Supporting Information (SI)

Dual EGFR and Telomerase Inhibitory Potential of New Triazole Tethered Schiff Bases Endowed with Apoptosis: Design, Synthesis, and Biological Assessments

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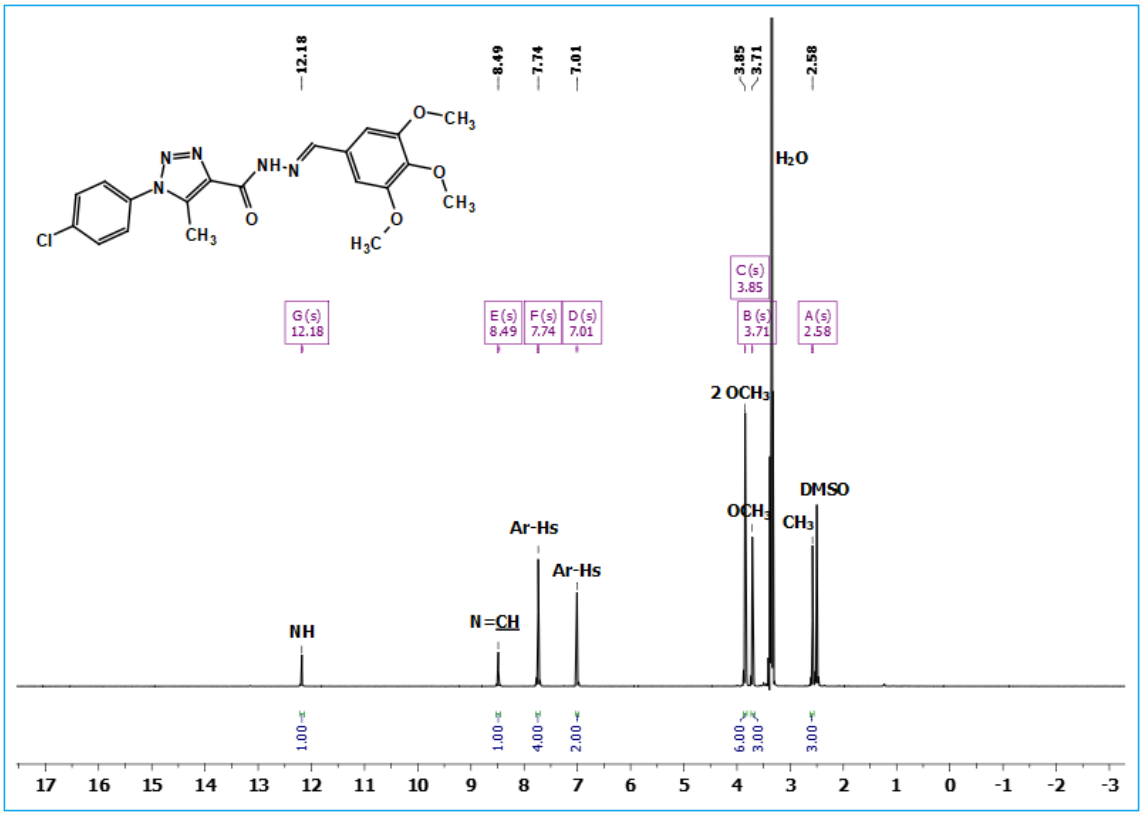


Figure S1. ^{1}H NMR spectrum (500 MHz, DMSO-d_6) for compound **5a**.

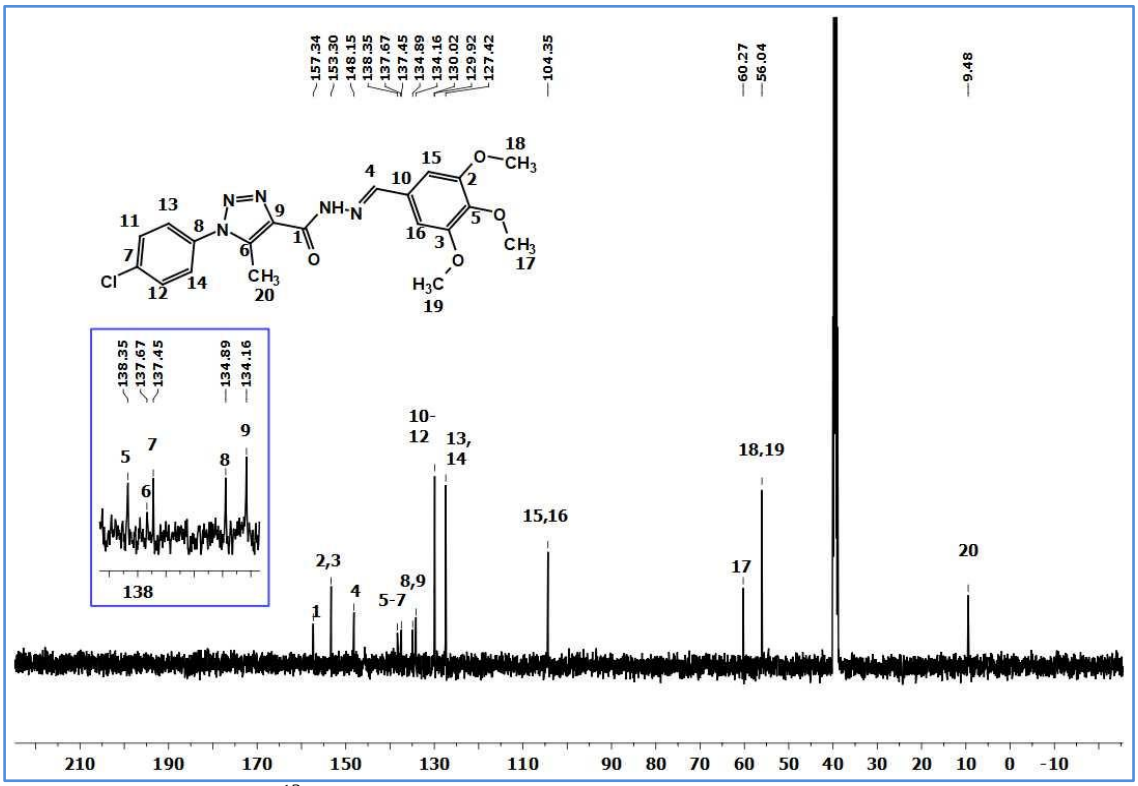


Figure S2. ^{13}C NMR spectrum (125 MHz, DMSO-d_6) for compound **5a**.

