92	38.16	51.85	25.31	20.92	15.63	47.33	57.90
HSF	4.62	23.17	3.17	11.26	22.08	4.49	4.84	20.76	2.40	19.52	26.79

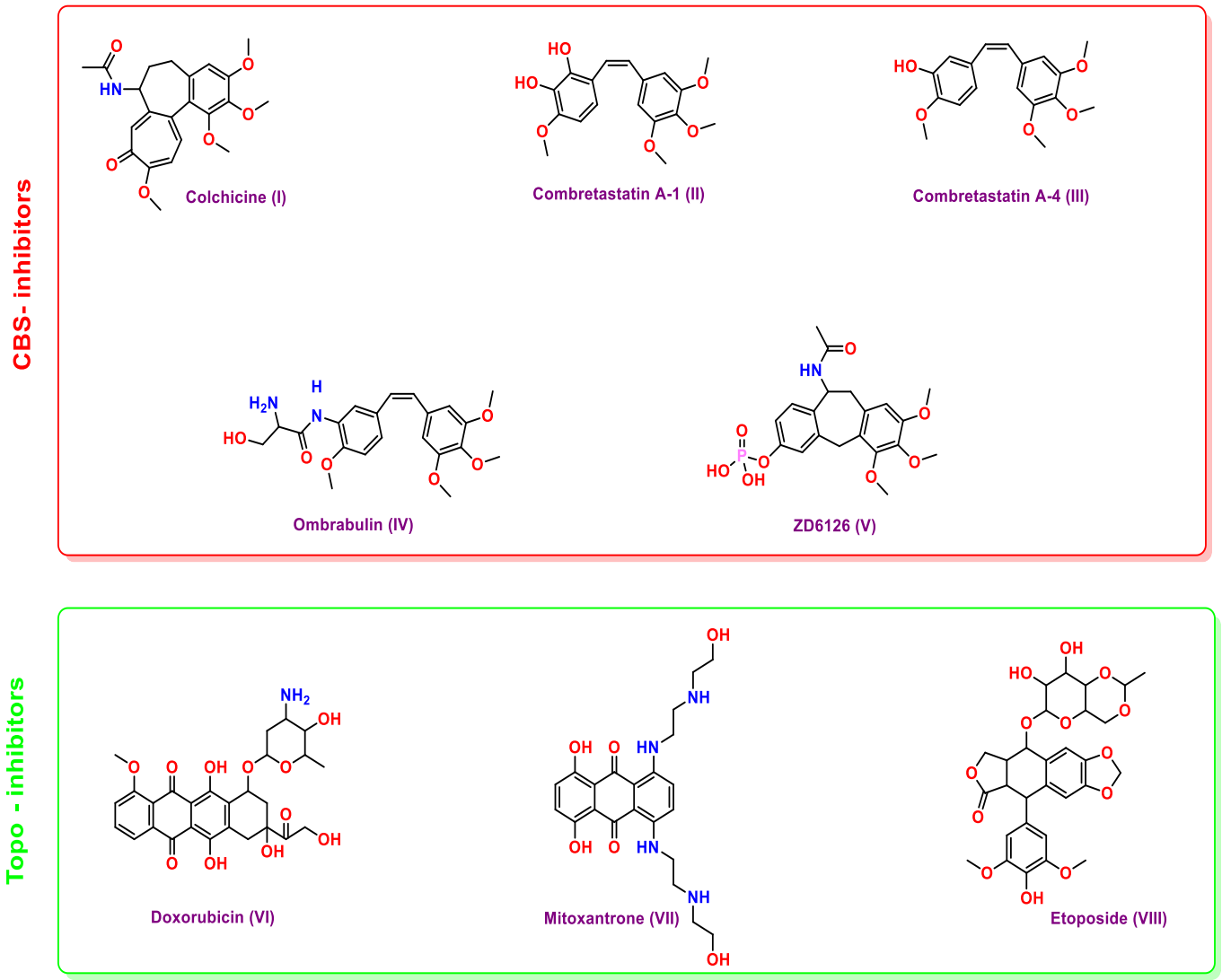


Figure S1. Some reported β-tubulin CBS inhibitors, Topo-II poisons (doxorubicin and mitoxantrone), and Topo-II catalytic inhibitors (etoposide).

FT-IR, ^{1}H NMR, and ^{13}C NMR spectral data of targets (2a-2k)