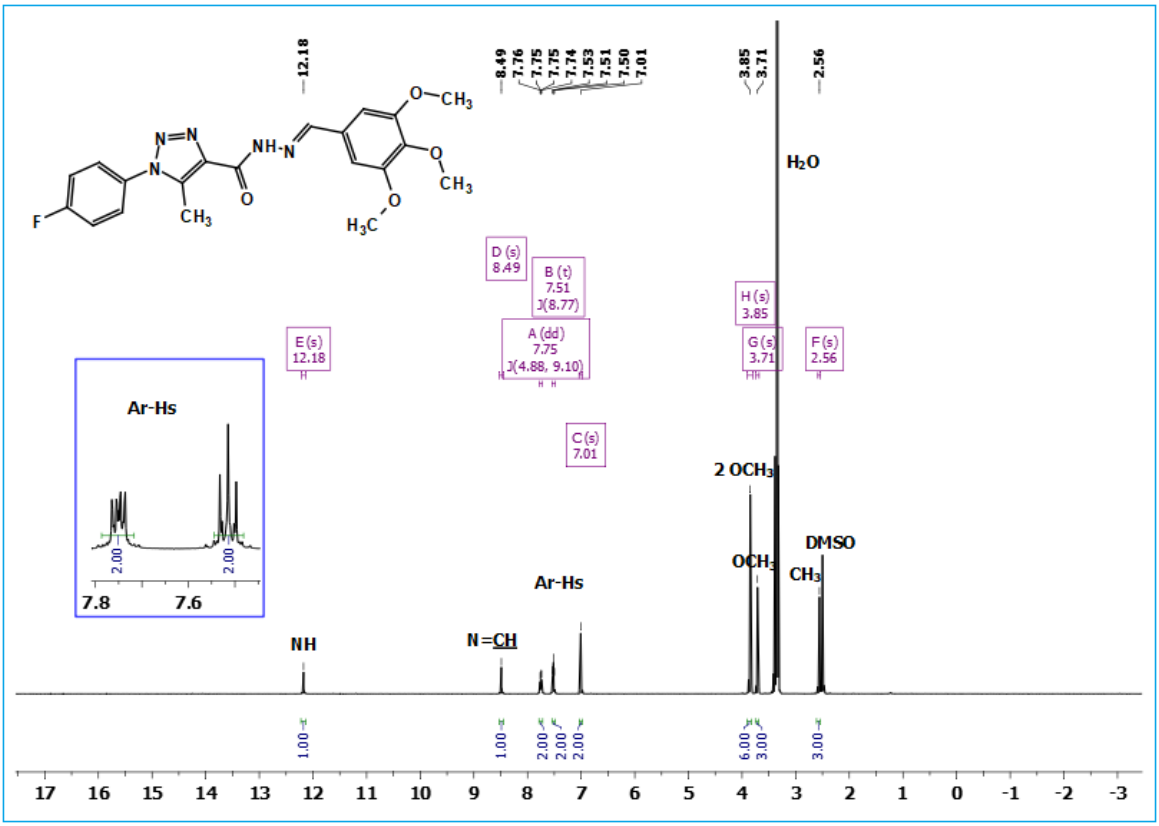


Figure S3. ^{1}H NMR spectrum (500 MHz, DMSO-d_6) for compound **5b**.

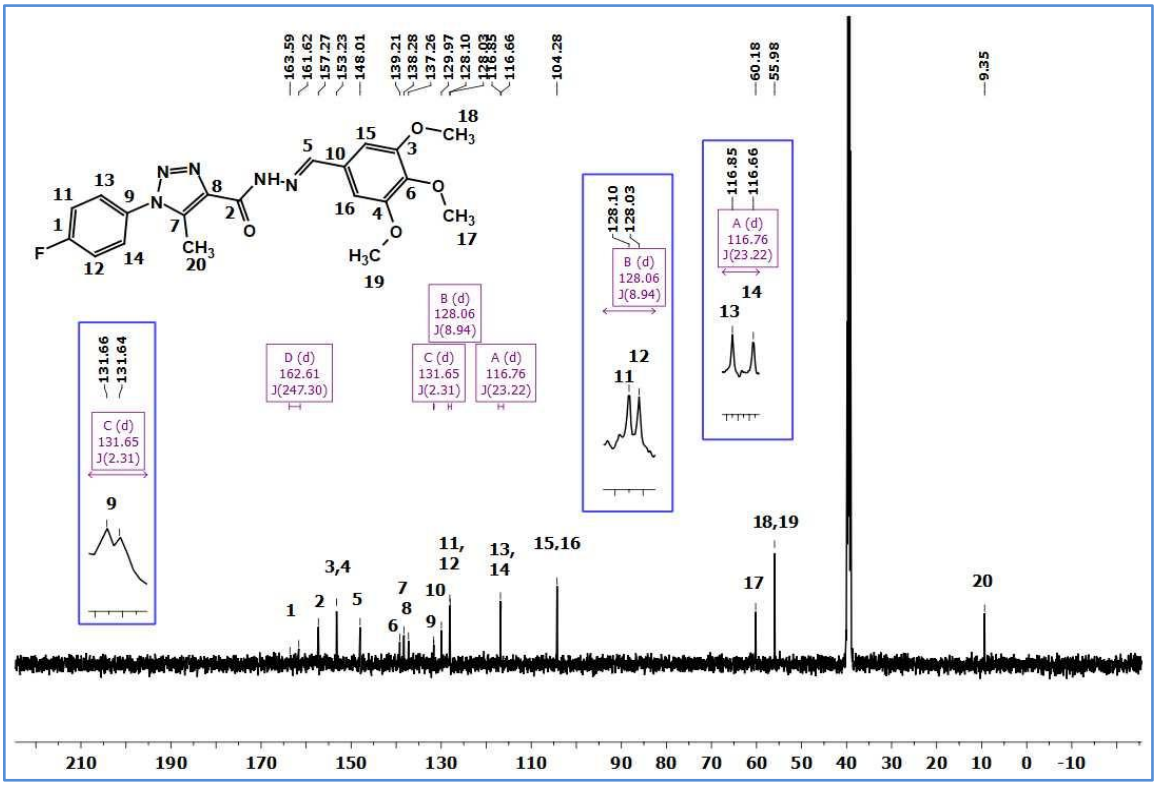


Figure S4. ^{13}C NMR spectrum (125 MHz, DMSO-d_6) for compound **5b**.

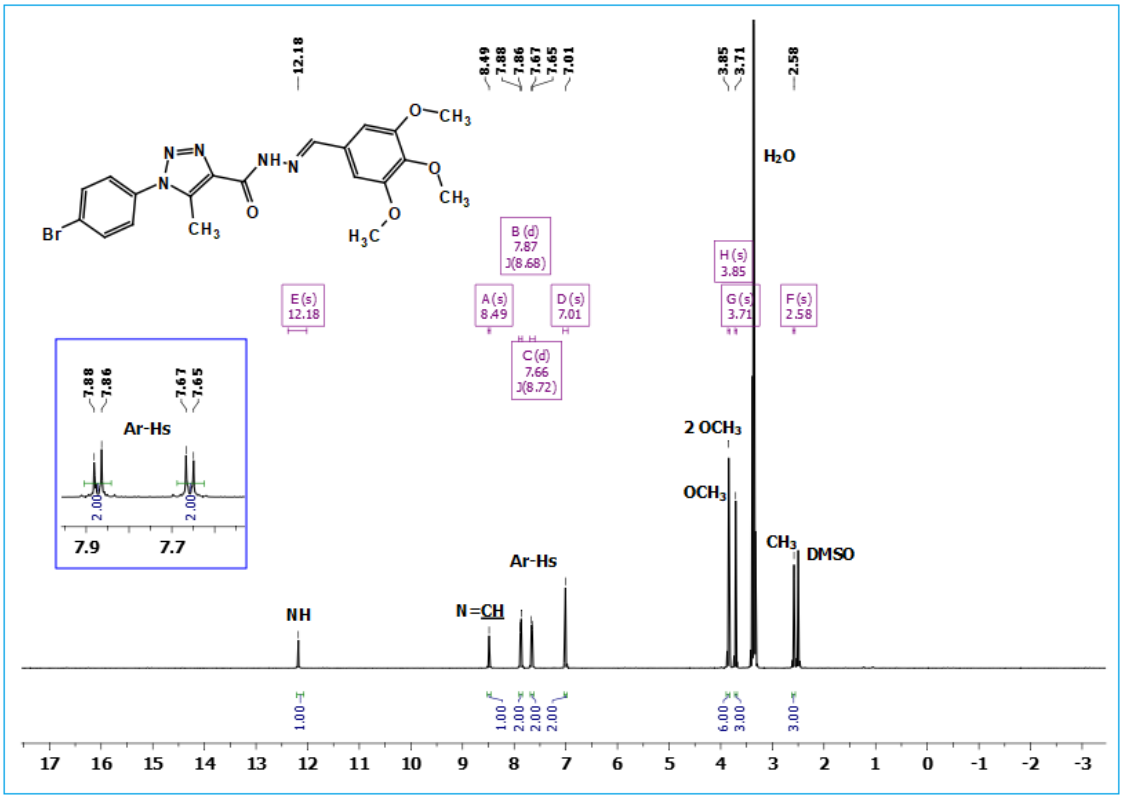


Figure S5. ^{1}H NMR spectrum (500 MHz, DMSO-d_6) for compound **5c**.

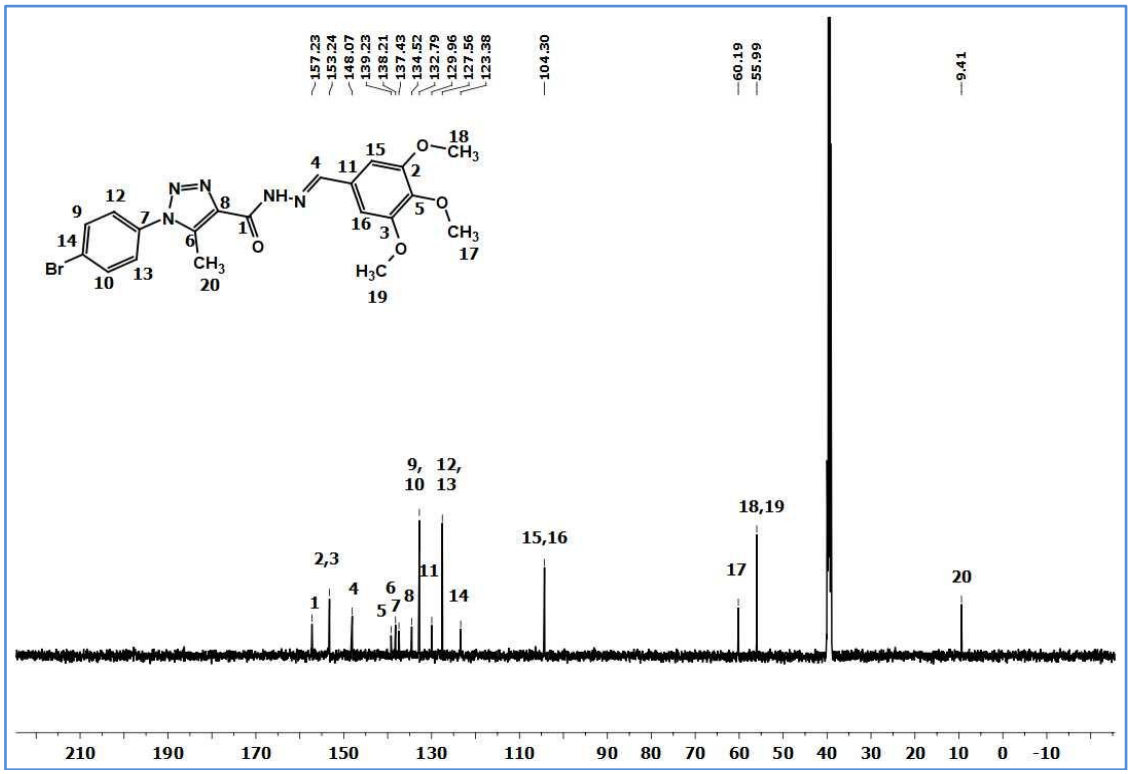


Figure S6. ^{13}C NMR spectrum (125 MHz, DMSO-d_6) for compound **5c**.