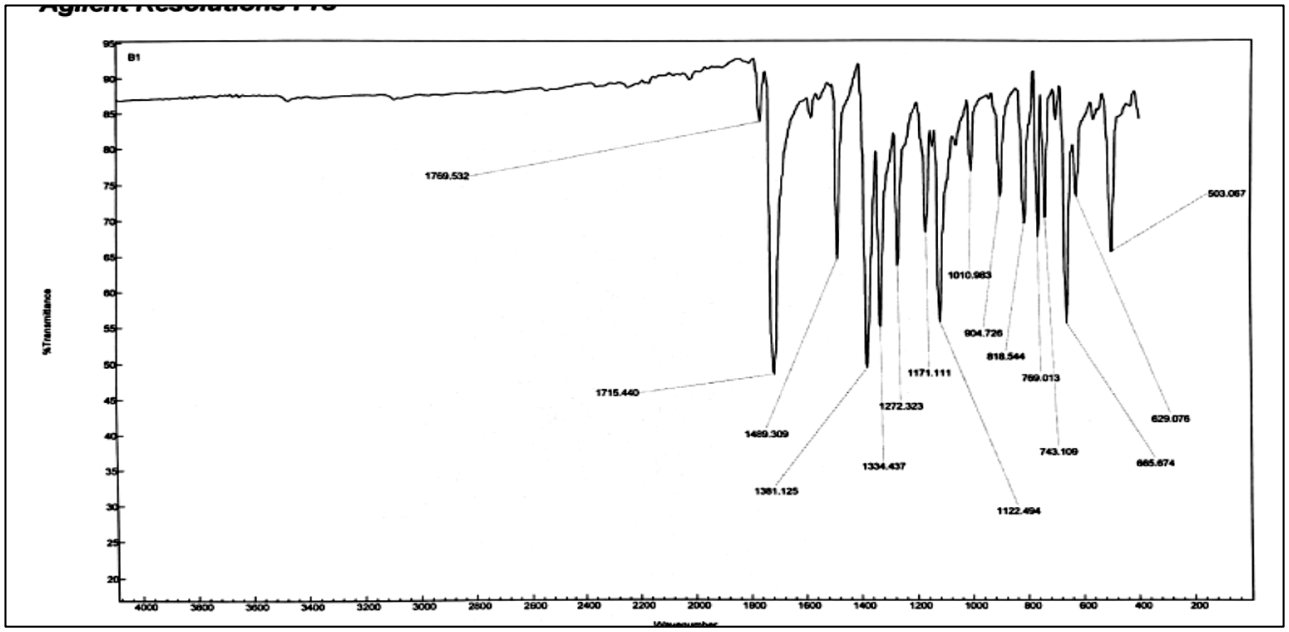


Figure S2. FTIR spectrum of compound **2a.**

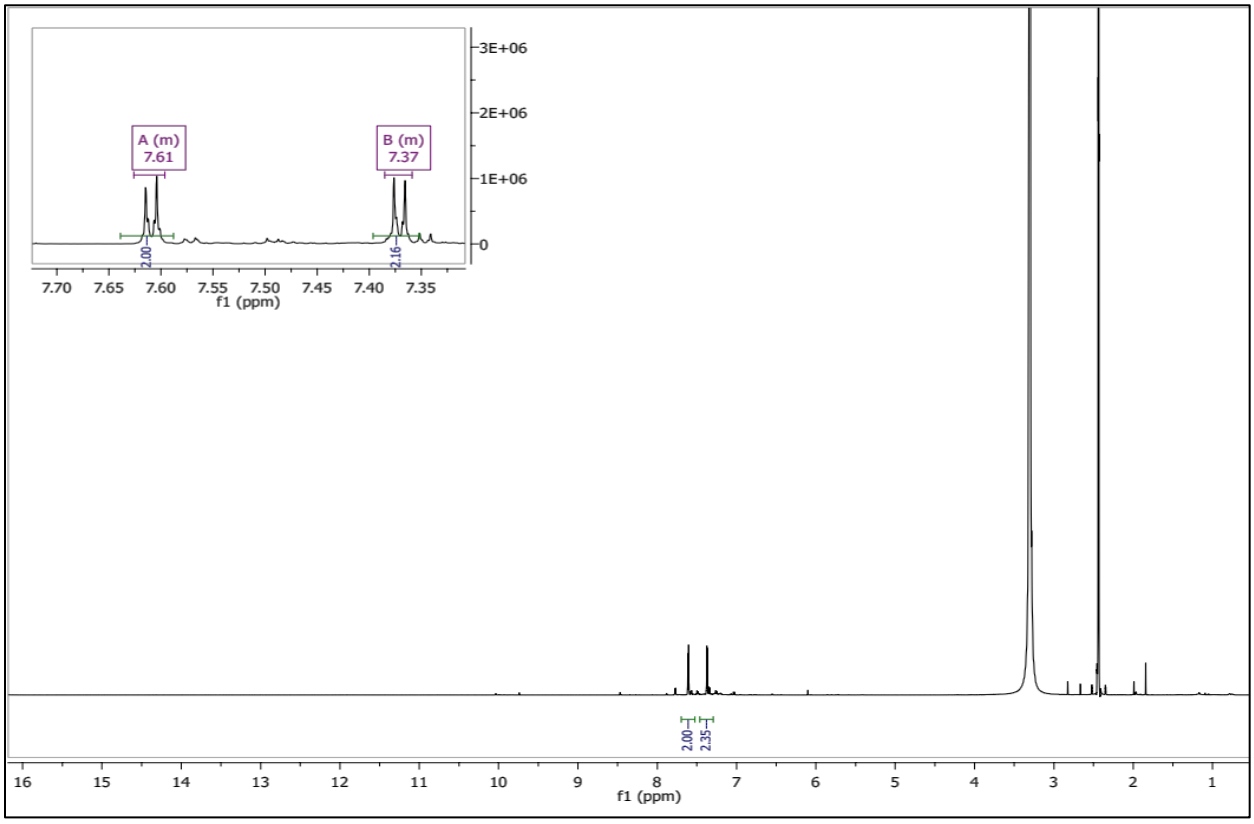


Figure S3. ^{1}H NMR (450 MHz, DMSO-d_6) spectrum of compound **2a.**

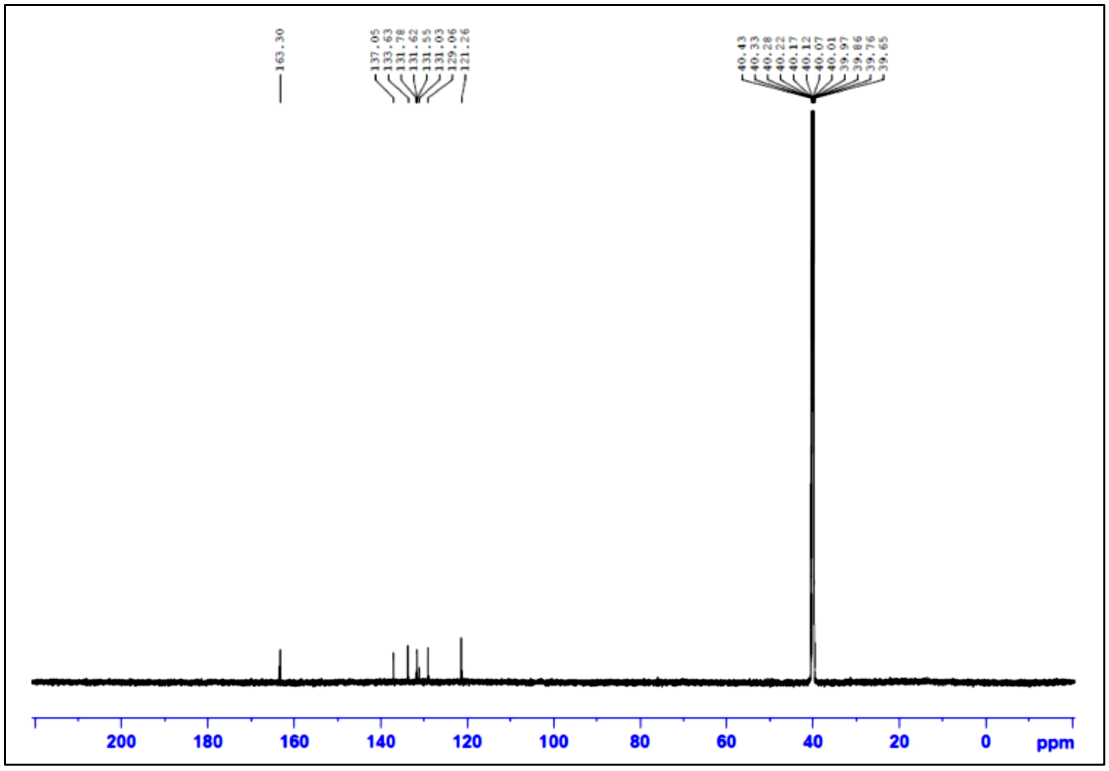


Figure S4. ^{13}C NMR (125 MHz, DMSO-d_6) spectrum of compound **2a.**

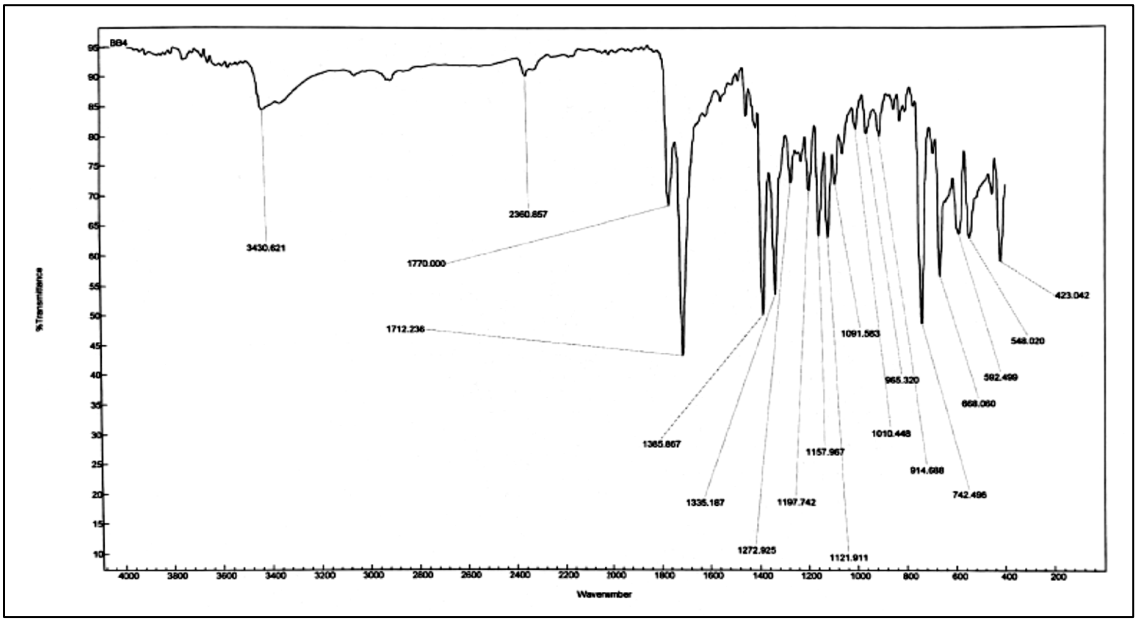


Figure S5. FTIR spectrum of compound **2b.**

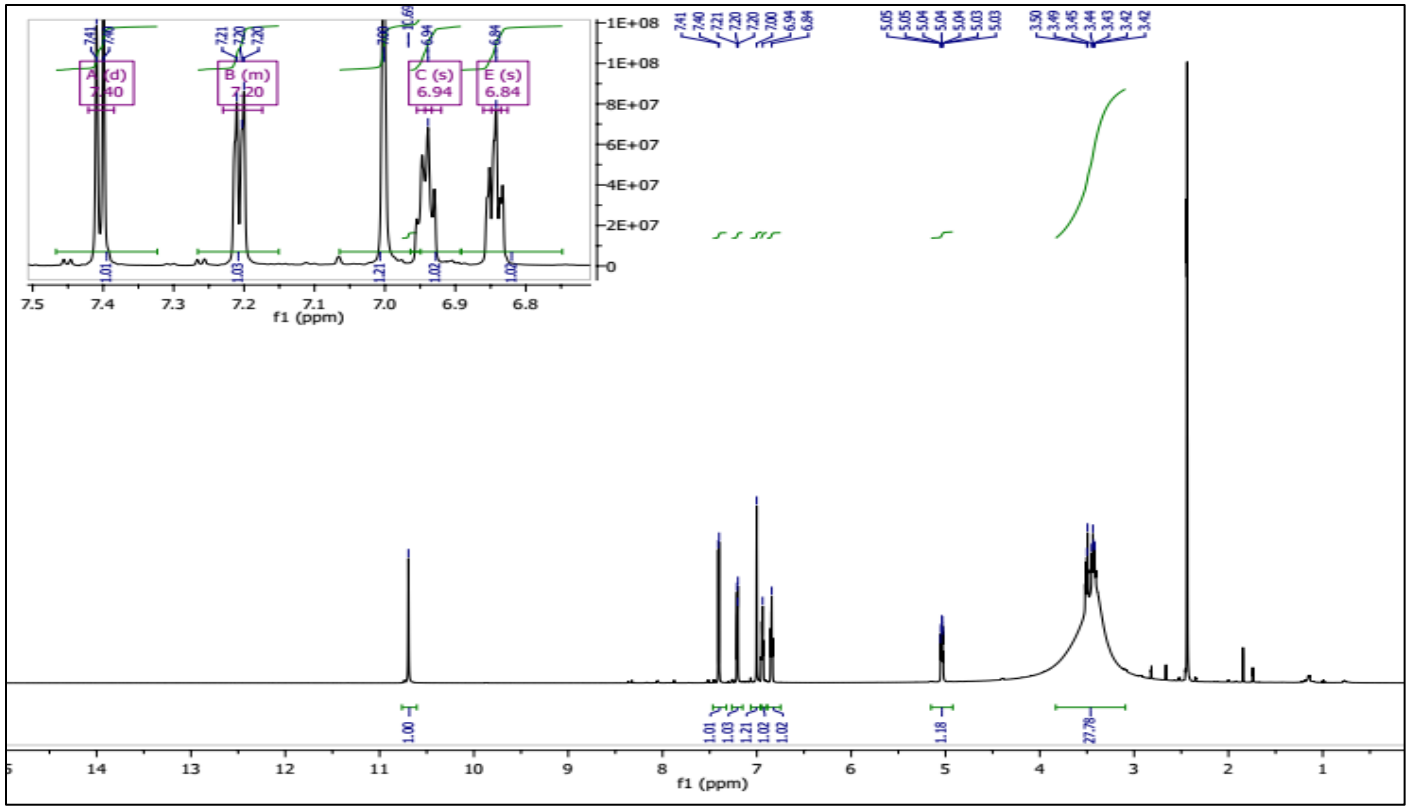


Figure S6. ^{1}H NMR (450 MHz, DMSO-d_6) spectrum of compound **2b.**

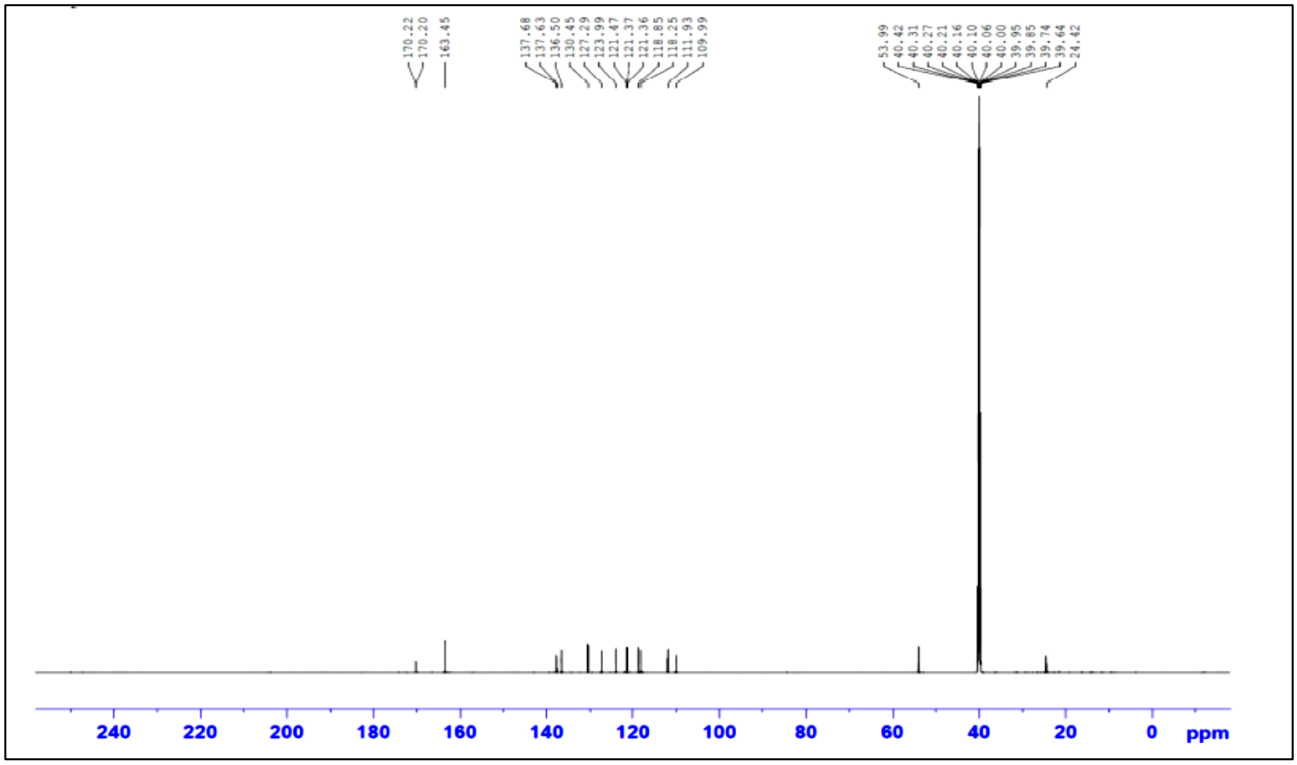


Figure S7. ^{13}C NMR (125 MHz, DMSO-d_6) spectrum of compound **2b.**

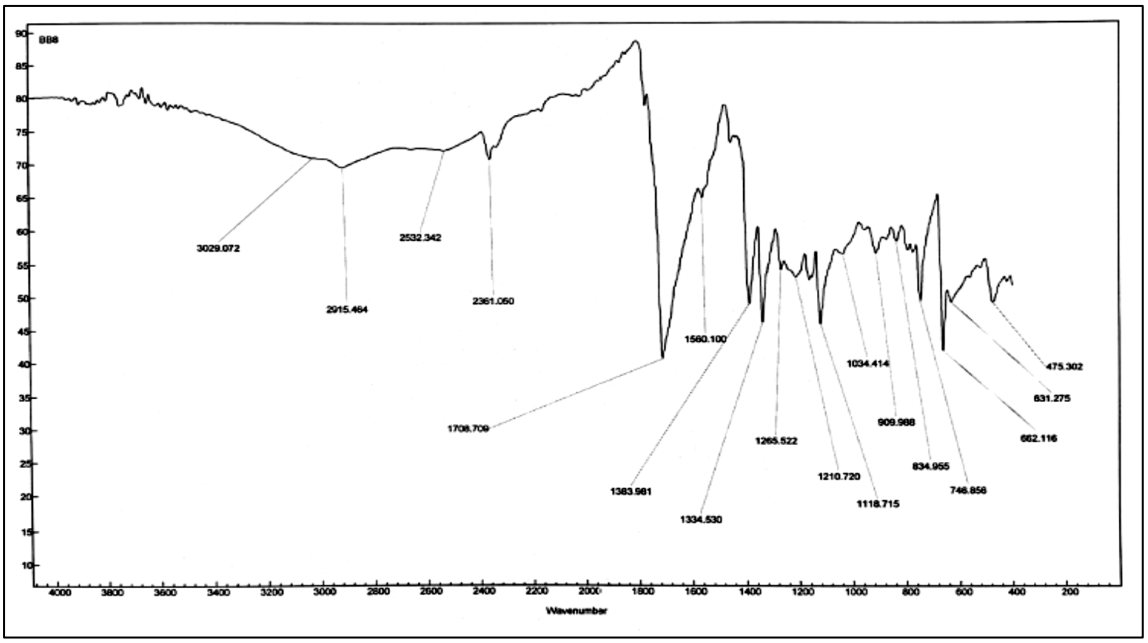


Figure S8. FTIR spectrum of compound **2c.**

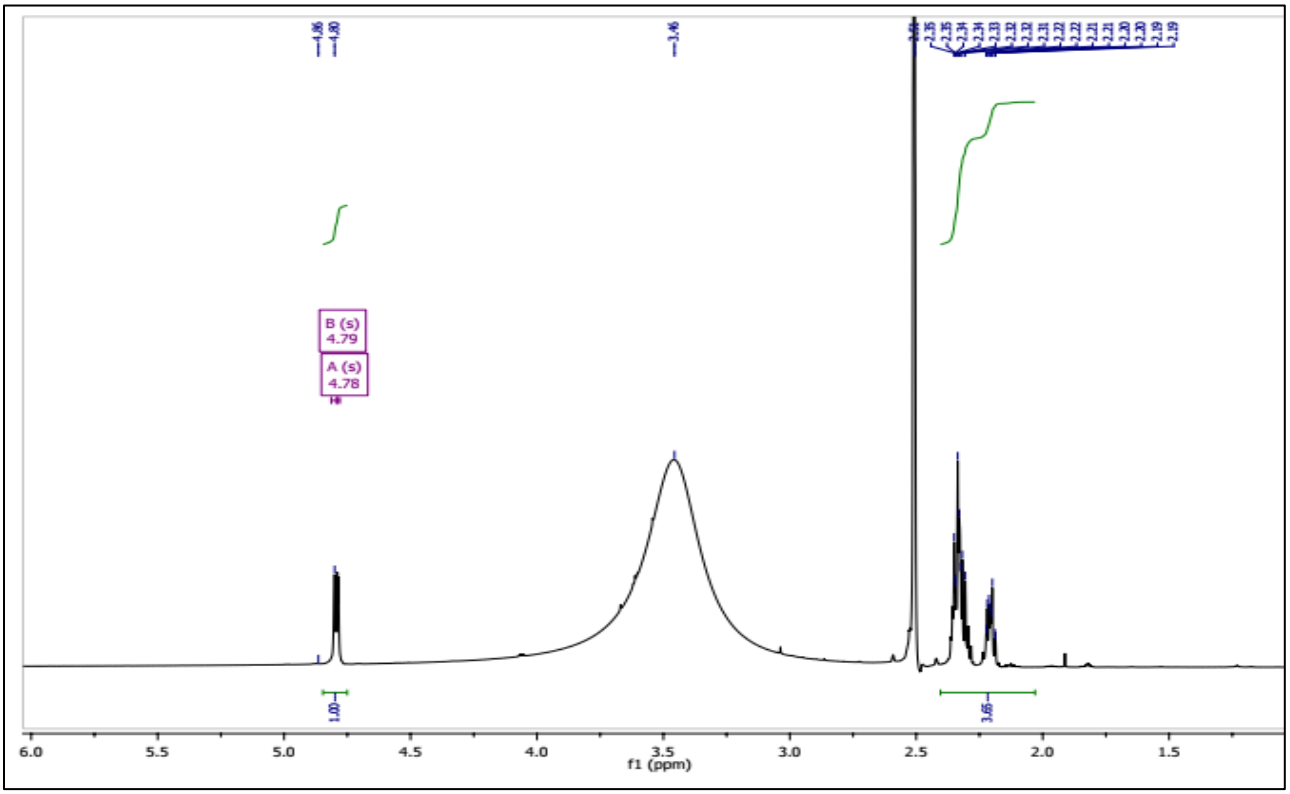


Figure S9. ^{1}H NMR (450 MHz, DMSO-d_6) spectrum of compound **2c.**