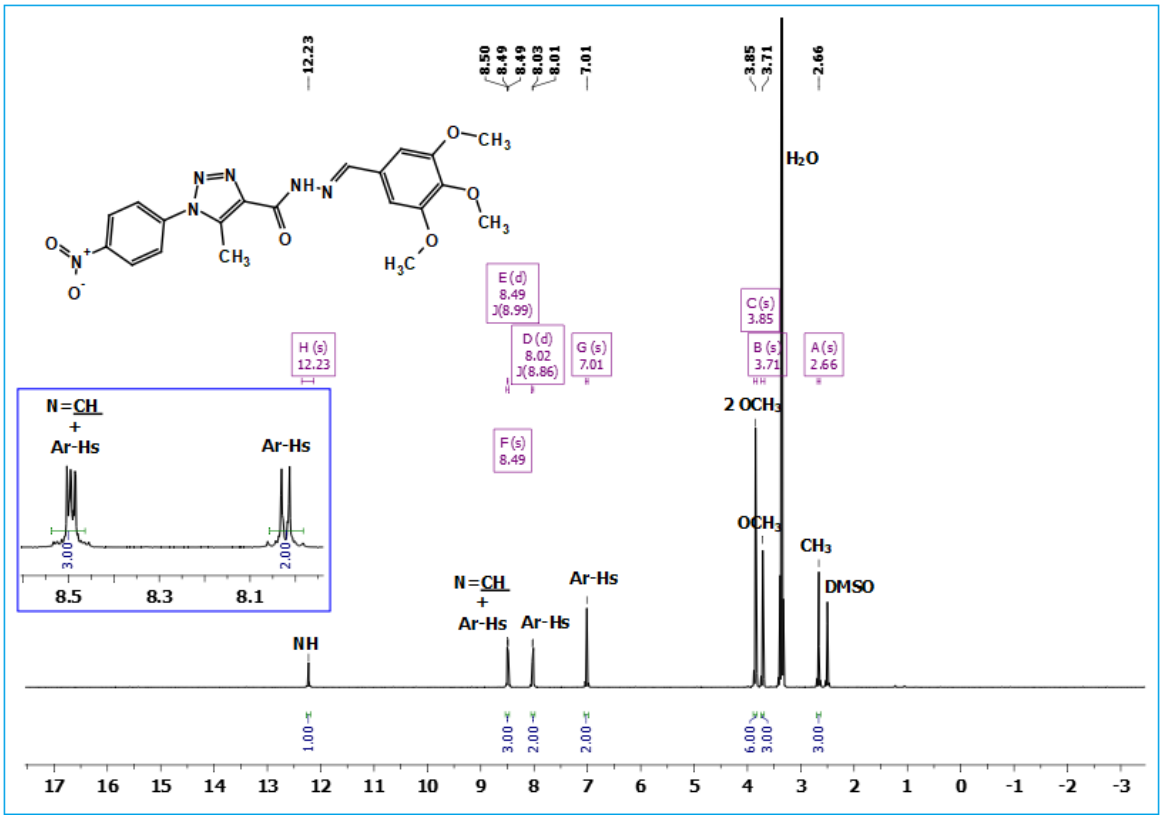


Figure S7. ^{1}H NMR spectrum (500 MHz, DMSO-d_6) for compound **5d**.

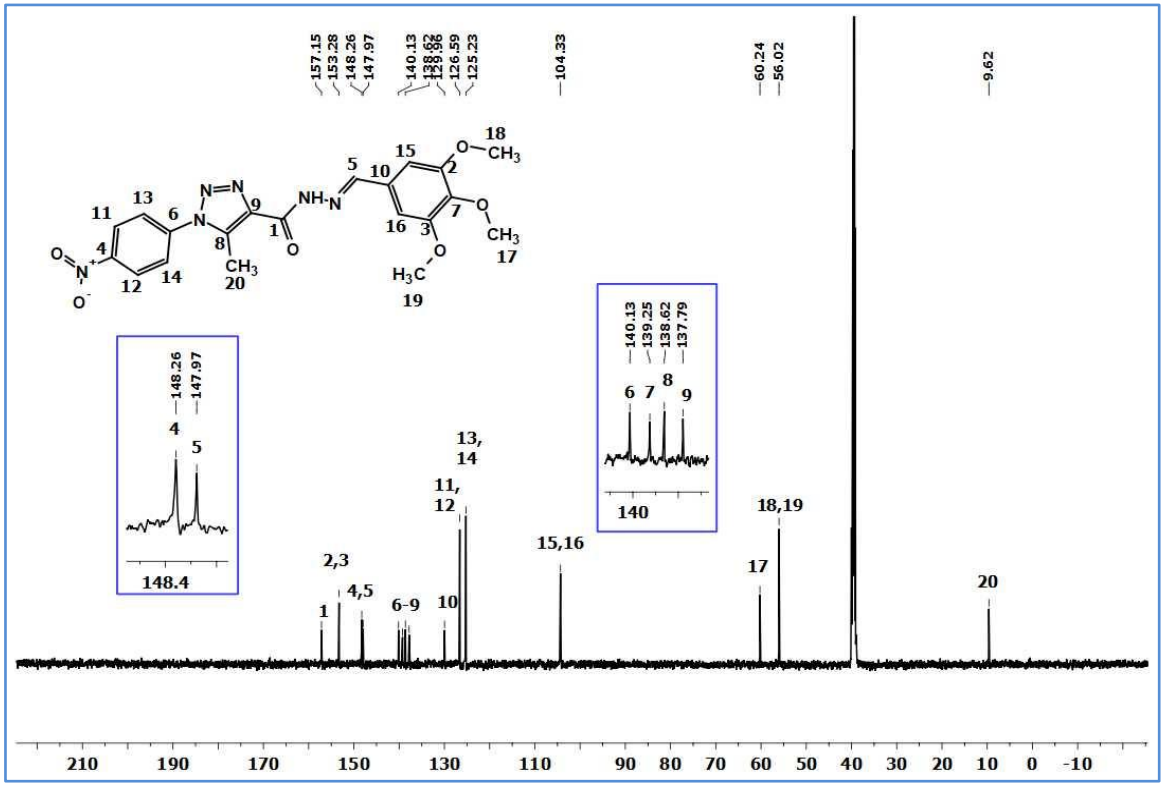


Figure S8. ^{13}C NMR spectrum (125 MHz, DMSO-d_6) for compound **5d**.

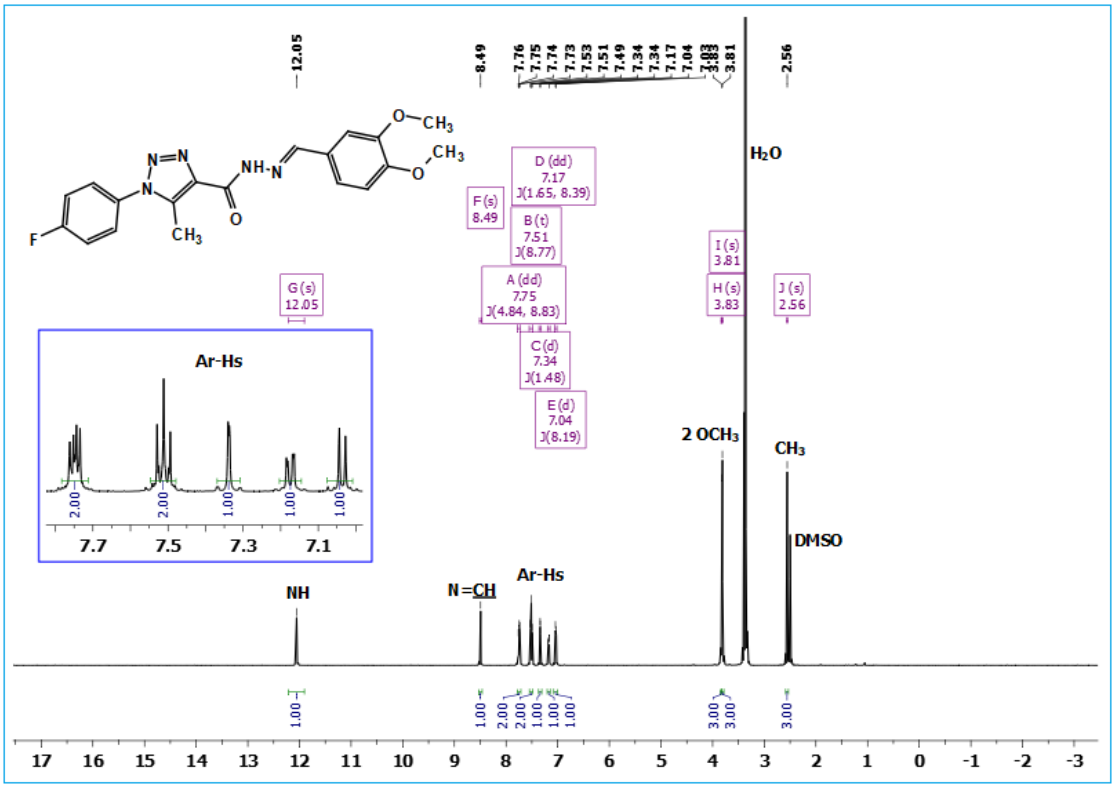


Figure S9. ^{1}H NMR spectrum (500 MHz, DMSO-d_6) for compound **5e**.

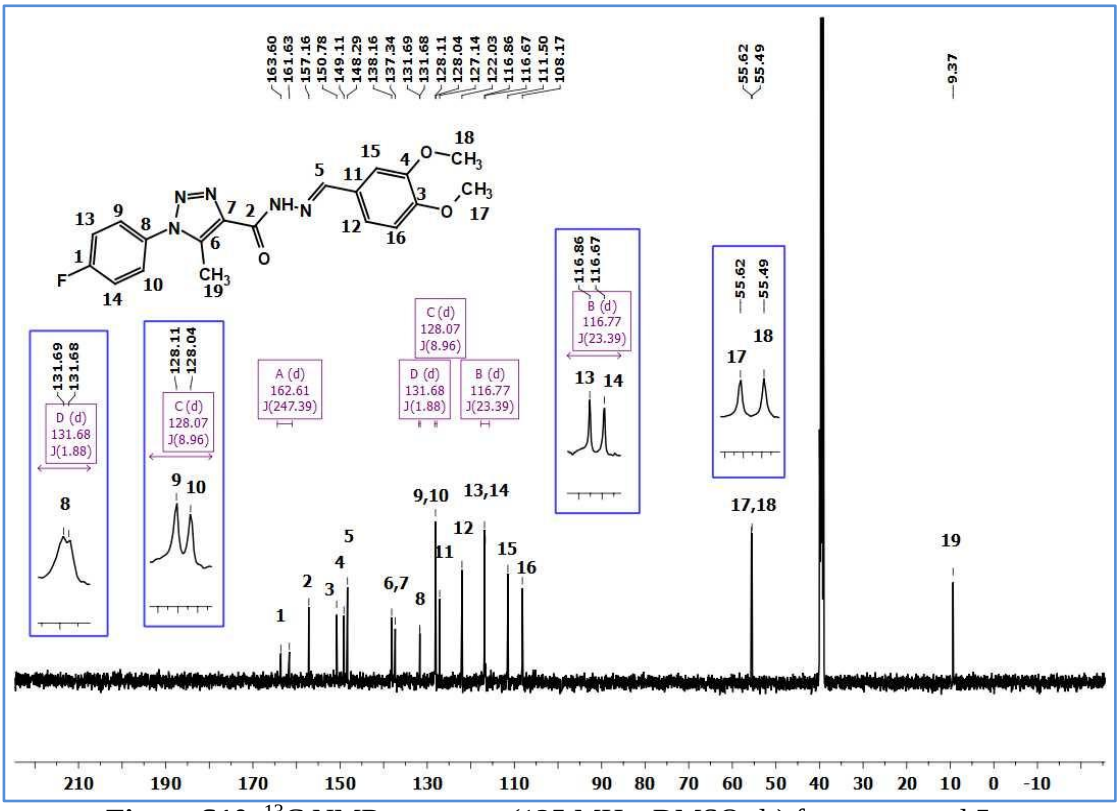


Figure S10. ^{13}C NMR spectrum (125 MHz, DMSO-d_6) for compound **5e**.

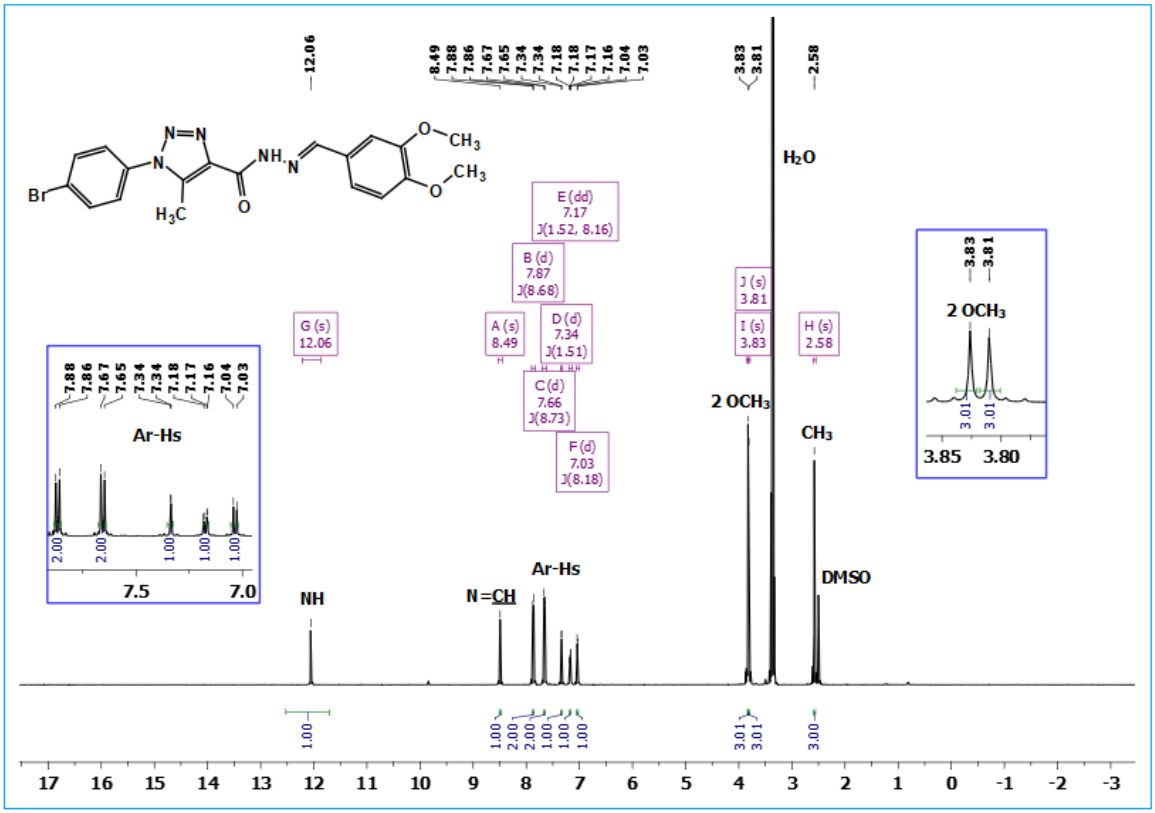


Figure S11. ^{1}H NMR spectrum (500 MHz, DMSO-d_6) for compound **5f**.