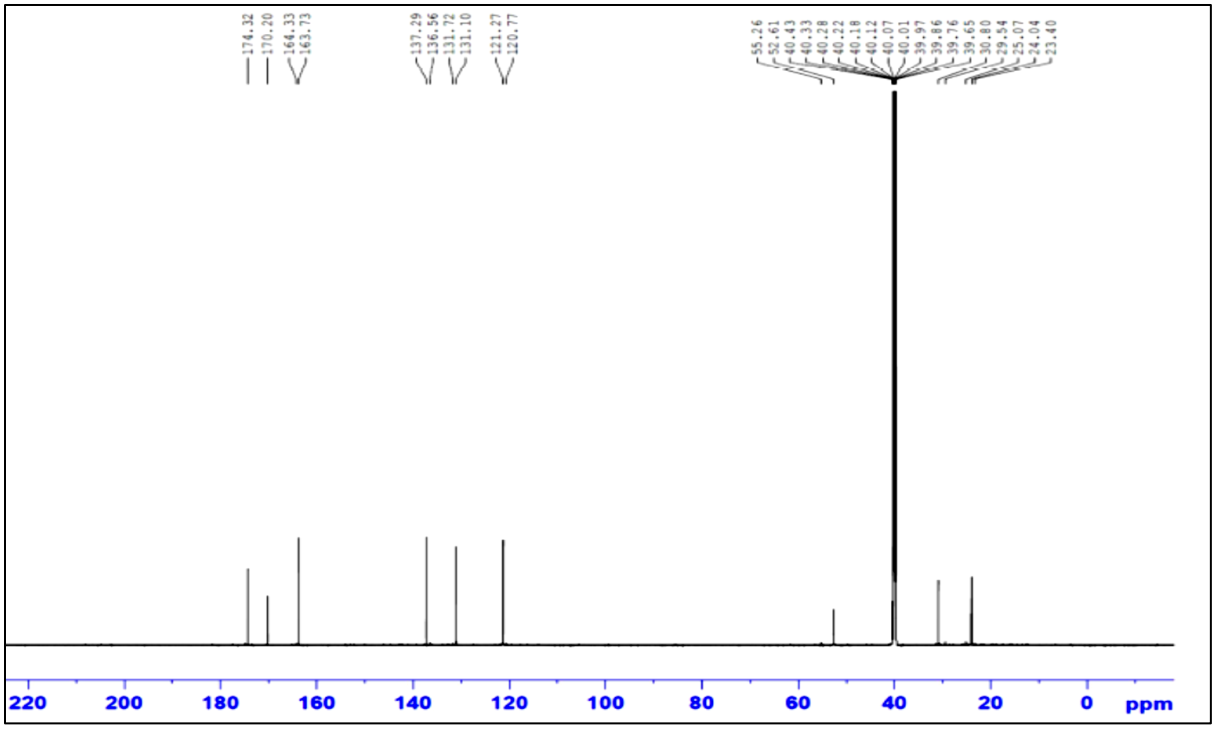


Figure S10. ^{13}C NMR (125 MHz, DMSO-$_6$) spectrum of compound **2c.**

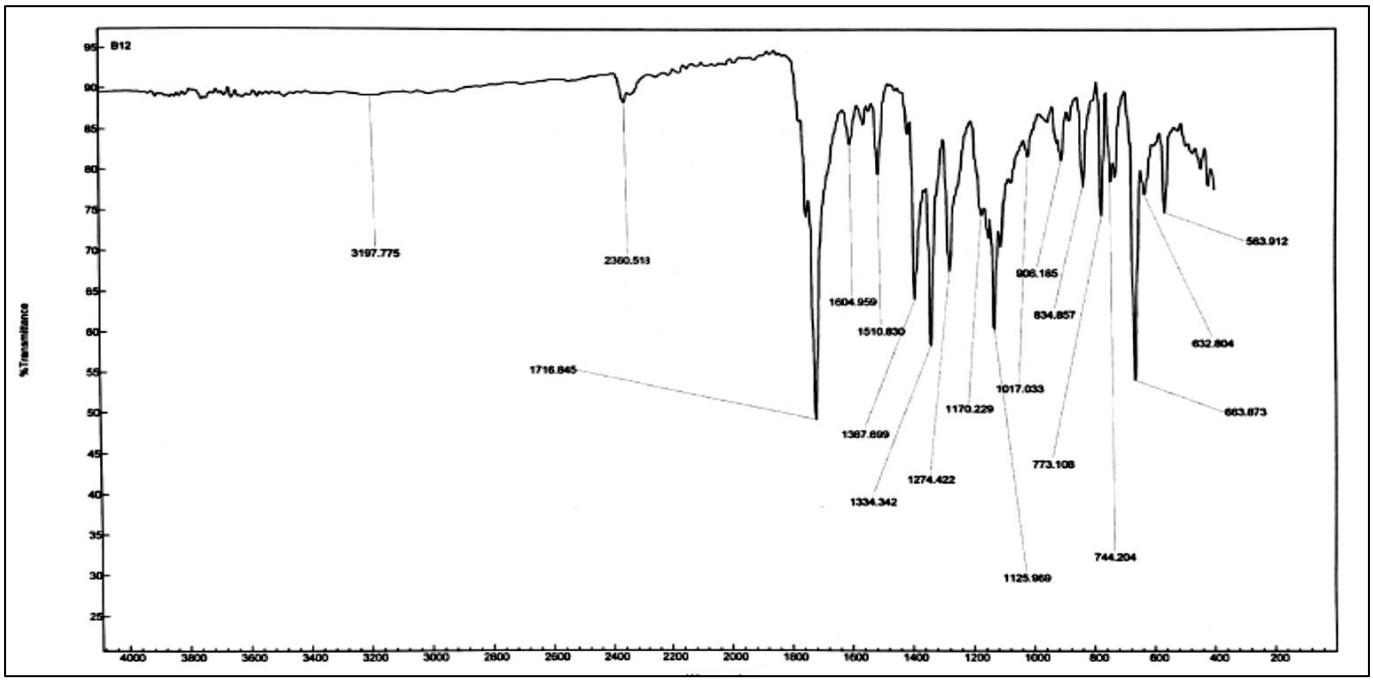


Figure S11. FTIR spectrum of compound **2d.**

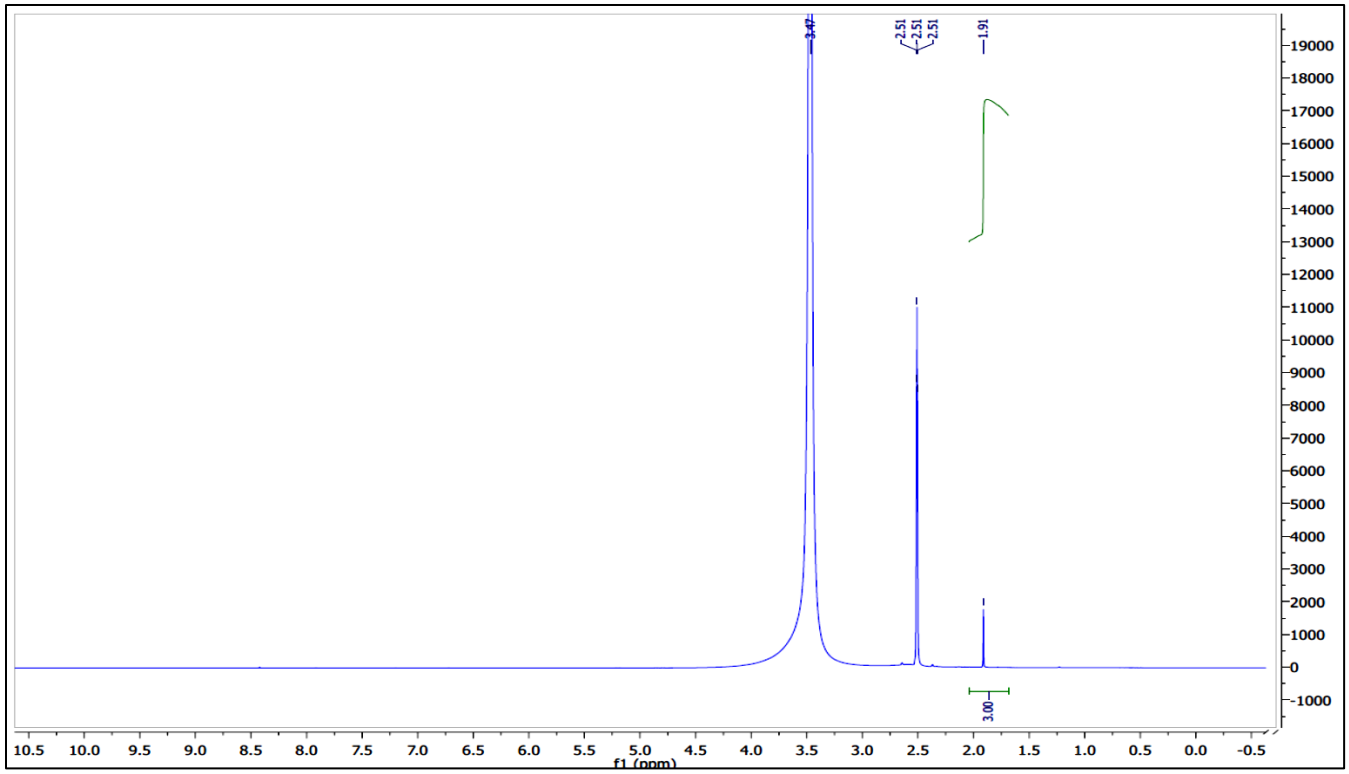


Figure S12. ^{1}H NMR (450 MHz, DMSO-d_6) spectrum of compound **2d.**

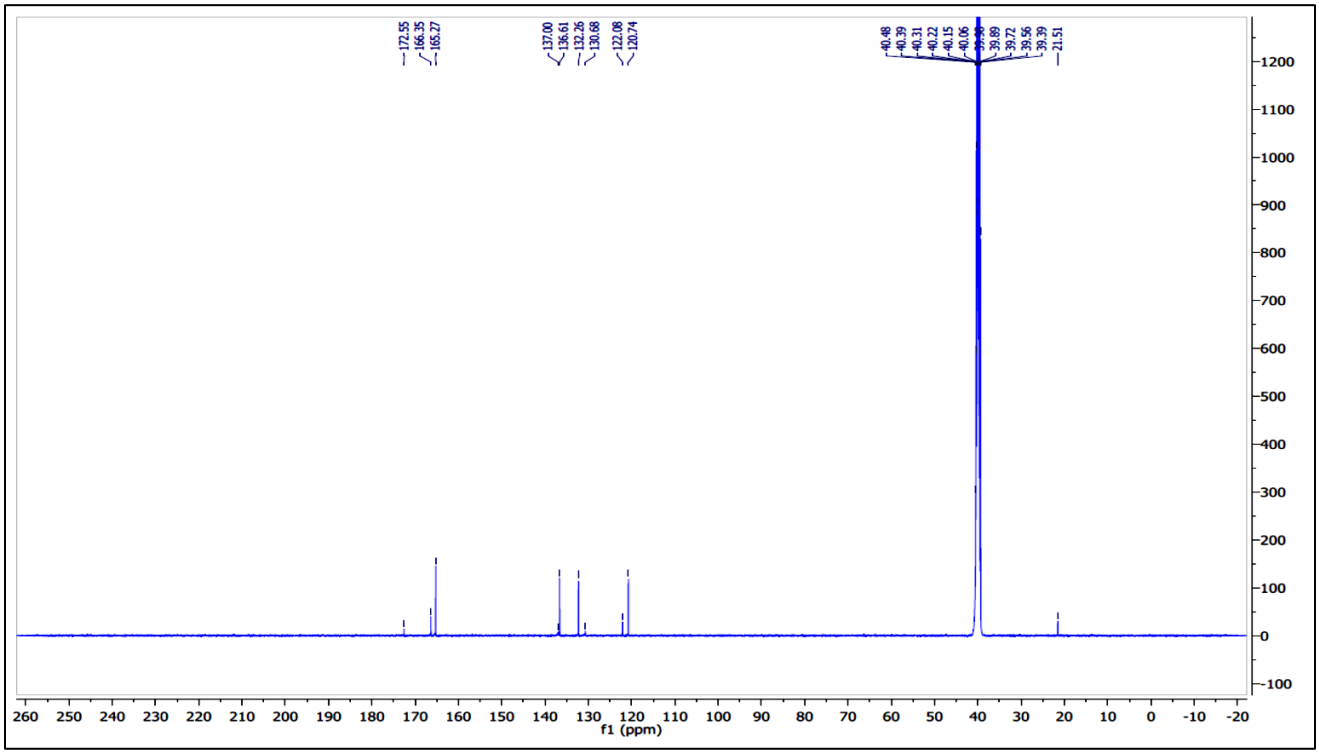


Figure S13. ^{13}C NMR (125 MHz, DMSO-d_6) spectrum of compound **2d.**

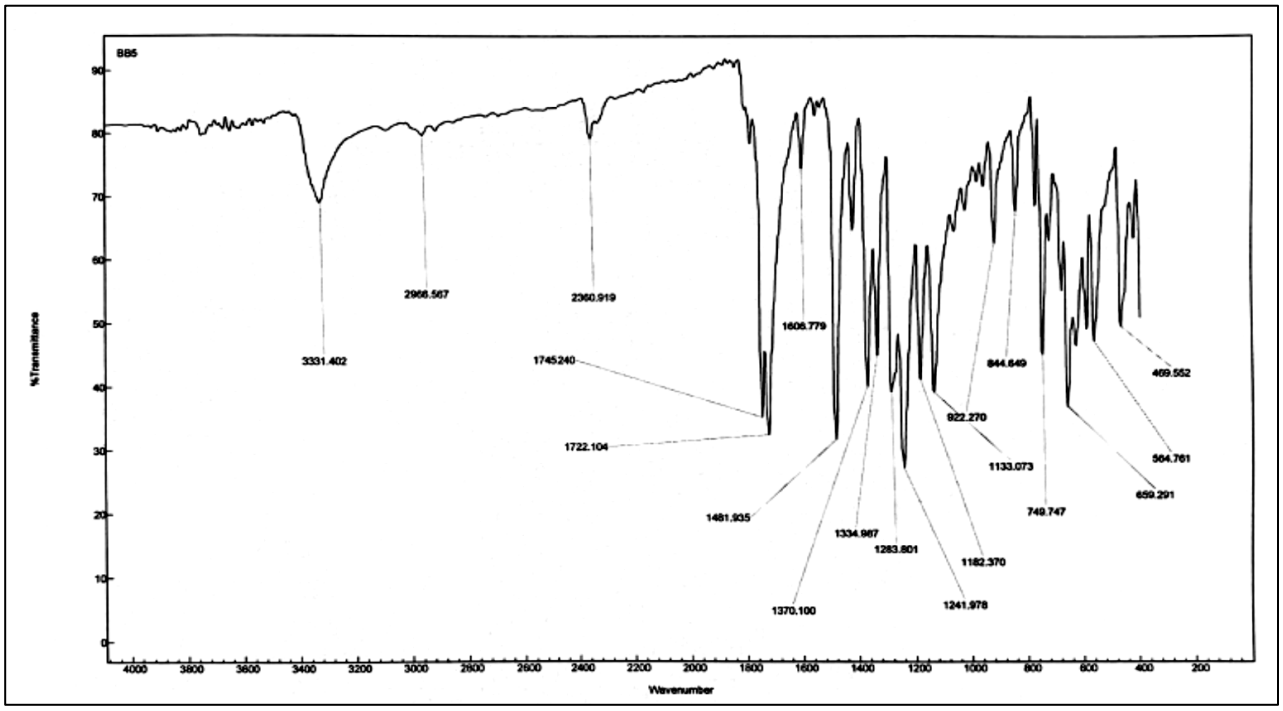


Figure S14. FTIR spectrum of compound **2f.**

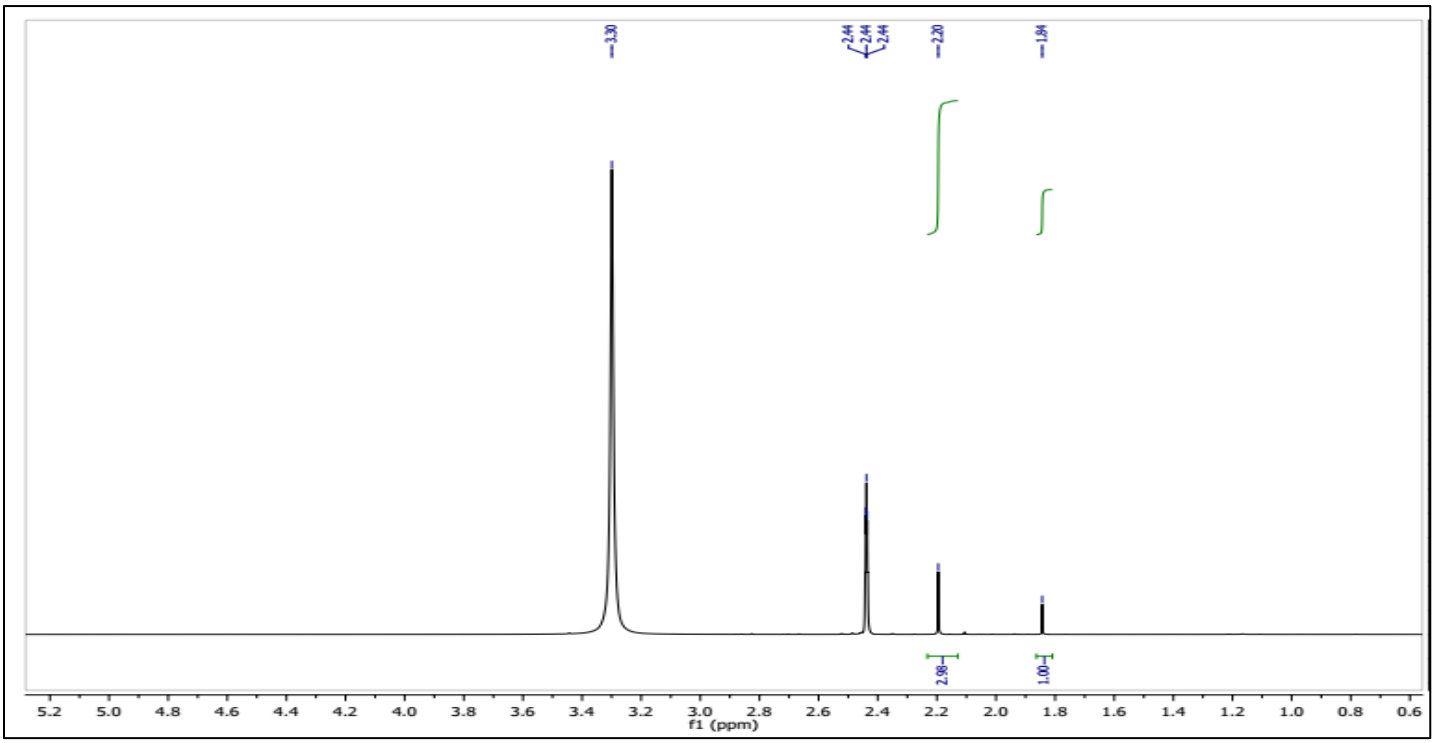


Figure S15. ^{1}H NMR (450 MHz, DMSO-d_6) spectrum of compound **2f.**

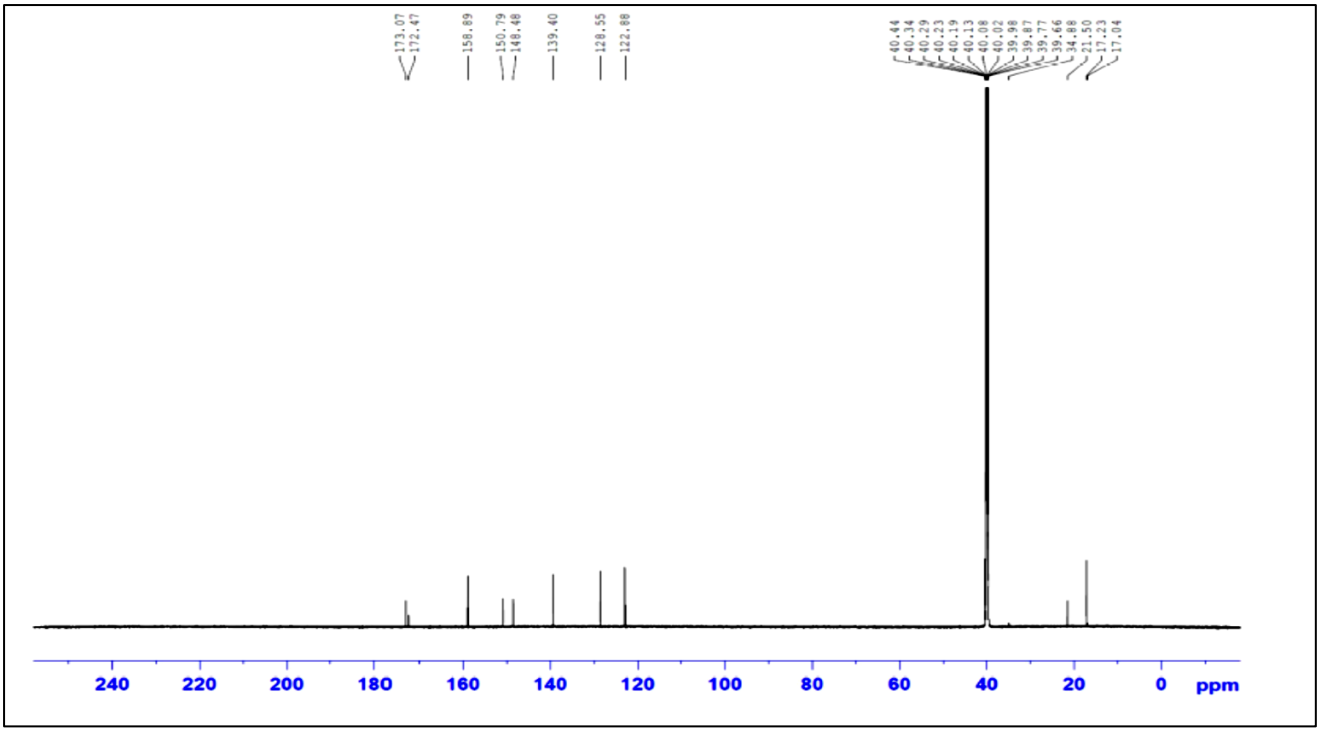


Figure S16. ^{13}C NMR (125 MHz, DMSO-d_6) spectrum of compound **2f.**

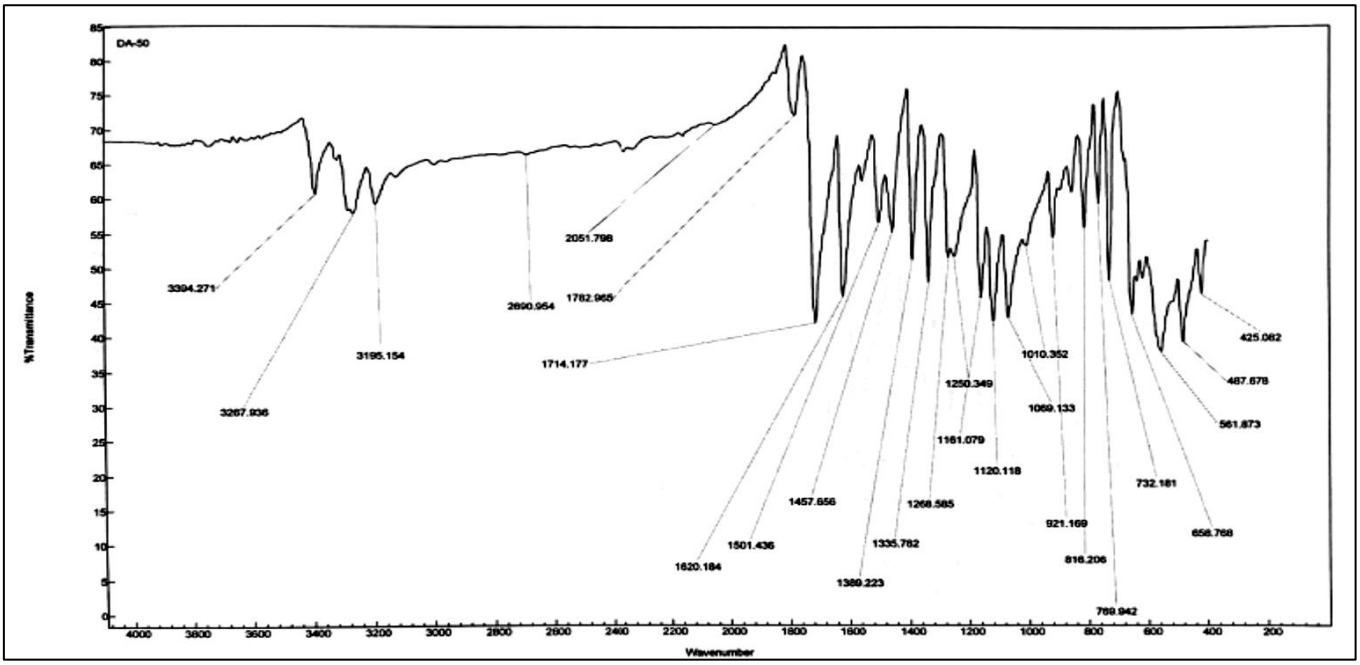