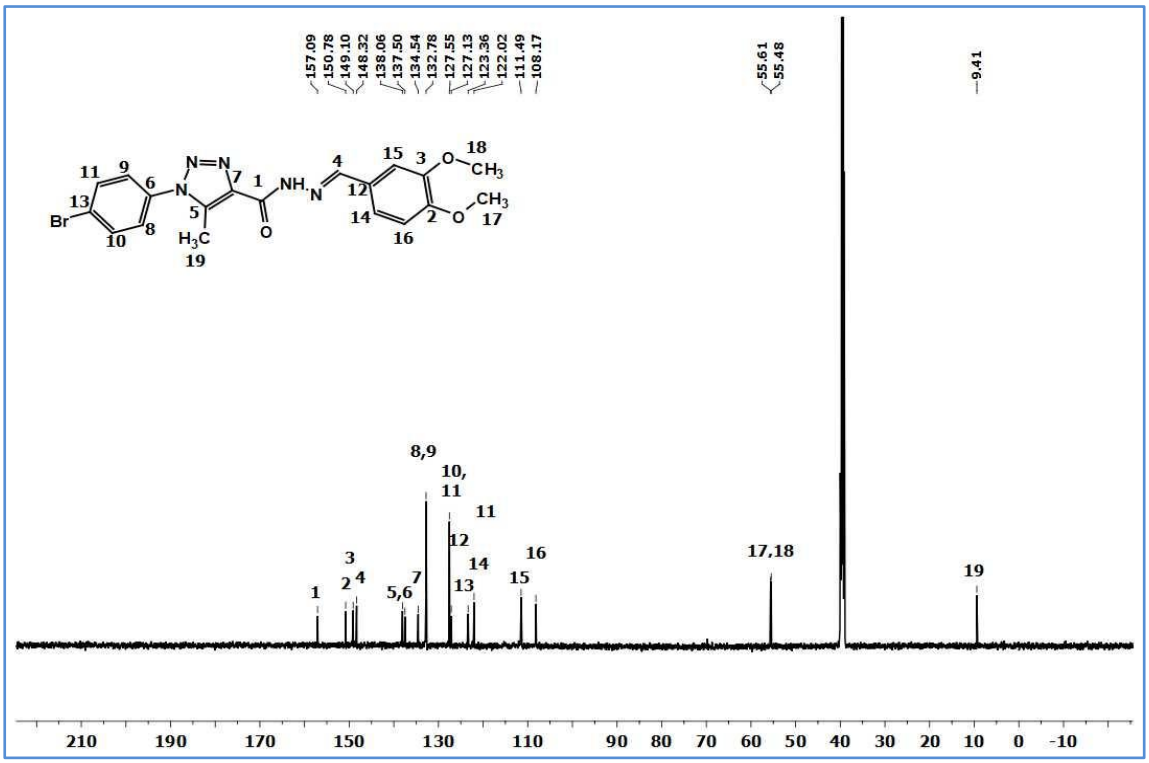


Figure S12. ^{13}C NMR spectrum (125 MHz, DMSO-d_6) for compound **5f**.

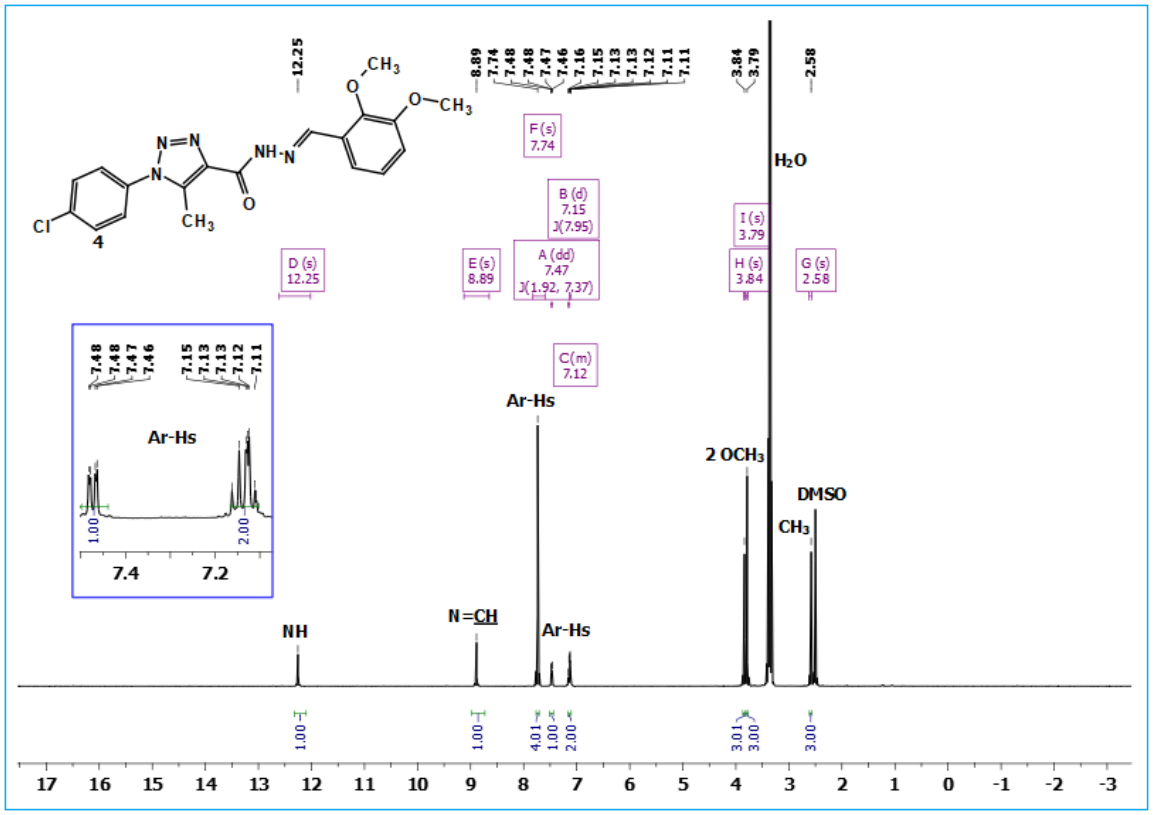


Figure S13. ^{1}H NMR spectrum (500 MHz, DMSO-d_6) for compound **5g**.