Figure S17. FTIR spectrum of compound **2g.**

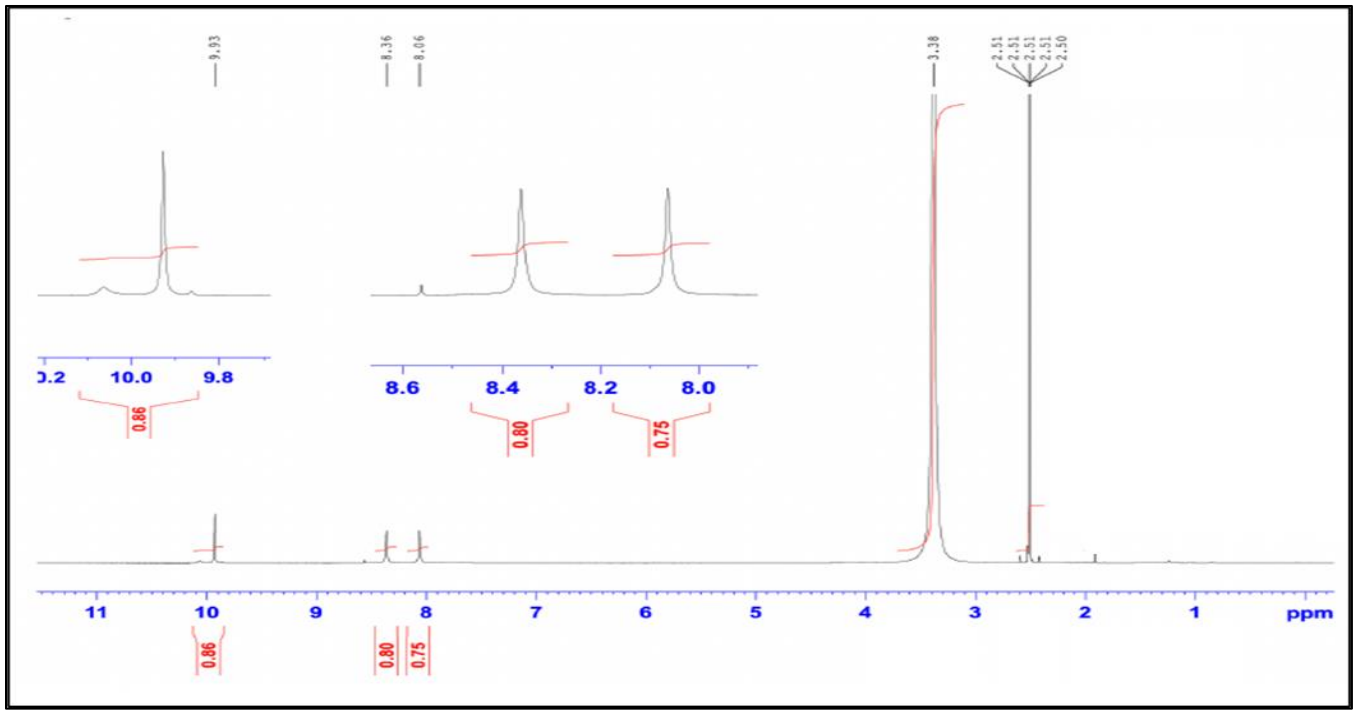


Figure S18. ^{1}H NMR (450 MHz, DMSO-d_6) spectrum of compound **2g.**

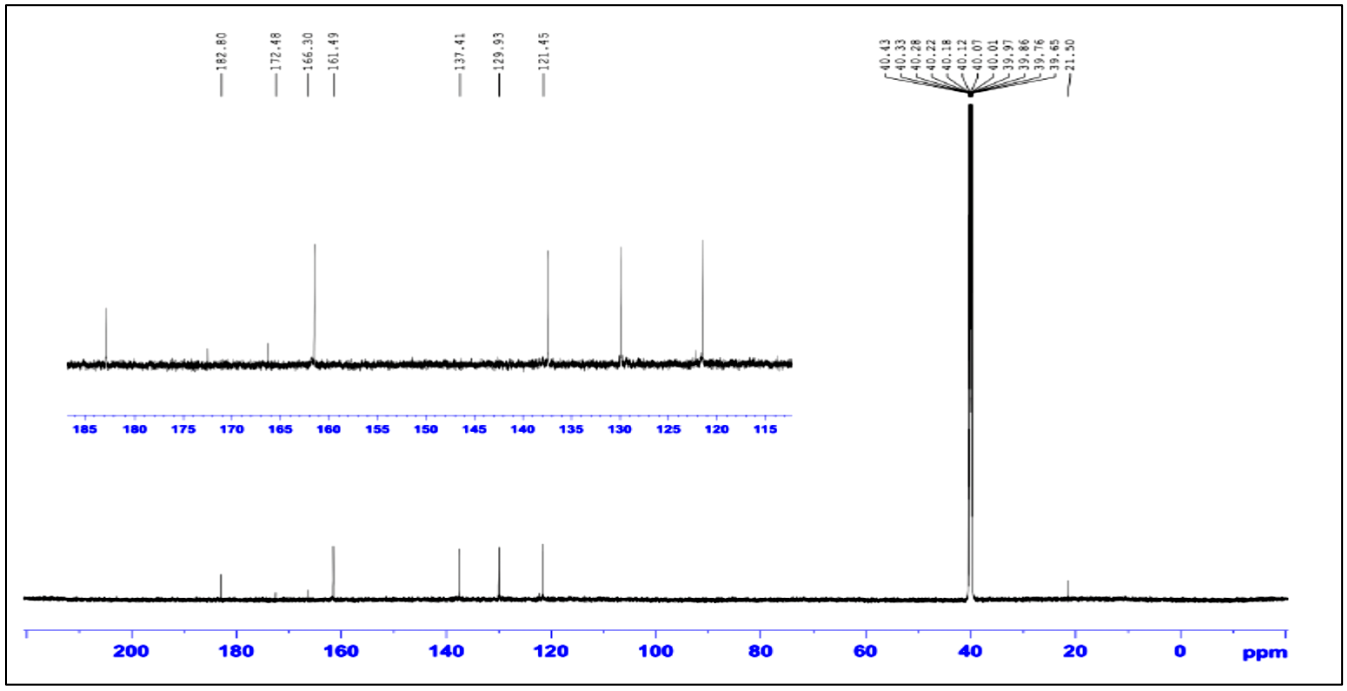


Figure S19. ^{13}C NMR (125 MHz, DMSO-d_6) spectrum of compound **2g.**

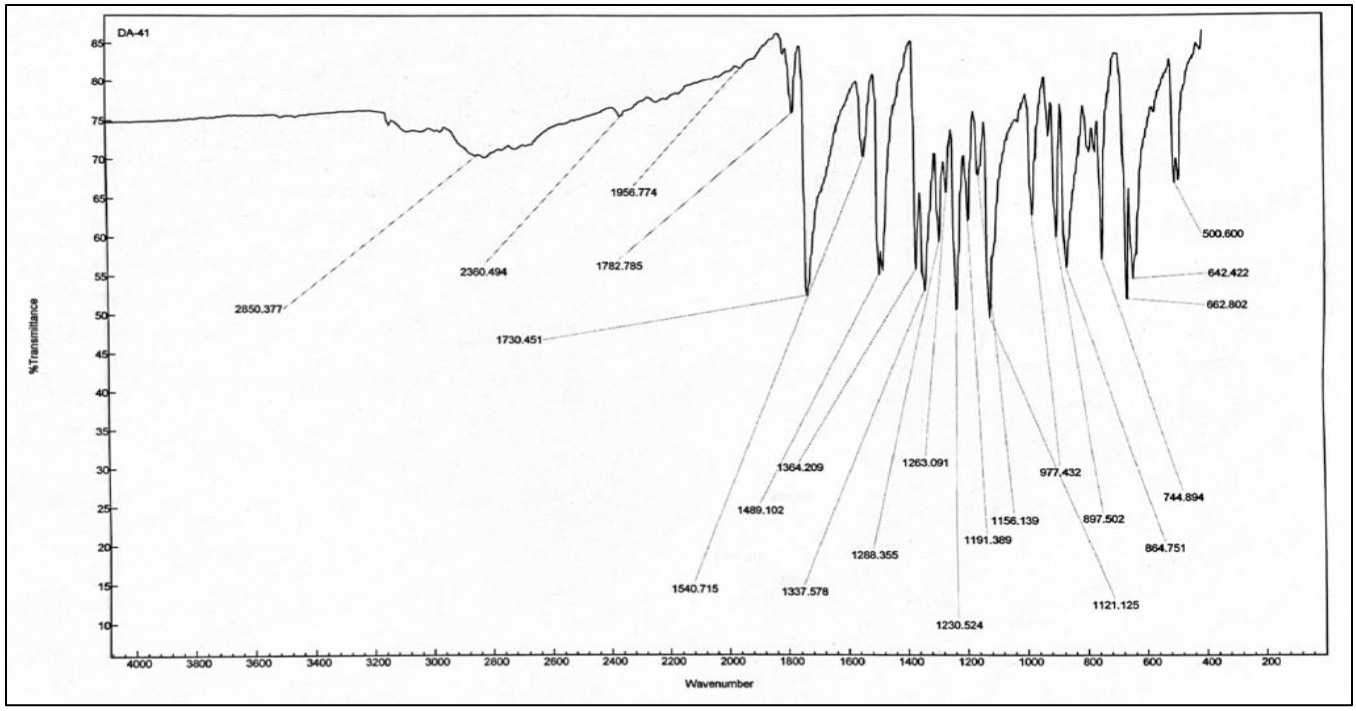


Figure S20. FTIR spectrum of compound **2h.**

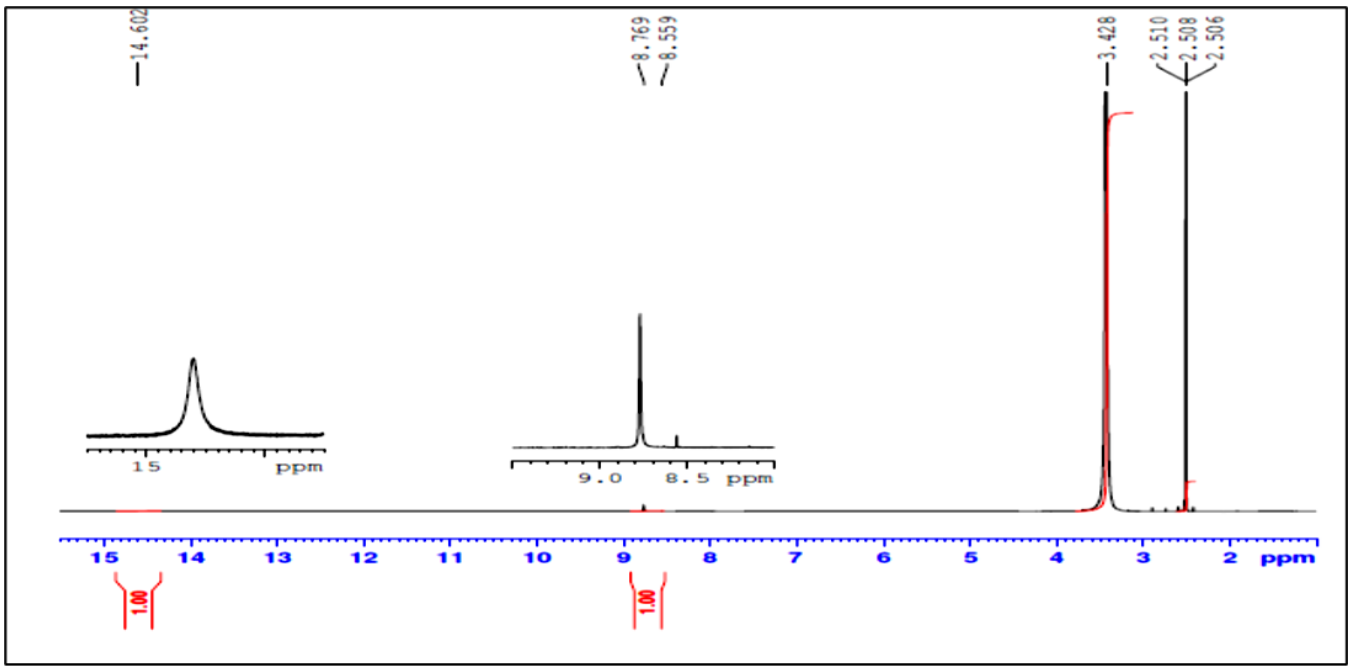


Figure S21. ^{1}H NMR (450 MHz, DMSO-d_6) spectrum of compound **2h.**

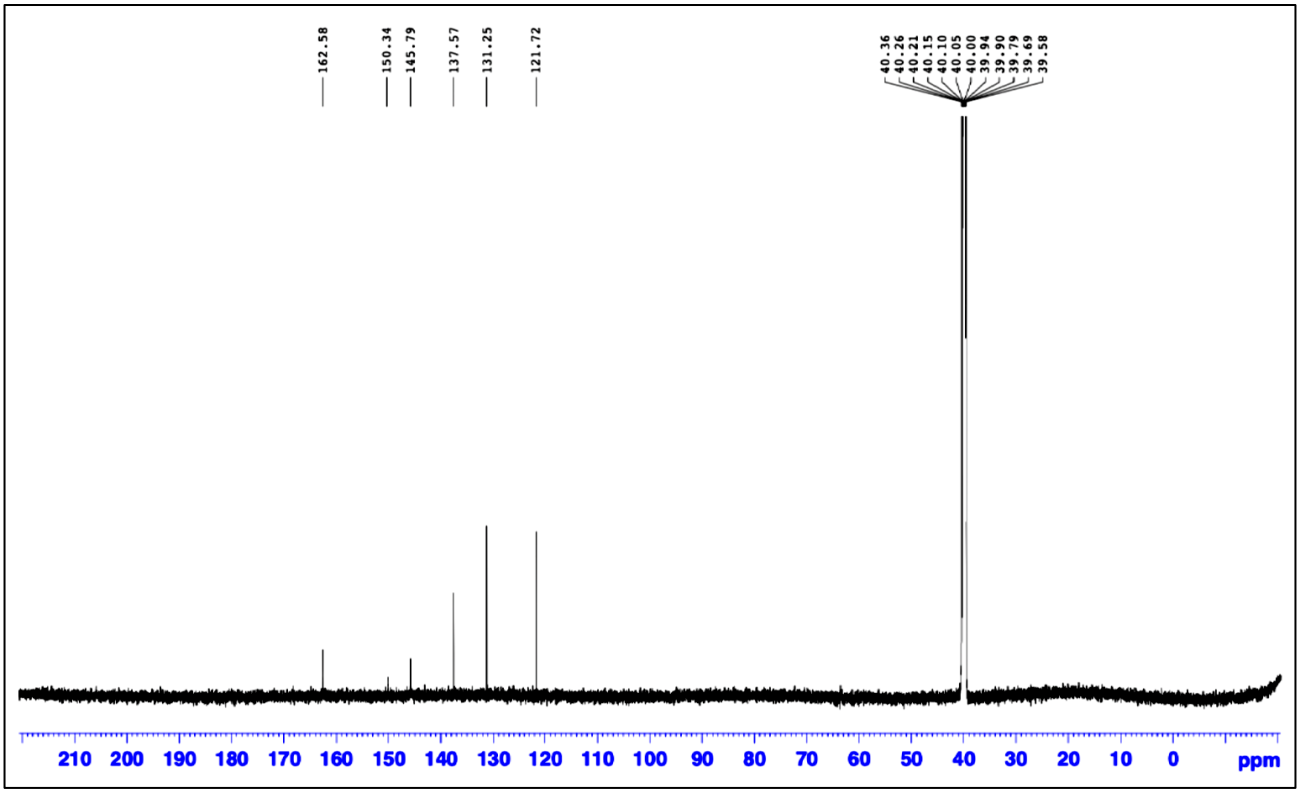


Figure S22. ^{13}C NMR (125 MHz, DMSO-d_6) spectrum of compound **2h.**

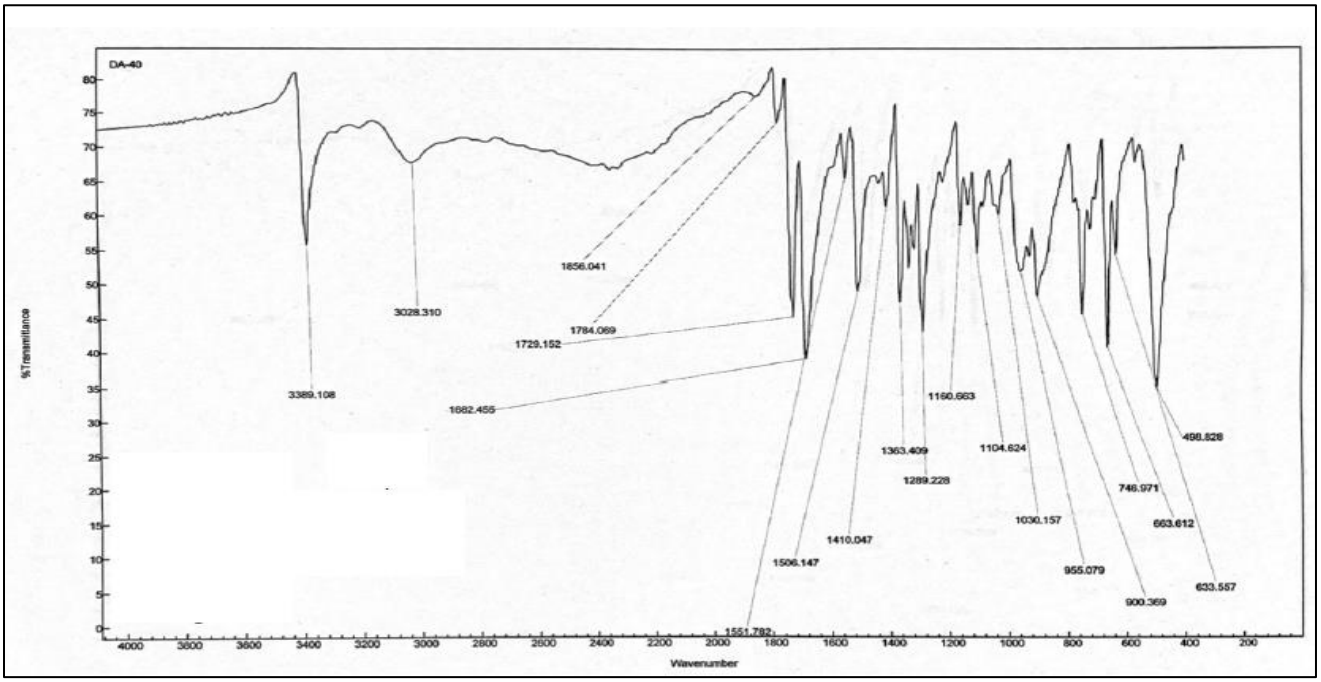


Figure S23. FTIR spectrum of compound **2i.**

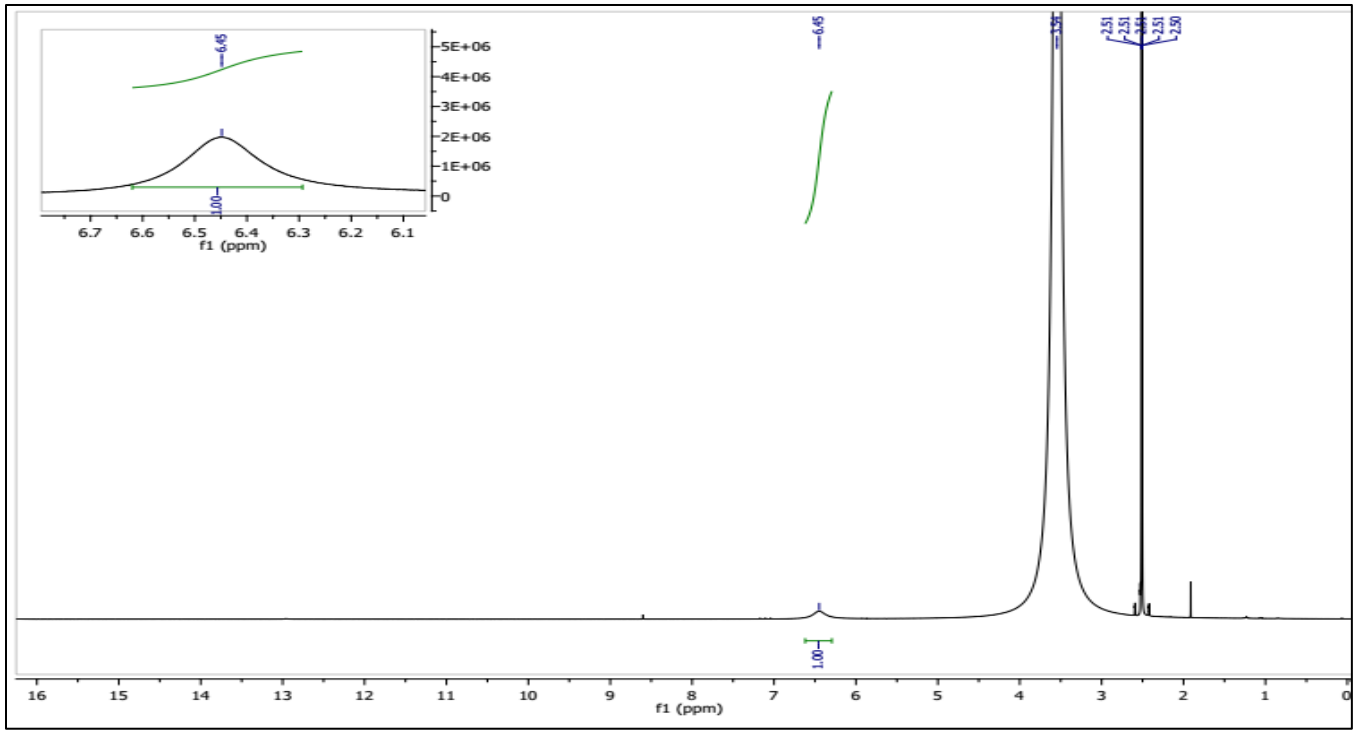


Figure S24. ^{1}H NMR (450 MHz, DMSO-d_6) spectrum of compound **2i.**

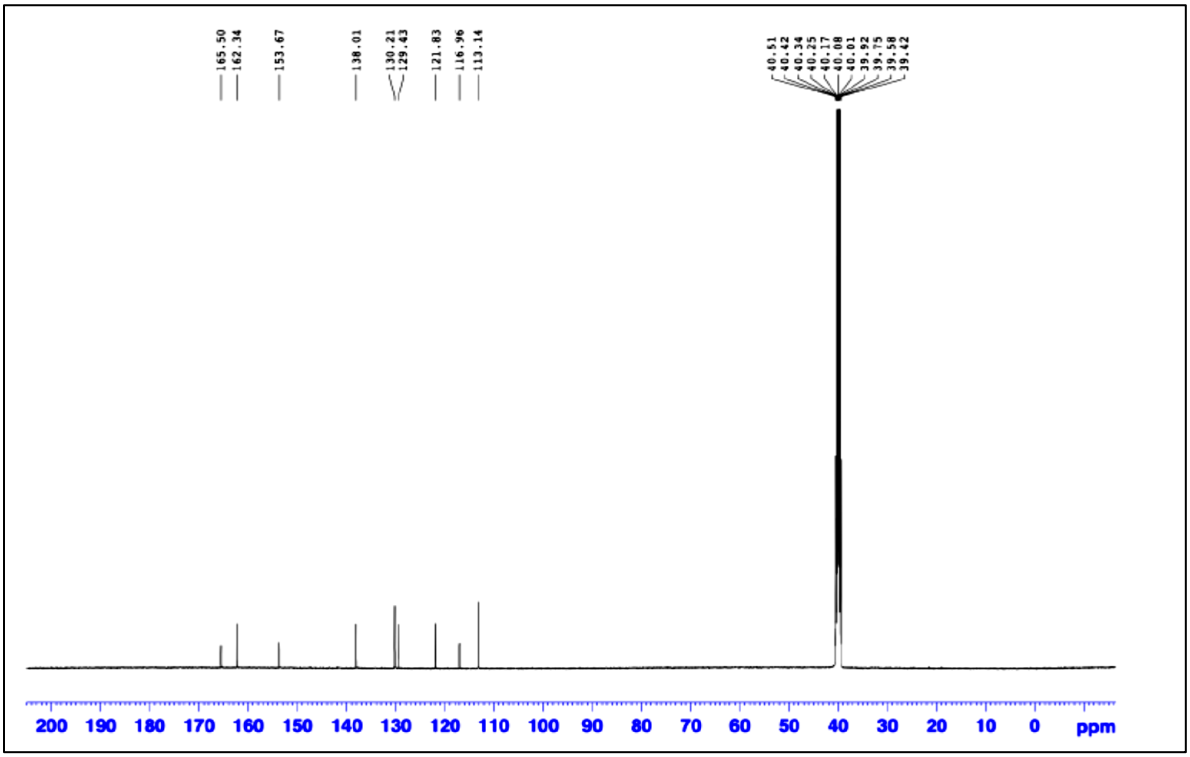


Figure S25. ^{13}C NMR (125 MHz, DMSO-d_6) spectrum of compound **2i.**

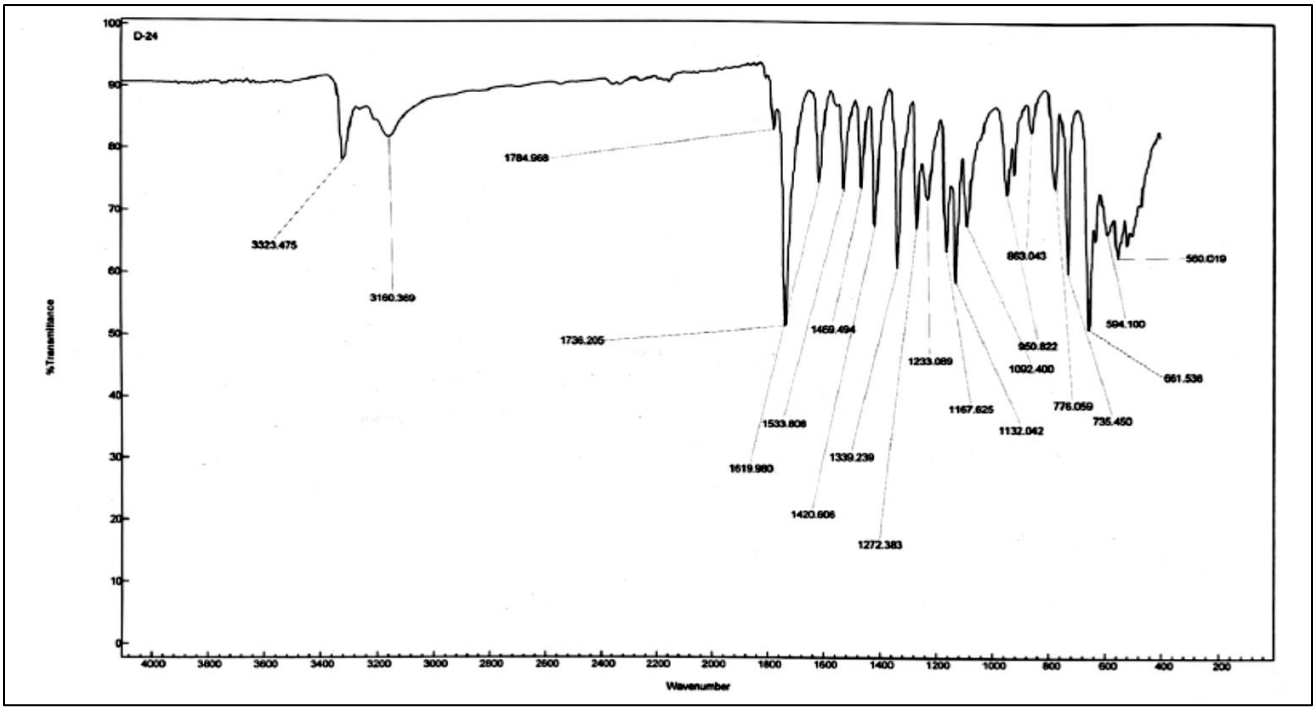


Figure S26. FTIR spectrum of compound **2j.**

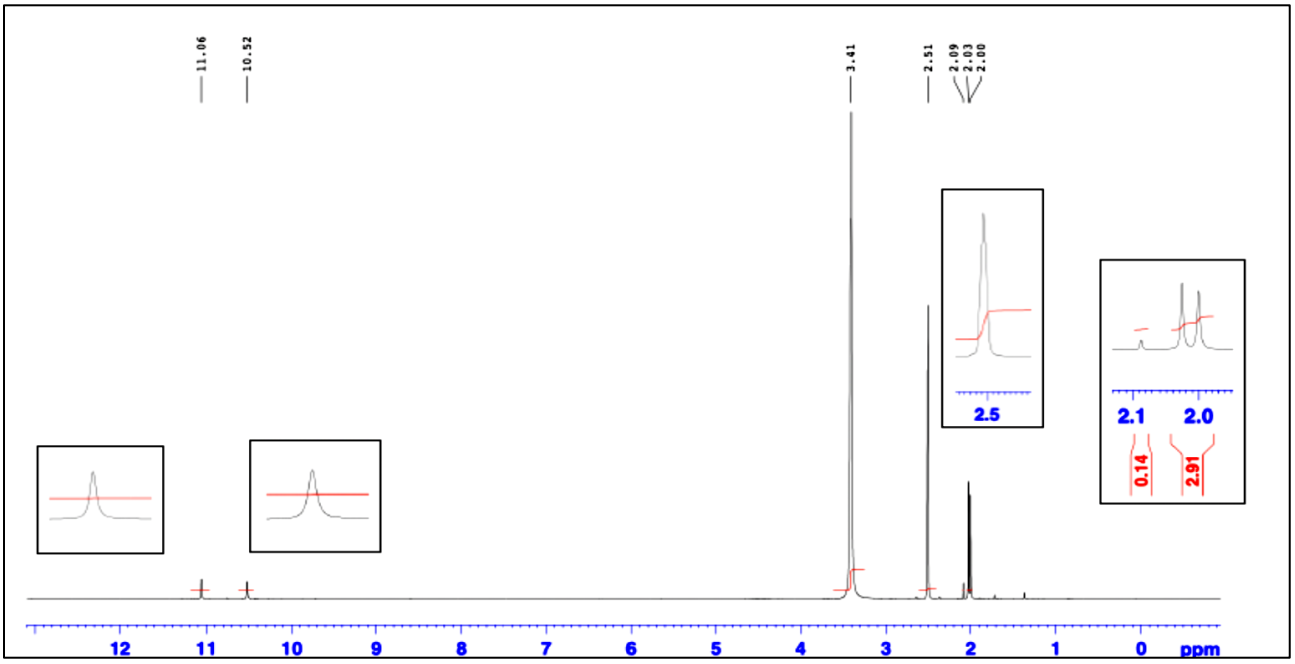


Figure S27. ^{1}H NMR (450 MHz, DMSO-d_6) spectrum of compound **2j.**

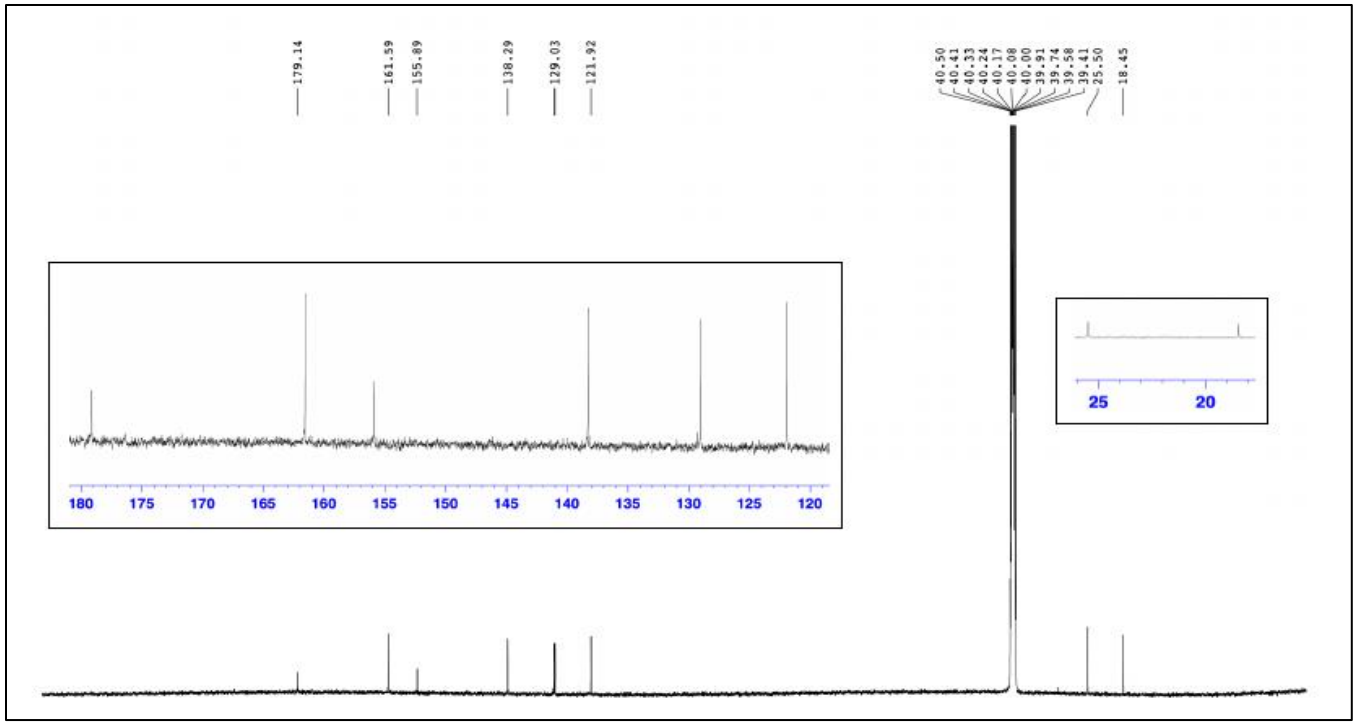


Figure S28. ^{13}C NMR (125 MHz, DMSO-d_6) spectrum of compound **2j.**

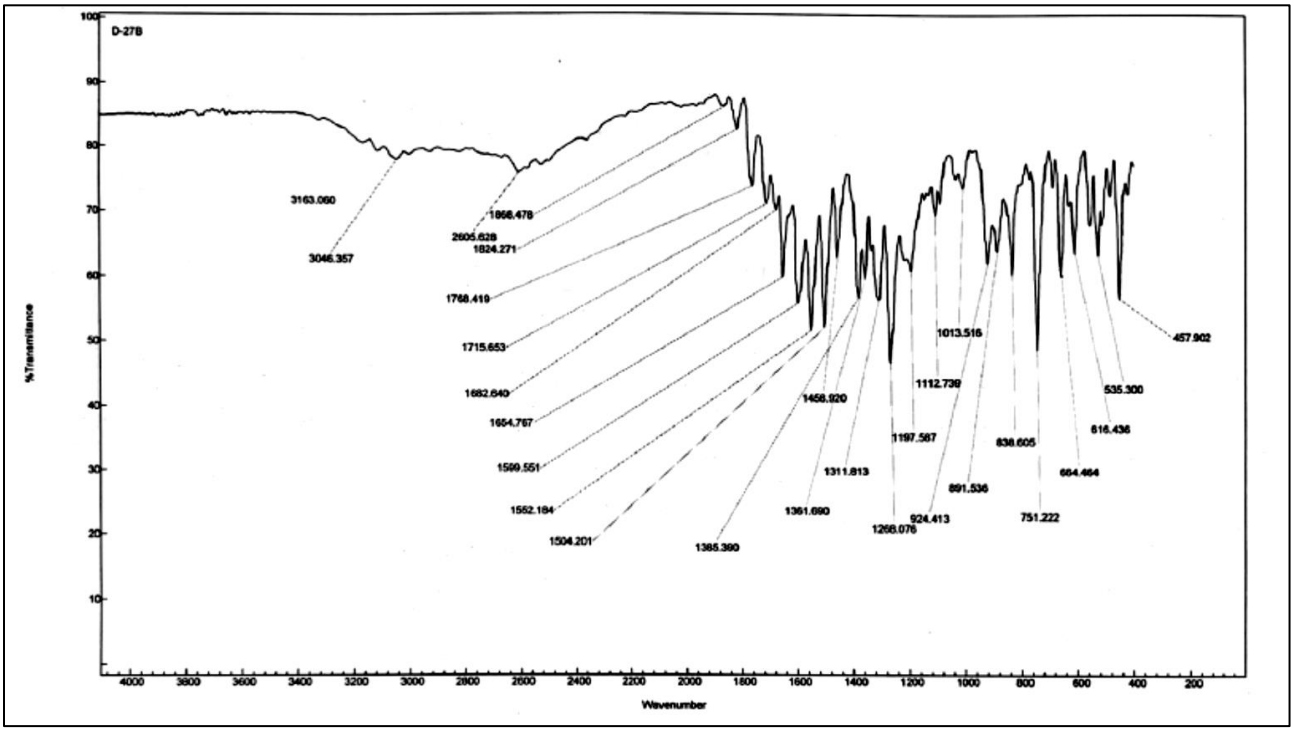


Figure S29. FTIR spectrum of compound **2k.**

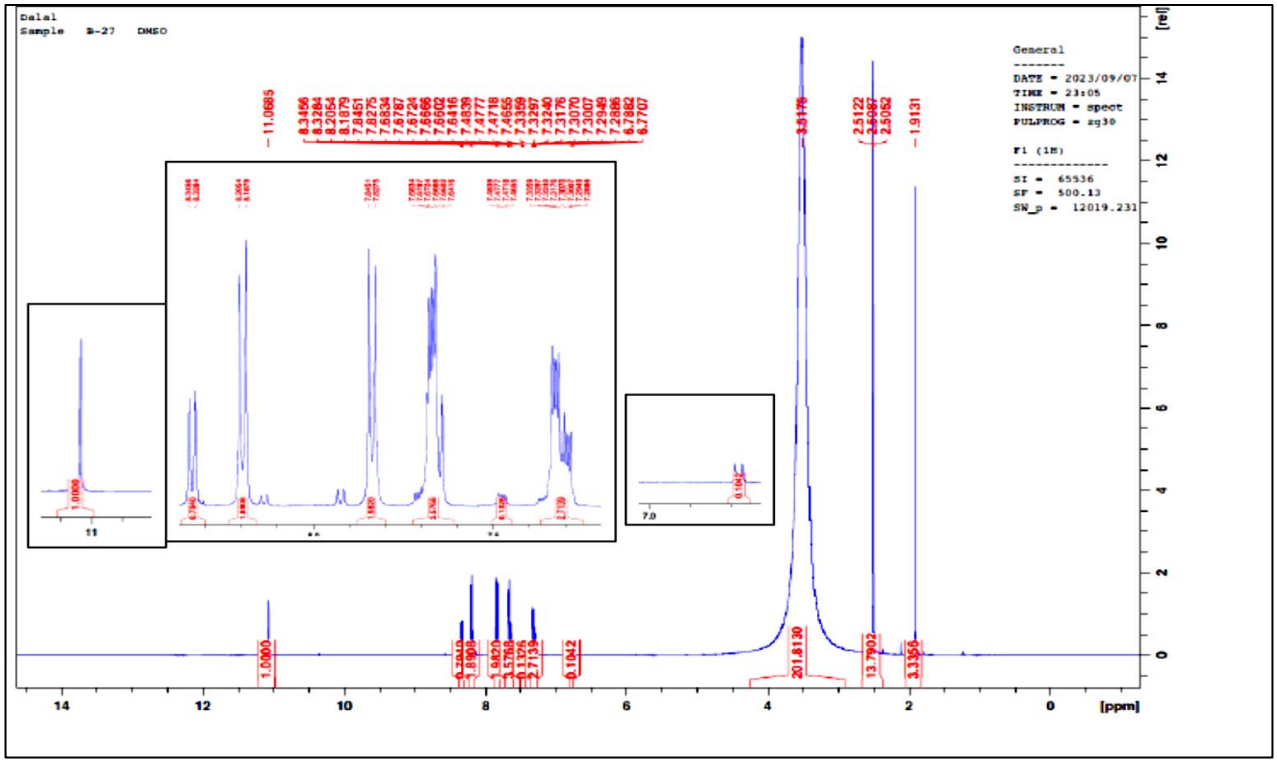


Figure S30. ^{1}H NMR (450 MHz, DMSO-d_6) spectrum of compound **2k.**

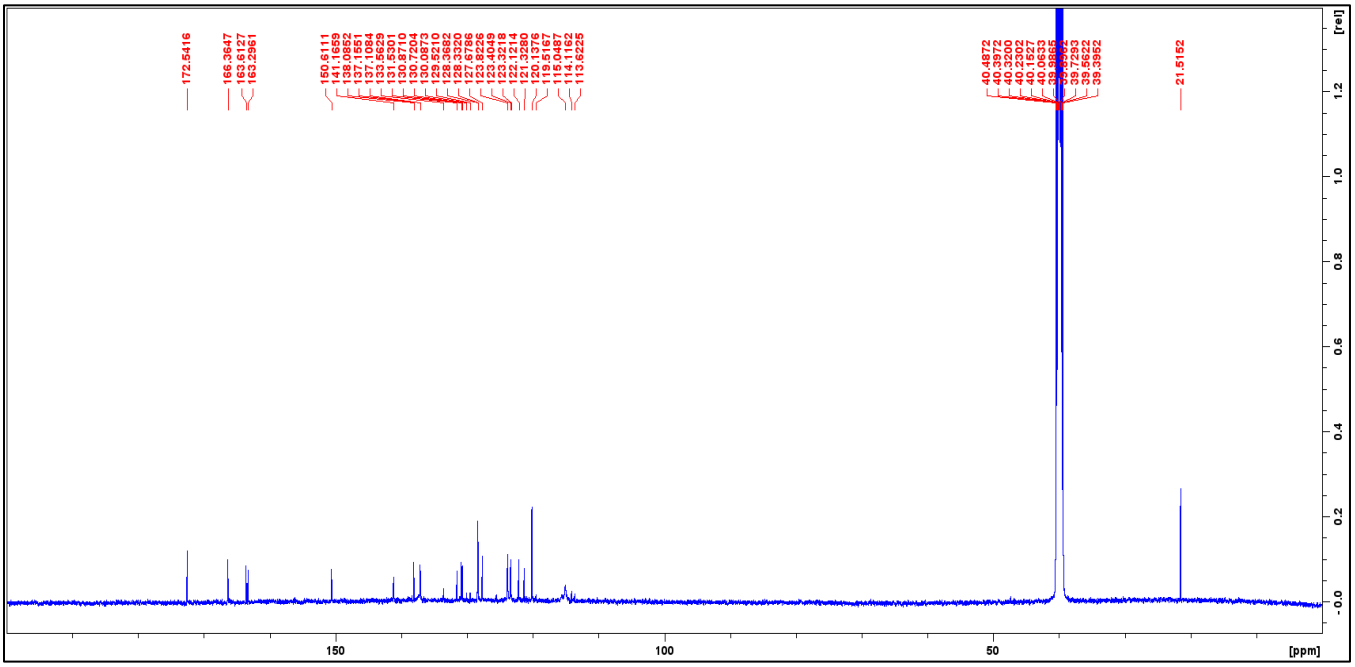


Figure S31. ^{13}C NMR (125 MHz, DMSO-d_6) spectrum of compound **2k.**

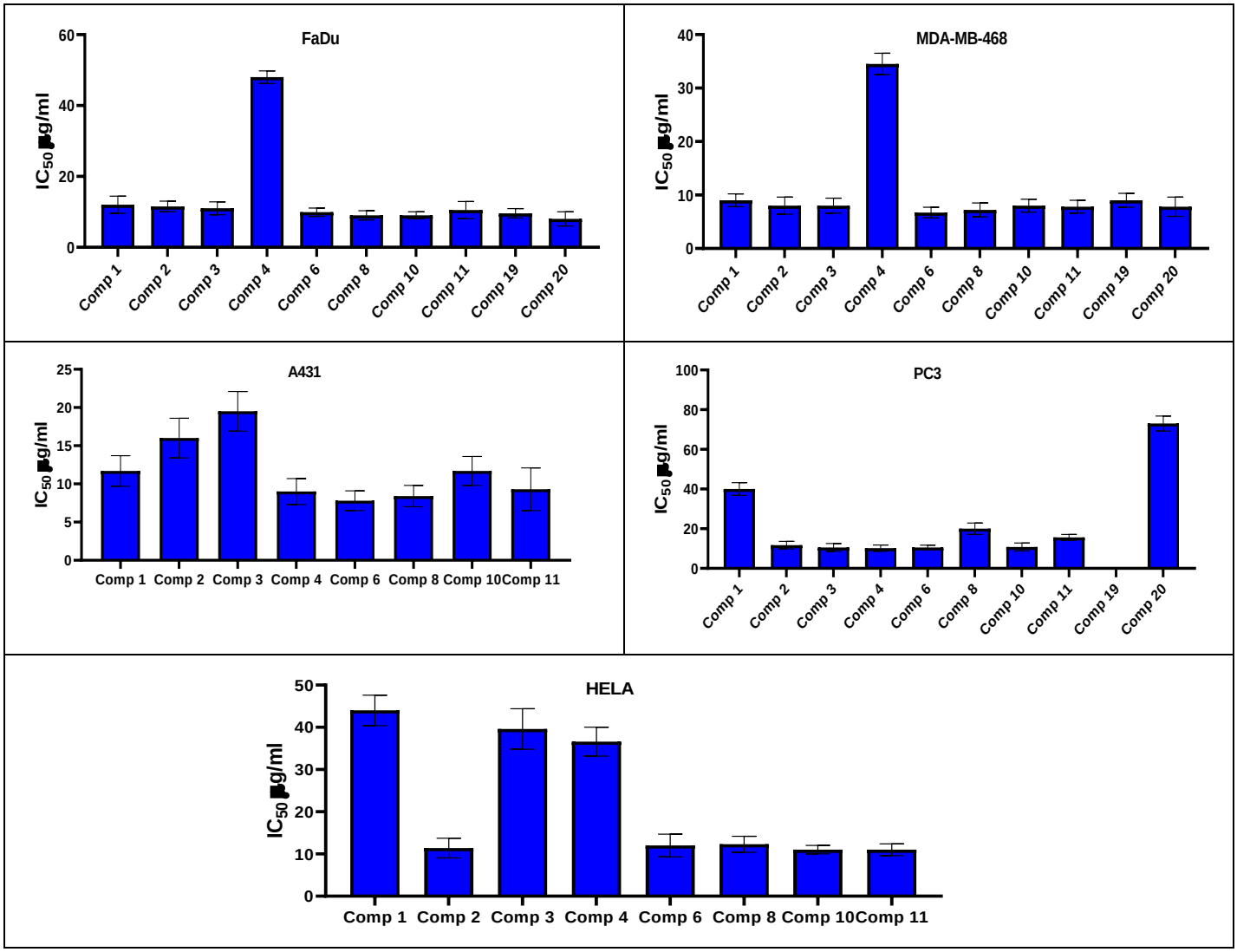
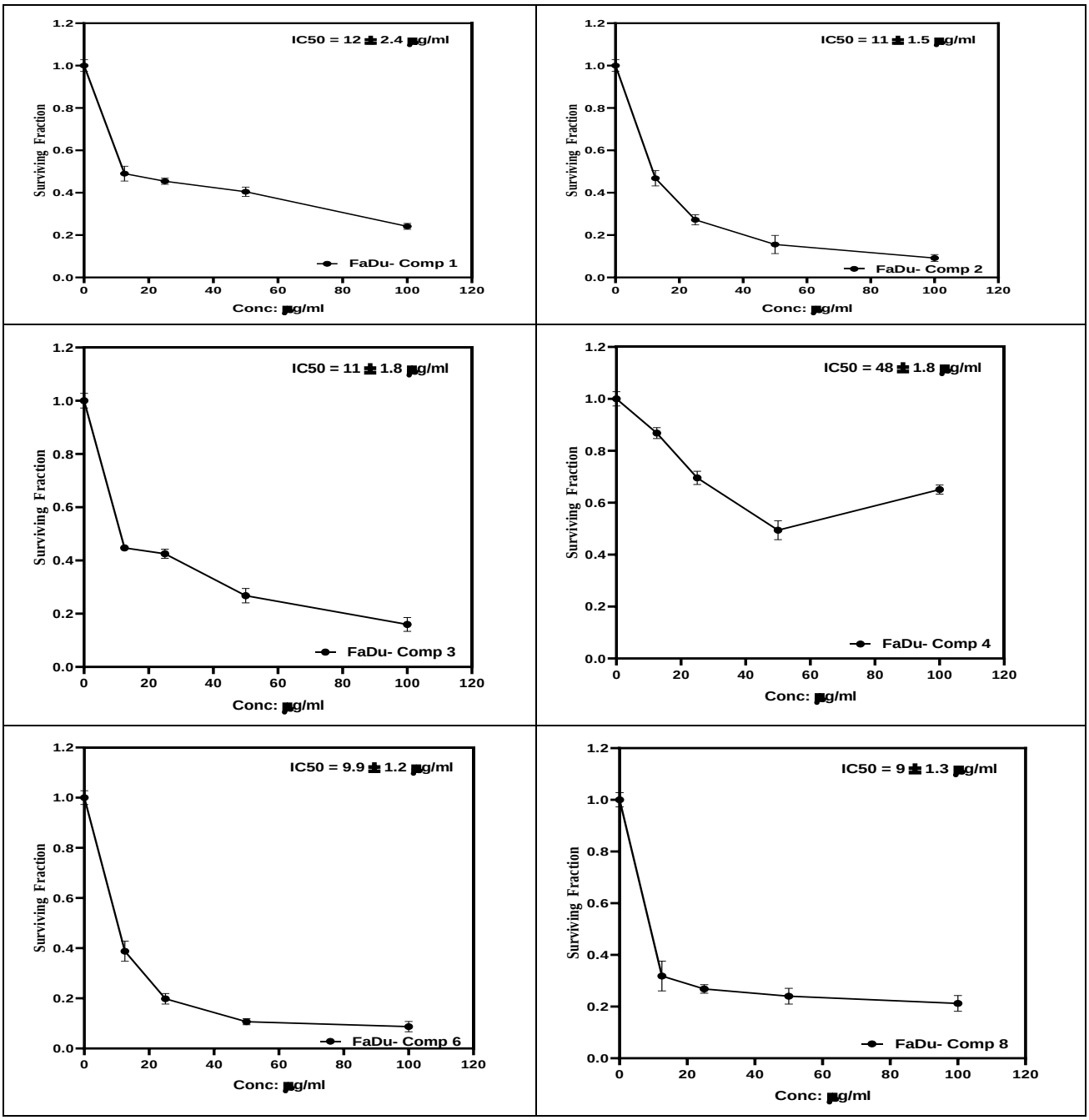