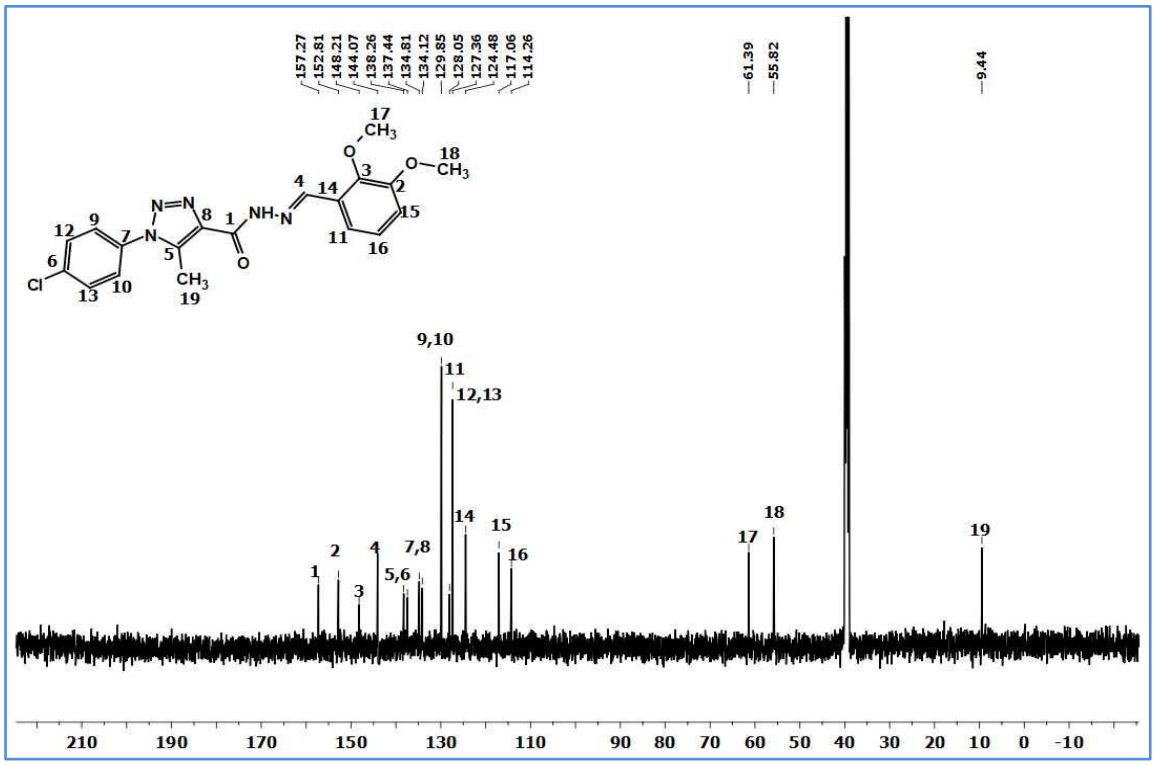


Figure S14. ^{13}C NMR spectrum (125 MHz, DMSO-d_6) for compound **5g**.

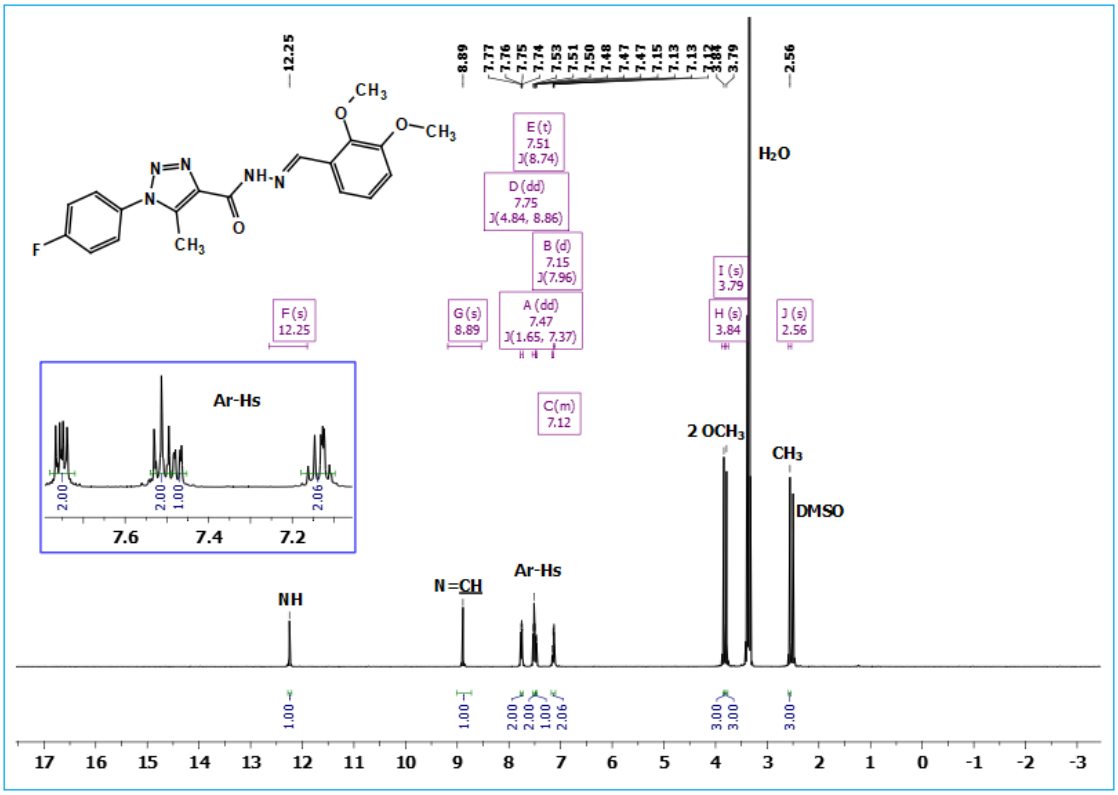


Figure S15. ^{1}H NMR spectrum (500 MHz, DMSO-d$_6$) for compound **5h**.