Figure S32. Cytotoxic inhibitory concentration 50 (IC_{50}) evaluation of the investigated compounds against FaDu, MDA-MB-468, A431, PC3, and HeLa cancer cell lines.


IC50 = 12 ± 2.4 µg/ml
Surviving Fraction
Conc: µg/ml
FaDu- Comp 1
IC50 = 11 ± 1.5 µg/ml
FaDu- Comp 2
IC50 = 11 ± 1.8 µg/ml
FaDu- Comp 3
IC50 = 48 ± 1.8 µg/ml
FaDu- Comp 4
IC50 = 9.9 ± 1.2 µg/ml
FaDu- Comp 6
IC50 = 9 ± 1.3 µg/ml
FaDu- Comp 8

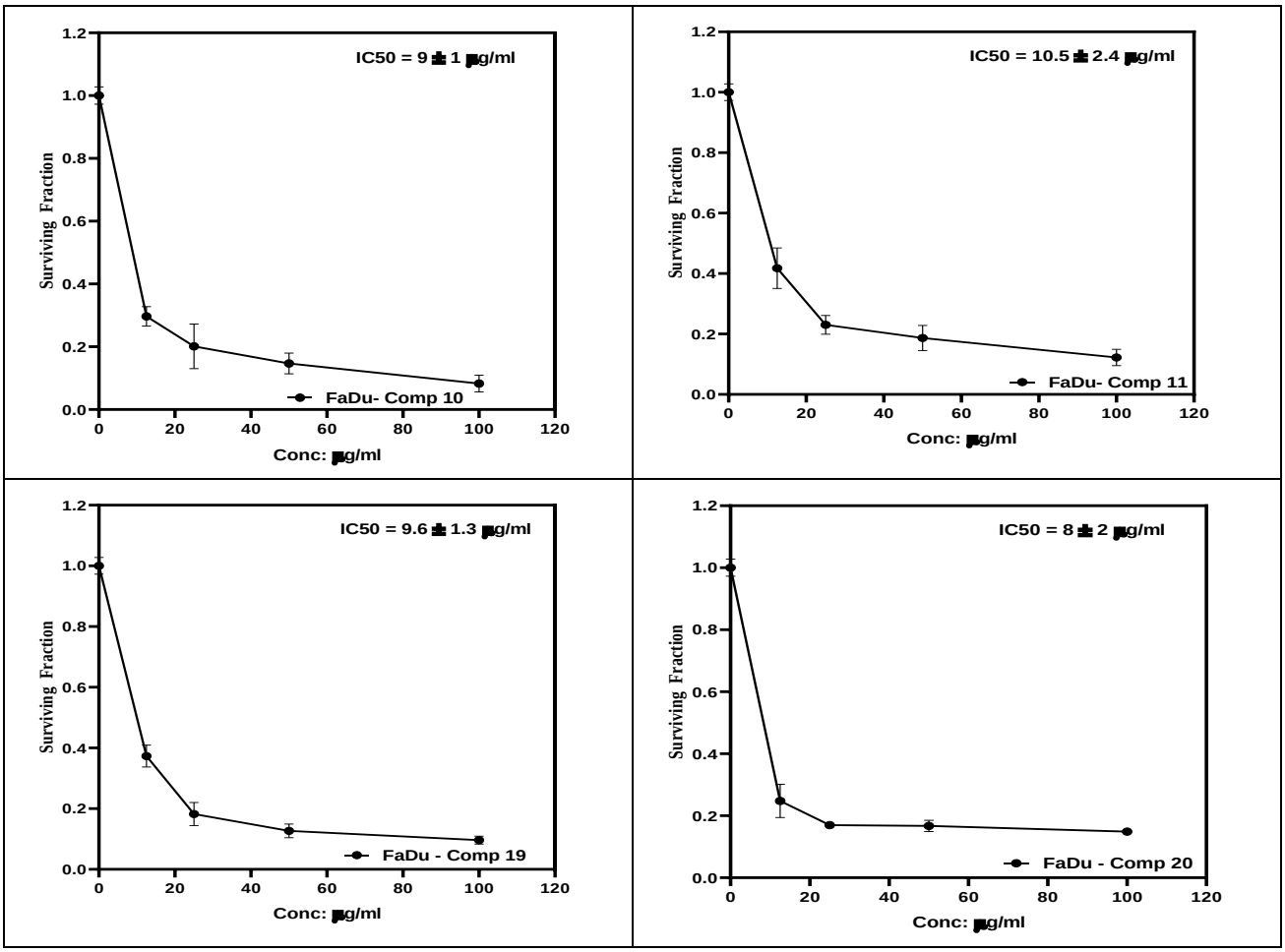
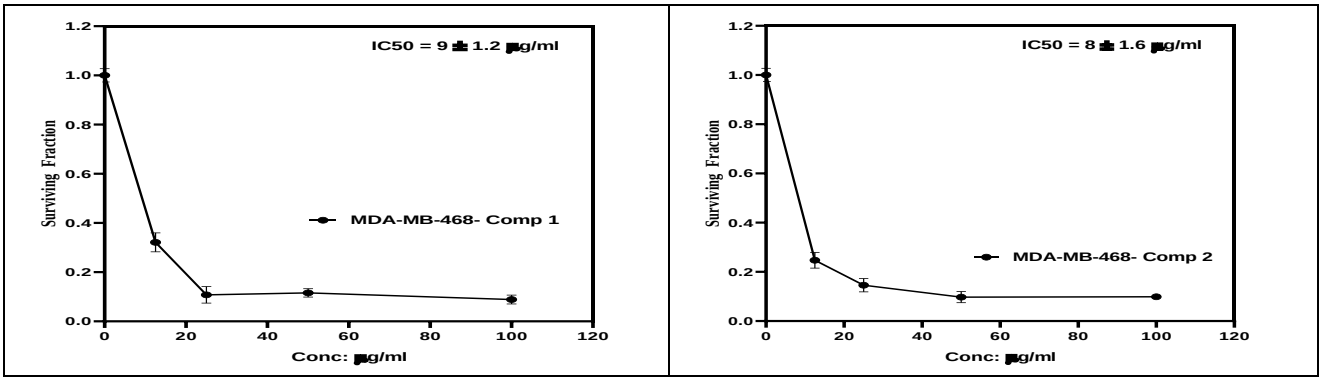


Figure S33. Cytotoxic inhibitory concentration 50 (IC_{50}) evaluation of the investigated compounds against FaDu cancer cell line.



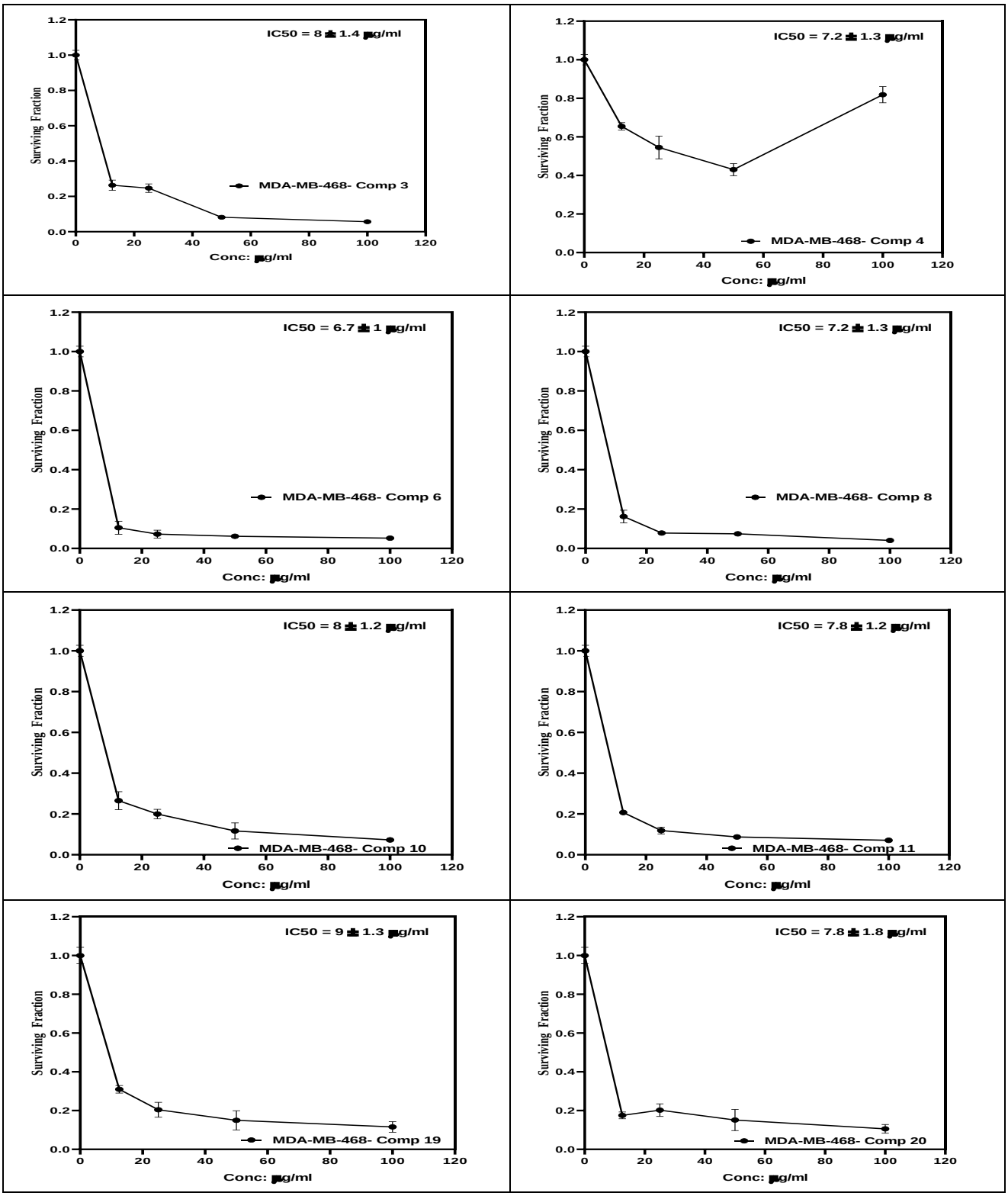


Figure S34. Cytotoxic inhibitory concentration 50 (IC_{50}) evaluation of the investigated compounds against MDA-MB-468 cancer cell line.

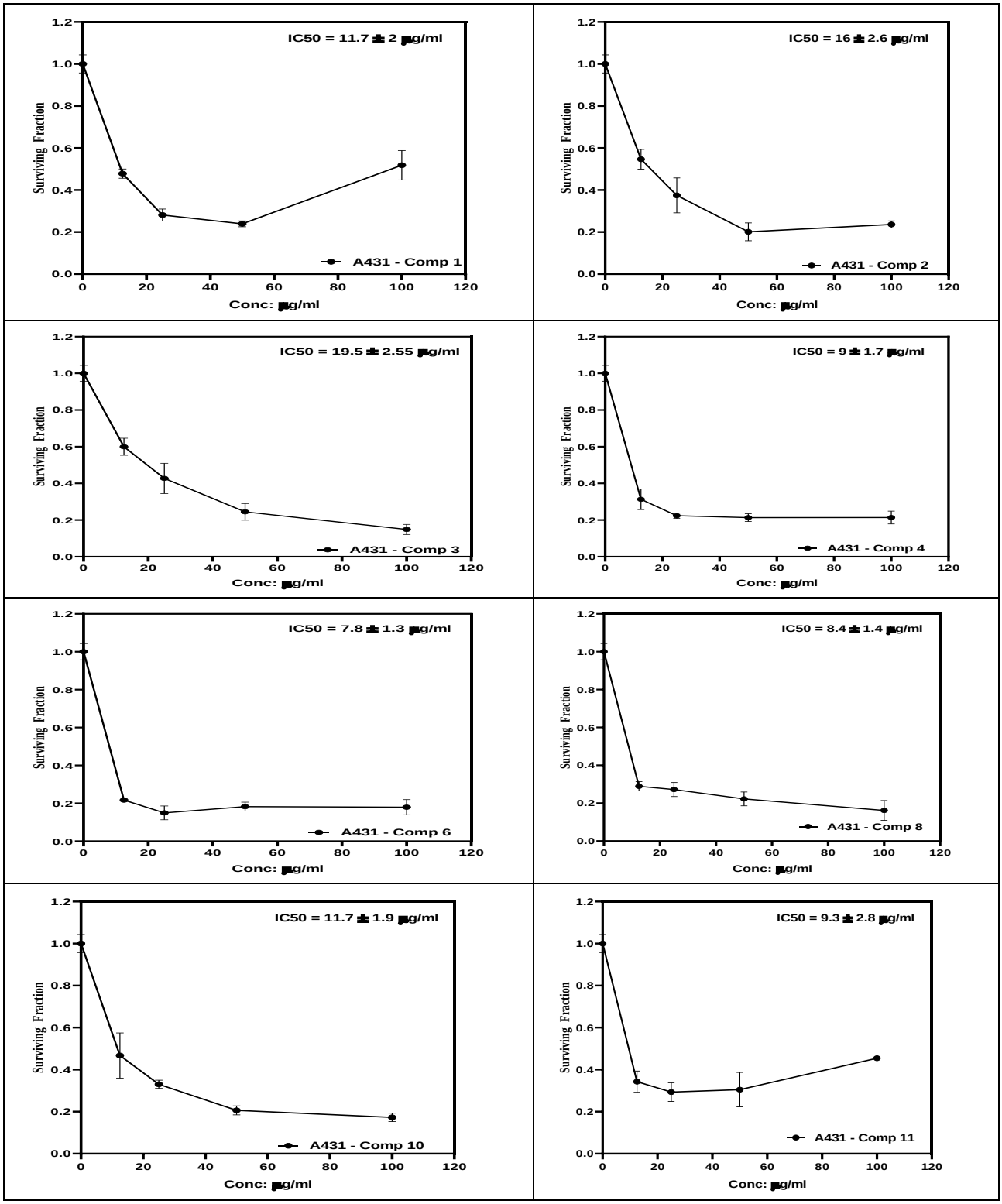
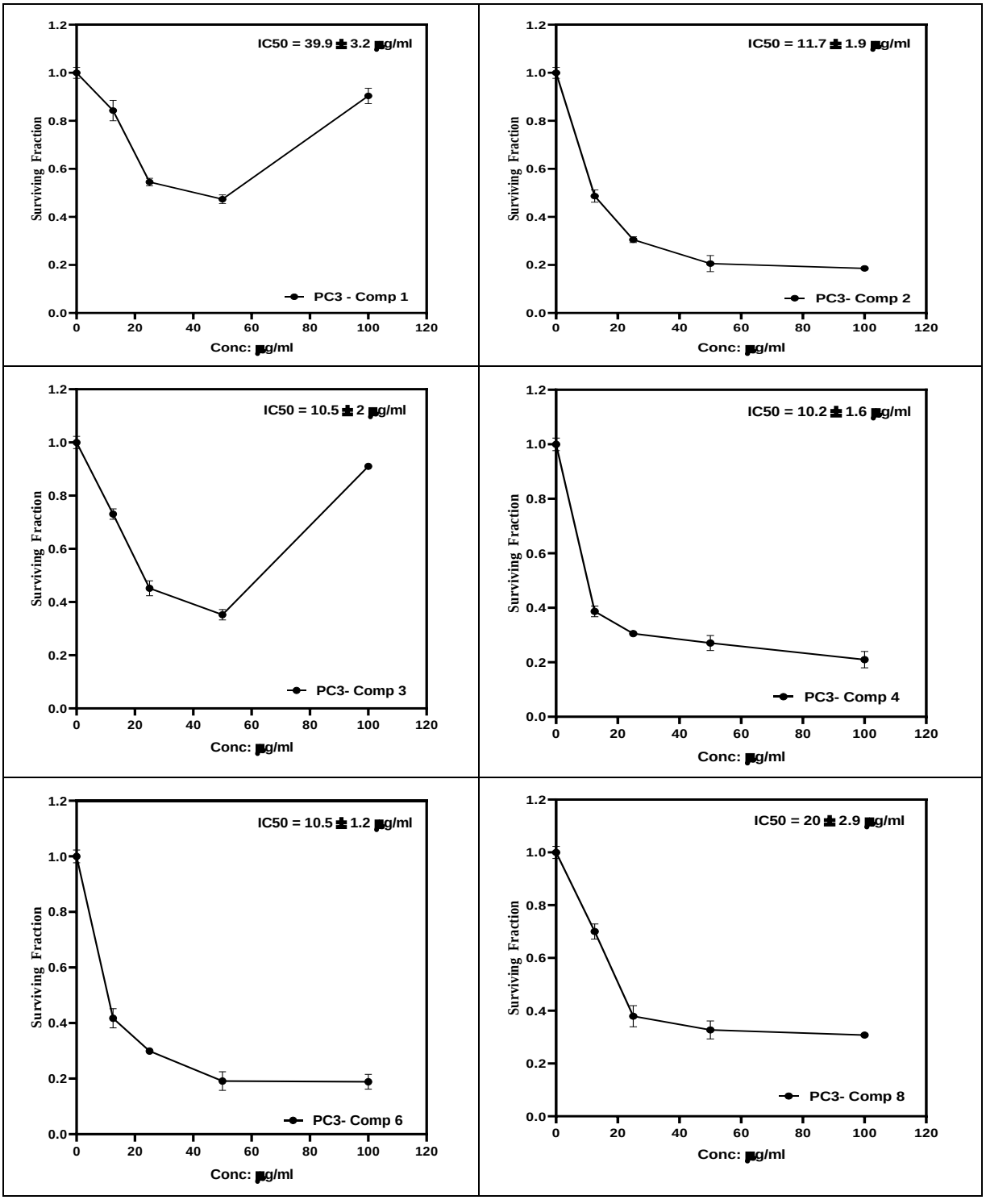


Figure S35. Cytotoxic inhibitory concentration 50 (IC_{50}) evaluation of the investigated compounds against A431 cancer cell line.


IC50 = 39.9 ± 3.2 µg/ml
Surviving Fraction
PC3 - Comp 1
Conc: µg/ml
IC50 = 11.7 ± 1.9 µg/ml
Surviving Fraction
PC3- Comp 2
Conc: µg/ml
IC50 = 10.5 ± 2 µg/ml
Surviving Fraction
PC3- Comp 3
Conc: µg/ml
IC50 = 10.2 ± 1.6 µg/ml
Surviving Fraction
PC3- Comp 4
Conc: µg/ml
IC50 = 10.5 ± 1.2 µg/ml
Surviving Fraction
PC3- Comp 6
Conc: µg/ml
IC50 = 20 ± 2.9 µg/ml
Surviving Fraction
PC3- Comp 8
Conc: µg/ml

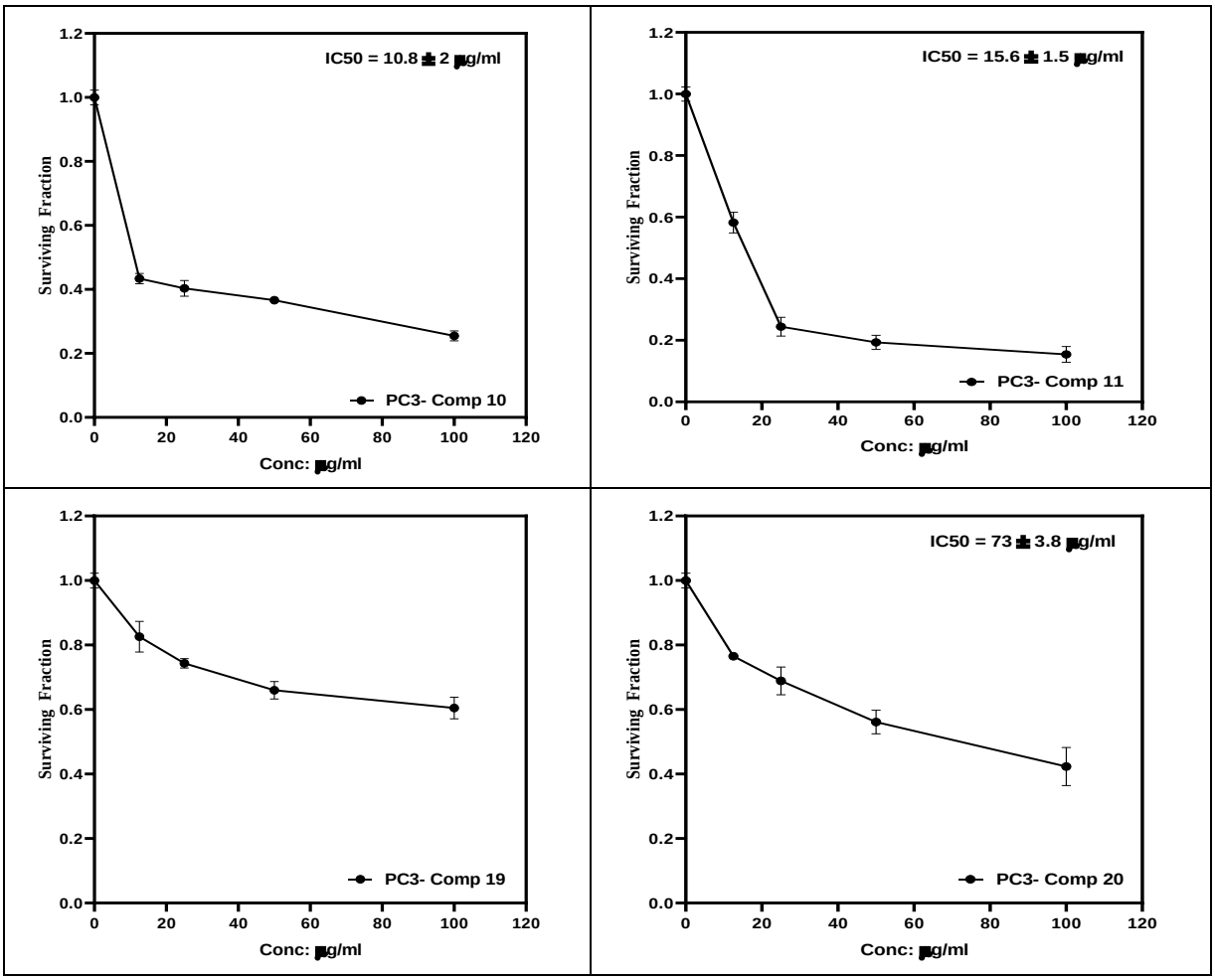
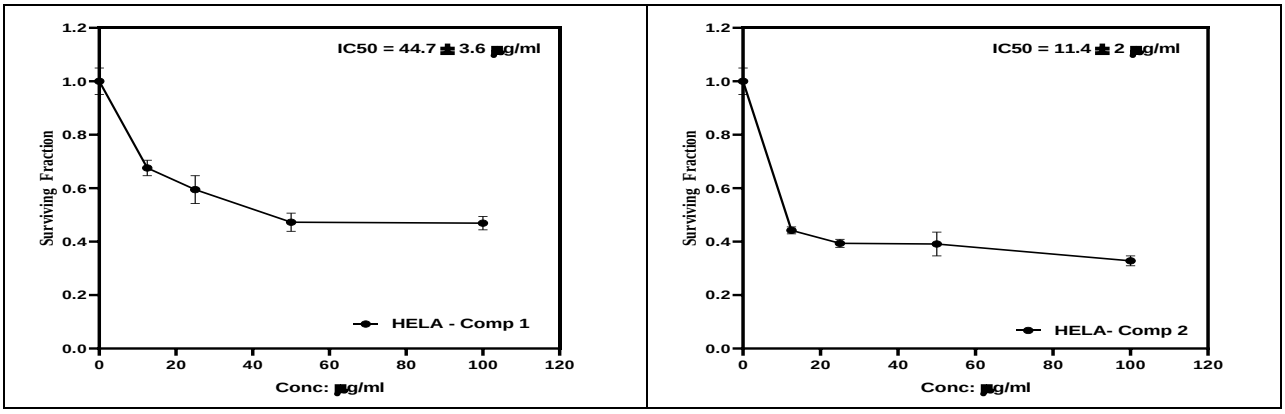


Figure S36. Cytotoxic inhibitory concentration 50 (IC_{50}) evaluation of the investigated compounds against PC3 cancer cell line.



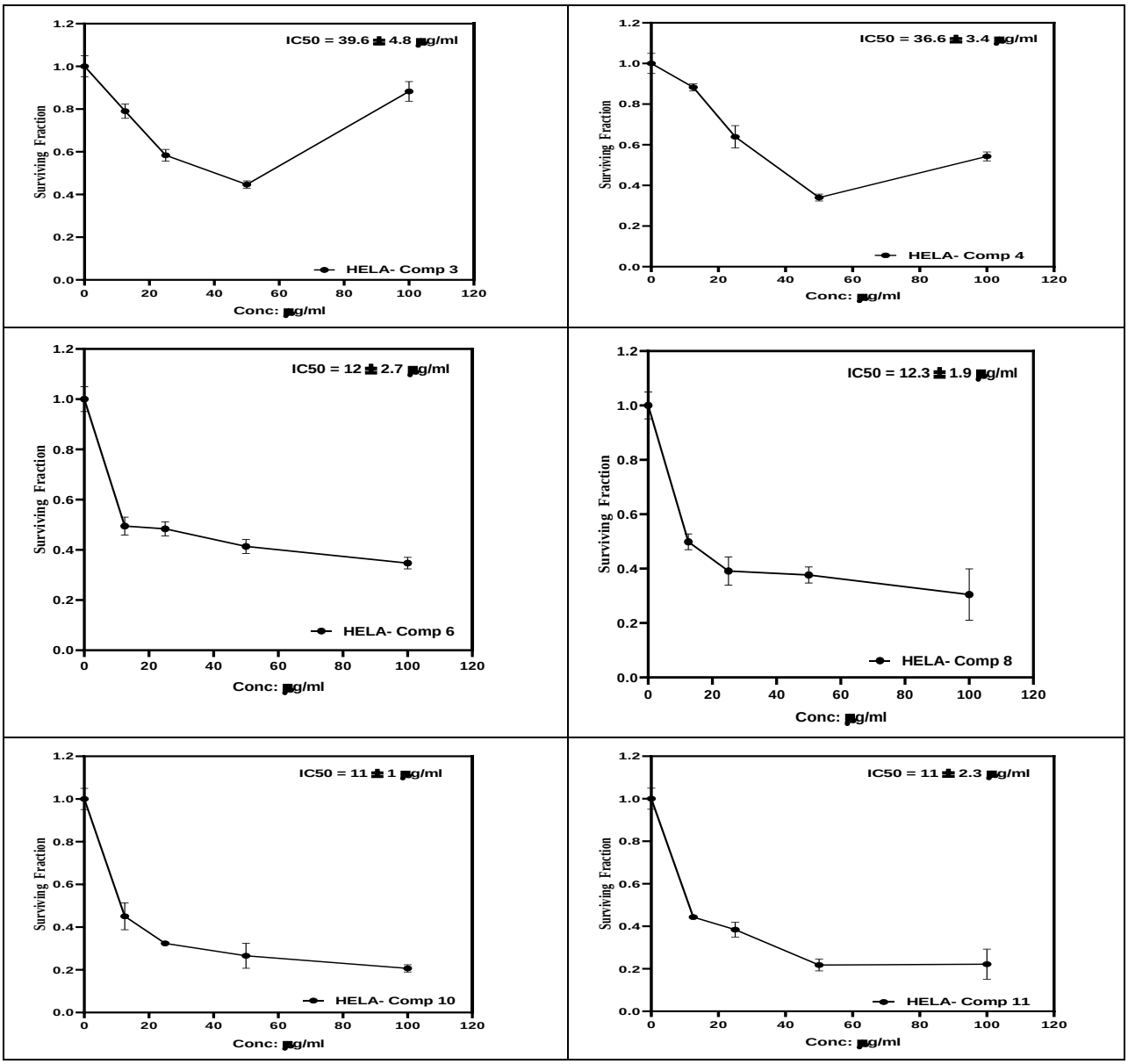


Figure S37. Cytotoxic inhibitory concentration 50 (IC_{50}) evaluation of the investigated compounds against HeLa cancer cell line.

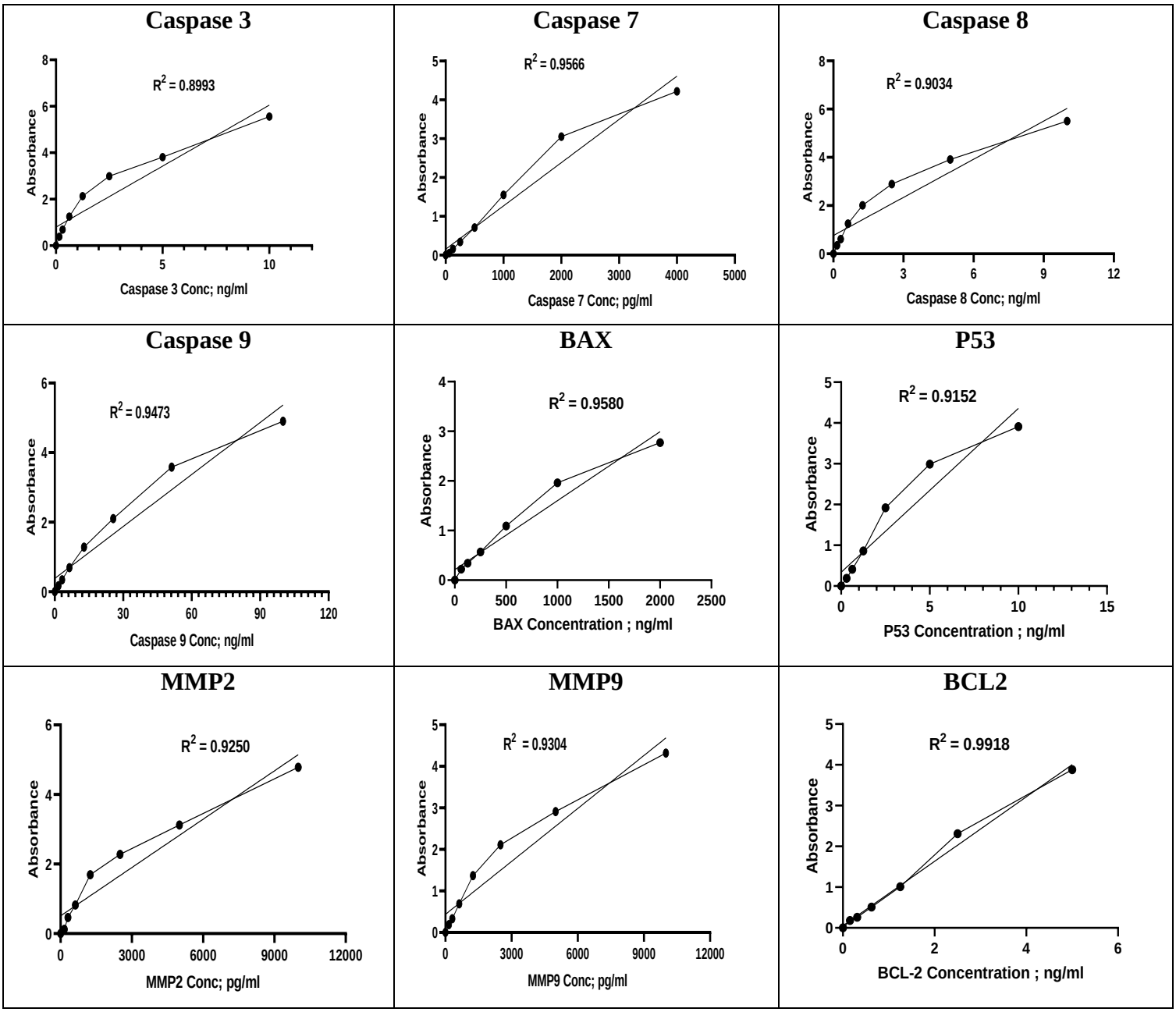


Figure S38. Compound **2f** protein expression levels for caspases 3, 7, 8, and 9, Bax, P53, MMP2, MMP9, and BCL-2 in both the treated and untreated MDA-MB-468 cancer cell line.

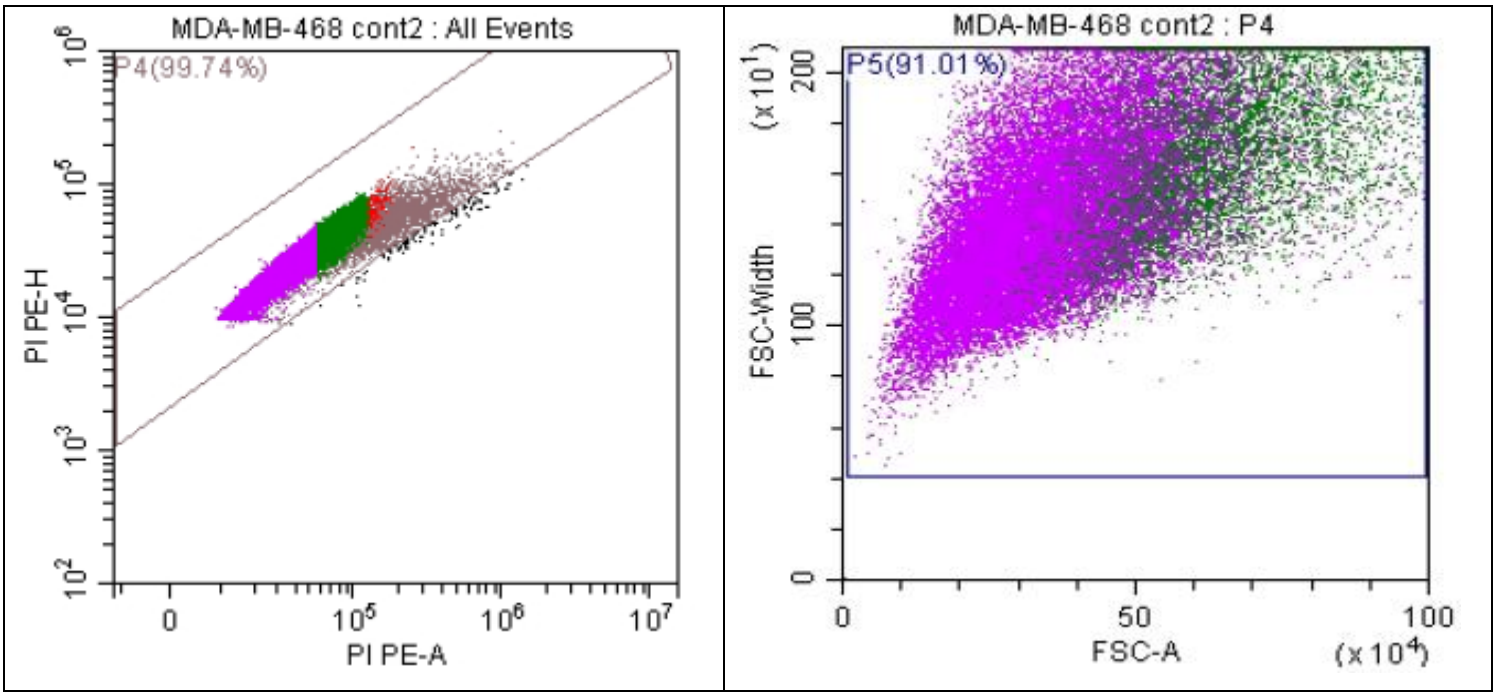


Figure S39. The main experiment of the cell cycle analysis (control) at MDA-MB-468 cancer cell line.

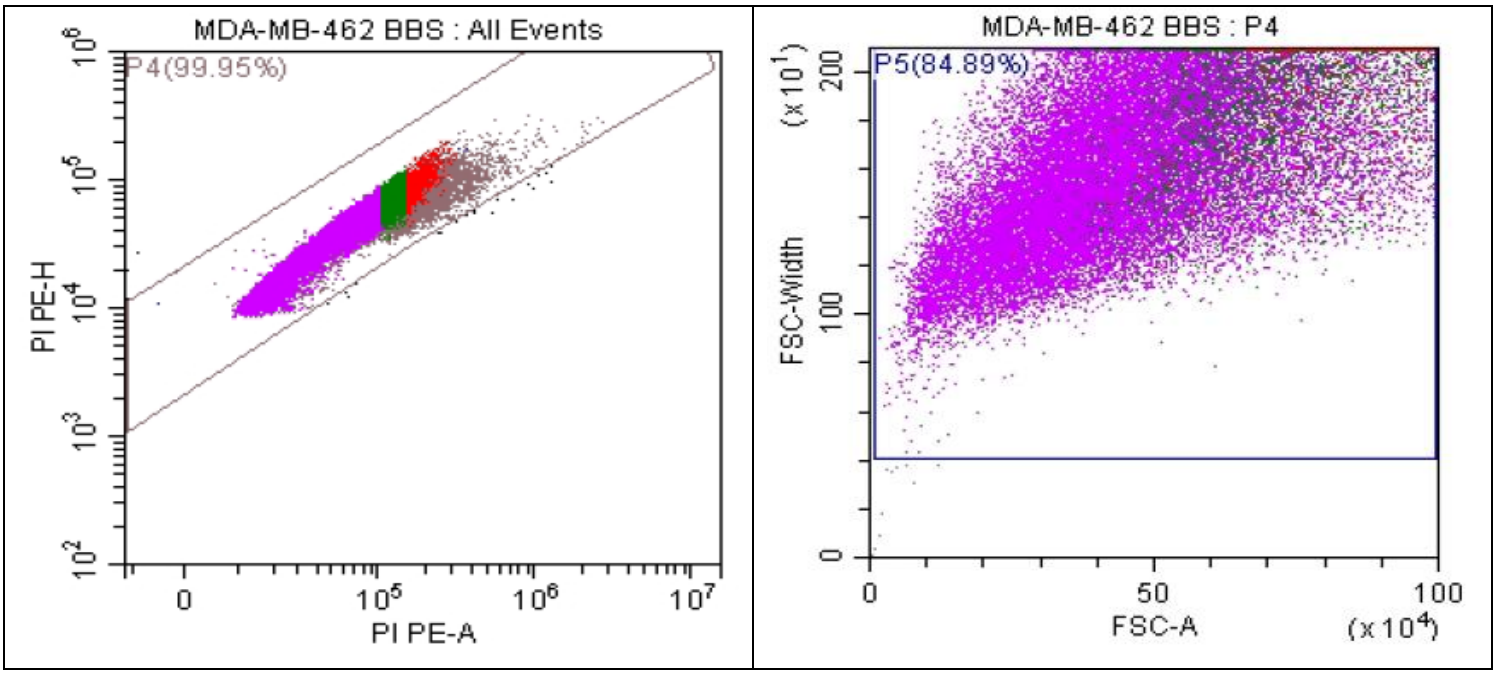


Figure S40. The main experiment of the cell cycle analysis (Compound **2f**) at MDA-MB-468 cancer cell line.

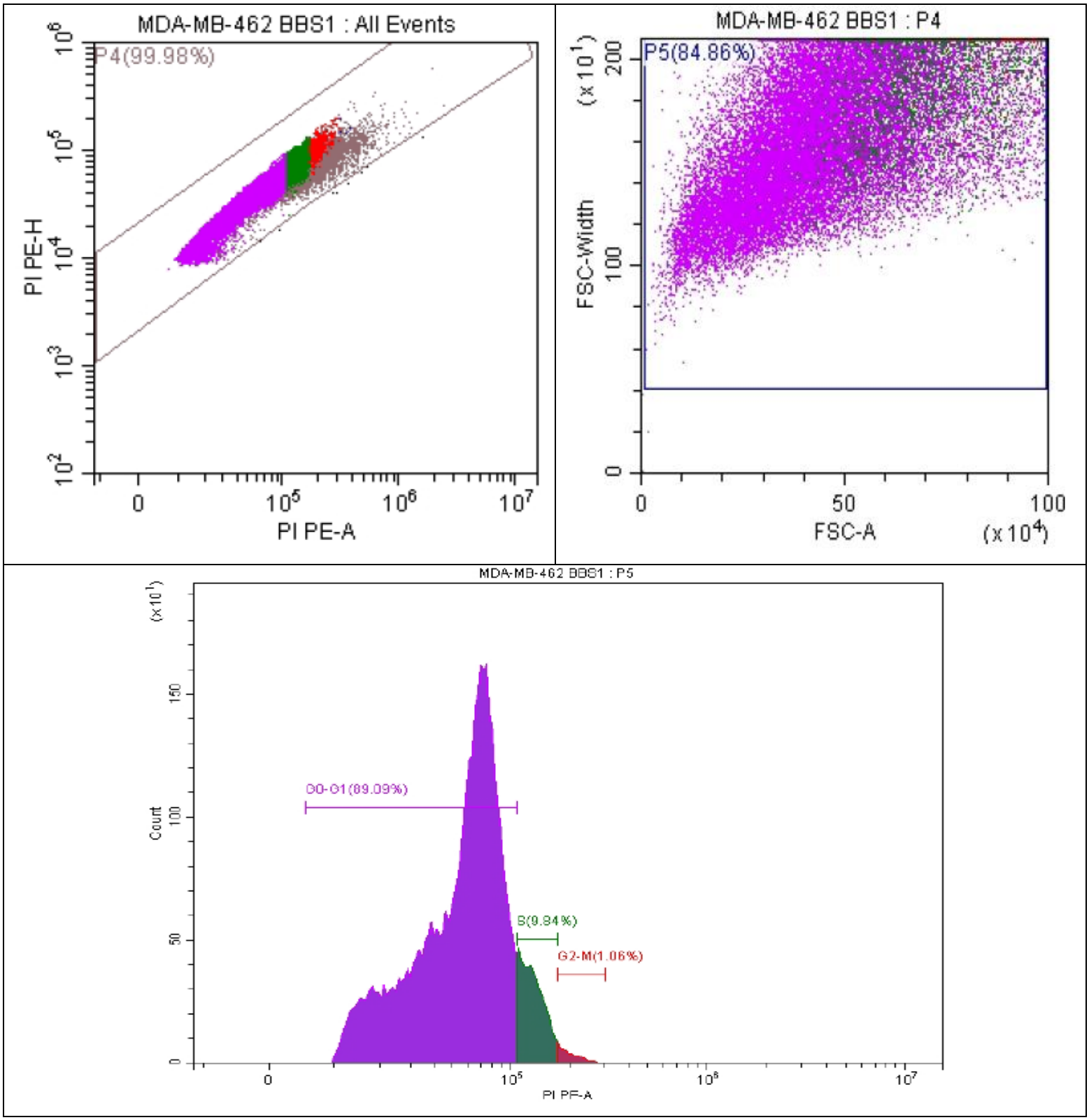


Figure S41. The repeated experiment of the cell cycle analysis (compound **2f**) at MDA-MB-468 cancer cell line.

Materials and Methods

SI1. Growth Inhibition% against human fifteen cancer cell lines at 100 µg/mL

The antitumor activities of compounds (**2a-k**) against all tested cell lines were evaluated by sulphorhodamine-B (SRB) assay[1]. Briefly, cells were seeded at a density of 3×10^3 cells/well in 96-well microtiter plates. They were left to attach for 24 h before incubation with the aforementioned compounds. Next, cells were treated with 100 µg/mL for **2a-k** candidates.

For each concentration, three wells were used and incubation was continued for 48 h. DMSO was used as a control vehicle (1 % v/v). At the end of incubation, cells were fixed with 20% trichloroacetic acid, and stained with 0.4% SRB dye. The optical density (O.D.) of each well was measured spectrophotometrically at 570 nm using an ELISA microplate reader (TECAN sunrise™, Germany). The mean survival fraction at each drug concentration was calculated as follows: O.D. of the treated cells/O.D. of the control cells. The IC_{50} (concentration that produces 50% of cell growth inhibition) value of each drug was calculated using sigmoidal dose-response curve-fitting models (Graph Pad Prizm software, version 8).

SI2. Cytotoxicity evaluation against FaDu, MDA-MB-468, A431, PC3, and HeLa cancer cell lines along with normal cell lines

The antitumor activities of compounds (**2a-k**) against FaDu, MDA-MB-468, A431, PC3, and HeLa cells, besides the normal OEC and HSF cell lines, were evaluated by sulphorhodamine-B (SRB) assay [1]. Briefly, cells were seeded at a density of 3×10^3 cells/well in 96-well microtiter plates. They were left to attach for 24 h before incubation with the aforementioned compounds. Next, cells were treated with different concentrations of 62.5, 12.5, 25, and 50 µg/mL for **2a-k** candidates.

For each concentration, three wells were used and incubation was continued for 48 h. DMSO was used as a control vehicle (1 % *v/v*). At the end of incubation, cells were fixed with 20% trichloroacetic acid, and stained with 0.4% SRB dye. The optical density (O.D.) of each well was measured spectrophotometrically at 570 nm using an ELISA microplate reader (TECAN sunrise™, Germany). The mean survival fraction at each drug concentration was calculated as follows: O.D. of the treated cells/O.D. of the control cells. The IC_{50} (concentration that produces 50% of cell growth inhibition) value of each drug was calculated using sigmoidal dose-response curve-fitting models (Graph Pad Prizm software, version 8).

SI3. Apoptotic markers assay (Enzyme-linked Immunosorbent assay)

The microplate provided in this kit has been pre-coated with an antibody specific to caspases 3, 7, 8, and 9, Bax, p53, MMP2, MMP9, and BCL-2. Standards or samples are then added to the appropriate microplate wells with a biotin-conjugated antibody specific to caspases 3, 7, 8, and 9, Bax, p53, MMP2, MMP9, and BCL-2. Next, Avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. After the TMB substrate solution is added, only those wells that contain caspases 3, 7, 8, and 9, Bax, p53, MMP2, MMP9, and BCL-2, biotin-conjugated antibody, and enzyme-conjugated Avidin will exhibit a color change. The enzyme-substrate reaction is terminated by the addition of sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450 nm ± 10 nm. The concentration of caspases 3, 7, 8, and 9, Bax, p53, MMP2, MMP9, and BCL-2 in the samples is then determined by comparing the O.D. of the samples to the standard curve. Average the duplicate readings for each standard, control, and sample, and subtract the average zero standard optical density. Construct a standard curve by plotting the mean O.D. and concentration for each standard and draw a best-fit curve through the points on the graph or create a standard curve on log-log graph paper with caspases 3, 7, 8, and 9, Bax, p53, MMP2, MMP9, and BCL-2 concentration on the y-axis and absorbance on the x-axis. Using some plot software, for instance, Curve Expert 1.30, is also recommended. If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

Procedure

1. Determine wells for diluted standard, blank, and sample (**2f**). Prepare 7 wells for standard, 1 well for blank. Add 100 μL each of dilutions of standard, blank, and samples into the appropriate wells. Cover with the Plate sealer. Incubate for 1 h at 37 ^{o}C.

2. Remove the liquid from each well, don't wash.

3. Add 100 μL of Detection Reagent A working solution to each well, cover the wells with the plate sealer, and incubate for 1 h at 37 ^{o}C.

4. Aspirate the solution and wash with 350 μL of 1× Wash Solution to each well using a squirt bottle, multi-channel pipette, manifold dispenser, or autowasher, and let it sit for 1~2 min. Remove the remaining liquid from all wells completely by snapping the plate onto absorbent paper. Totally wash 3 times. After

the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against absorbent paper.

5. Add 100 μL of Detection Reagent B working solution to each well, cover the wells with the plate sealer, and incubate for 30 min at 37 ºC.

6. Repeat the aspiration/wash process for a total of 5 times as conducted in step 4.

7. Add 90 μL of Substrate Solution to each well. Cover with a new Plate sealer. Incubate for 10-20 min at 37 ºC (Don't exceed 30 min). Protect from light. The liquid will turn blue with the addition of a Substrate Solution.

8. Add 50 μL of Stop Solution to each well. The liquid will turn yellow with the addition of the stop solution. Mix the liquid by tapping the side of the plate. If the color change does not appear uniform, gently tap the plate to ensure thorough mixing.

9. Remove any drop of water and fingerprint on the bottom of the plate and confirm there is no bubble on the surface of the liquid. Then, run the microplate reader and conduct measurement at 450 nm immediately.

SI4. Colchicine-binding site assay

The assay procedure is straightforward if the set-up protocols are performed exactly as described. There are aliquots of biotin tubulin and GTP to be made as these are labile components that require -70 °C storage for increased stability and shelf life, which leads to improved reproducibility. As an alternative biotin tubulin is available as 20ug aliquots (T333-A or T333-B) which can be used one vial at a time for small numbers of assays. Colchicine binding is sensitive to temperature so it is important to perform the assay at 37 °C in a reproducible manner. The basic approach to the assay is as follows:

1. Set up a scintillation plate reader and warm the plate in the reader to 37 °C (CDS15 only).
2. Prepare compounds (**2b**, **2f**, **2h**, **2i**, and **2k**) to 20x strength (1mM) in G-PEM buffer plus 10% DMSO.
3. Prepare tritiated colchicine. Assay life 24 h.
4. Prepare biotin tubulin on ice. Assay life 4 h (four h).
5. Prepare streptavidin beads. Assay life 24 h.
6. Prepare bead/tubulin mixture. Assay life 24 h.
7. Pipette 10ul of the compound into the wells.
8. Pipette 10ul of tritiated colchicine into the wells.

9. Pipette 180ul of bead/tubulin into the wells.

10. Incubate for 45 min at 37 °C. 11. Read plate.

12. Extract the raw data and calculate percent inhibition.

SI5. Human Topoisomerase II ELISA Kit assay

This assay employs the quantitative sandwich enzyme immunoassay technique. Antibody specific for Top. IIB has been pre-coated onto a microplate. Standards and samples (**2b**, **2f**, **2h**, **2i**, and **2k**) are pipetted into the wells and any Top. IIB present is bound by the immobilized antibody. After removing any unbound substances, a biotin-conjugated antibody specific for Top. IIB is added to the wells. After washing, avidin-conjugated Horseradish Peroxidase (HRP) is added to the wells. Following a wash to remove any unbound avidin-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of Top. IIB bound in the initial step. The color development is stopped and the intensity of the color is measured. The minimum detectable dose of human Top. IIB is typically less than 4.69 pg/mL. The sensitivity of this assay, or Lower Limit of Detection (LLD) was defined as the lowest protein concentration that could be differentiated from zero. It was determined the mean O.D value of 20 replicates of the zero-standard added by their three standard deviations.

This assay has high sensitivity and excellent specificity for the detection of human Top. IIB. No significant cross-reactivity or interference between human Top. IIB and analogues were observed.

SI6. Cell cycle analysis

Triple-negative breast cancer cells (MDA-MB-468) were grown in six-well plates (each one contains 2 x 105 cells per well) containing 10% fetal bovine serum and incubated for 24 h at 37 ºC and 5% CO_2. The medium was replaced with (DMSO 1% *v/v*) containing the 3.1 µM of compound (**2f**), then incubated for 48 h, collected, and washed with cold phosphate-buffered saline (PBS). After fixation of the collected cells with ice-cold absolute ethanol (70%), the cells were rinsed with PBS then stained with the DNA fluorochrome PI and kept for 15 min at 37 ºC. Then samples were analyzed with a FACS Caliber flow cytometer.

References

1. Skehan P, Storeng R, Scudiero D *et al.* New colorimetric cytotoxicity assay for anticancer-drug screening. *JNCI: Journal of the National Cancer Institute*, 82(13), 1107-1112 (1990).