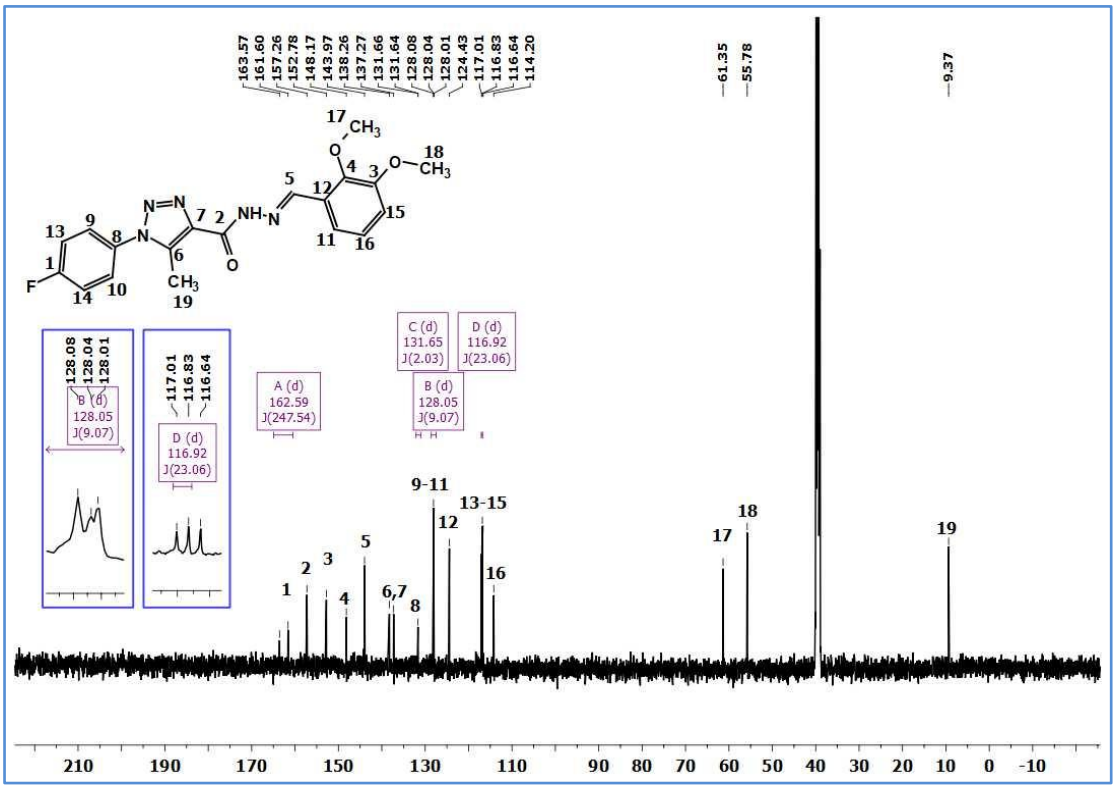


Figure S16. ^{13}C NMR spectrum (125 MHz, DMSO-d$_6$) for compound **5h**

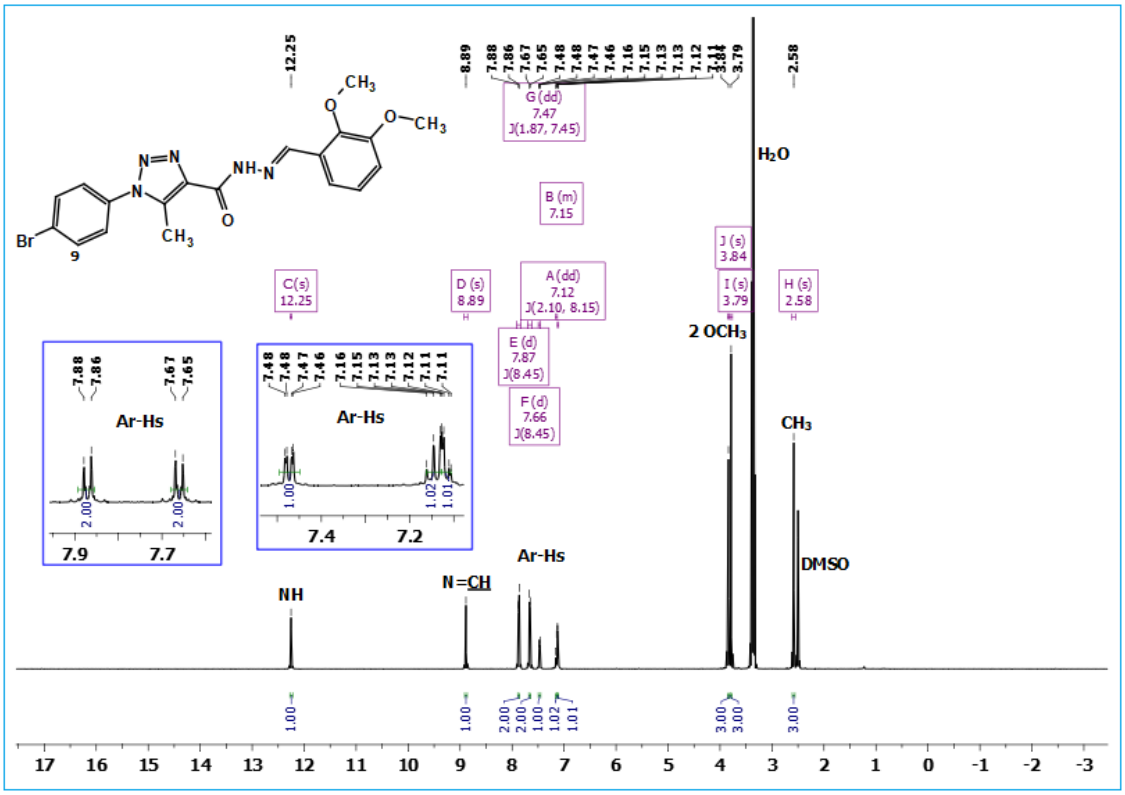


Figure S17. ^{1}H NMR spectrum (500 MHz, DMSO-d_6) for compound **5i**.

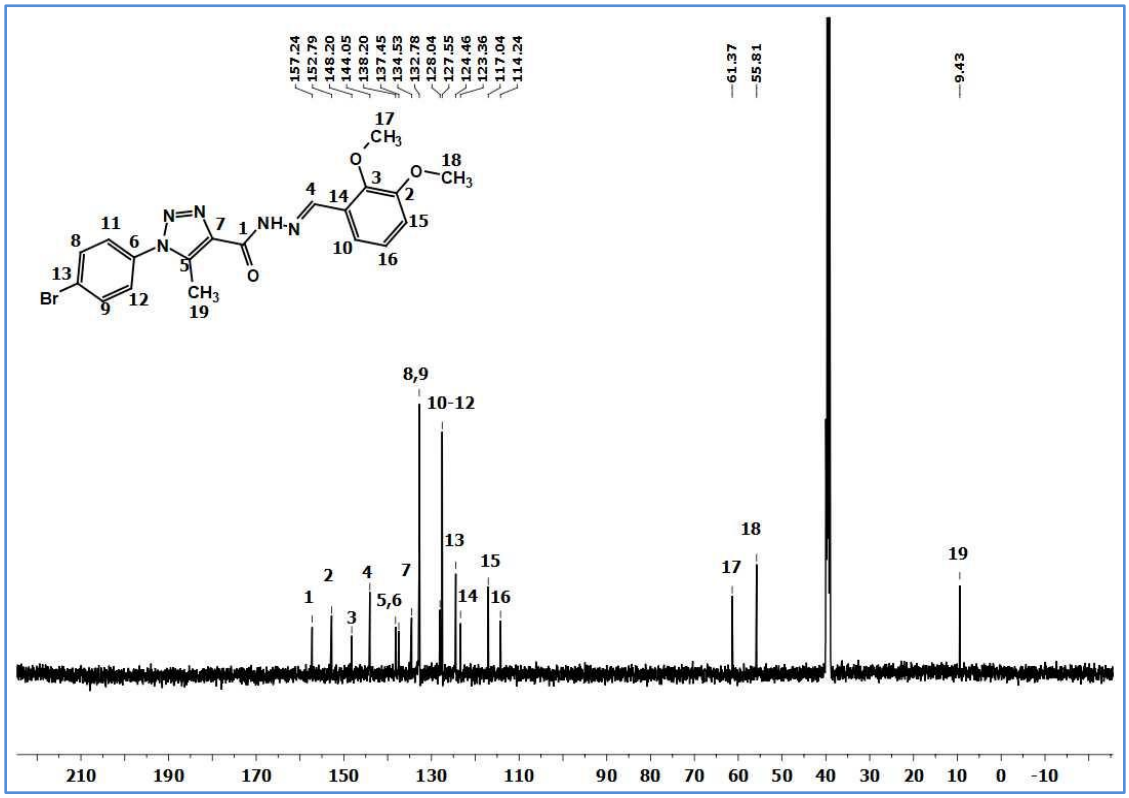


Figure S18. ^{13}C NMR spectrum (125 MHz, DMSO-d_6) for compound **5i**.

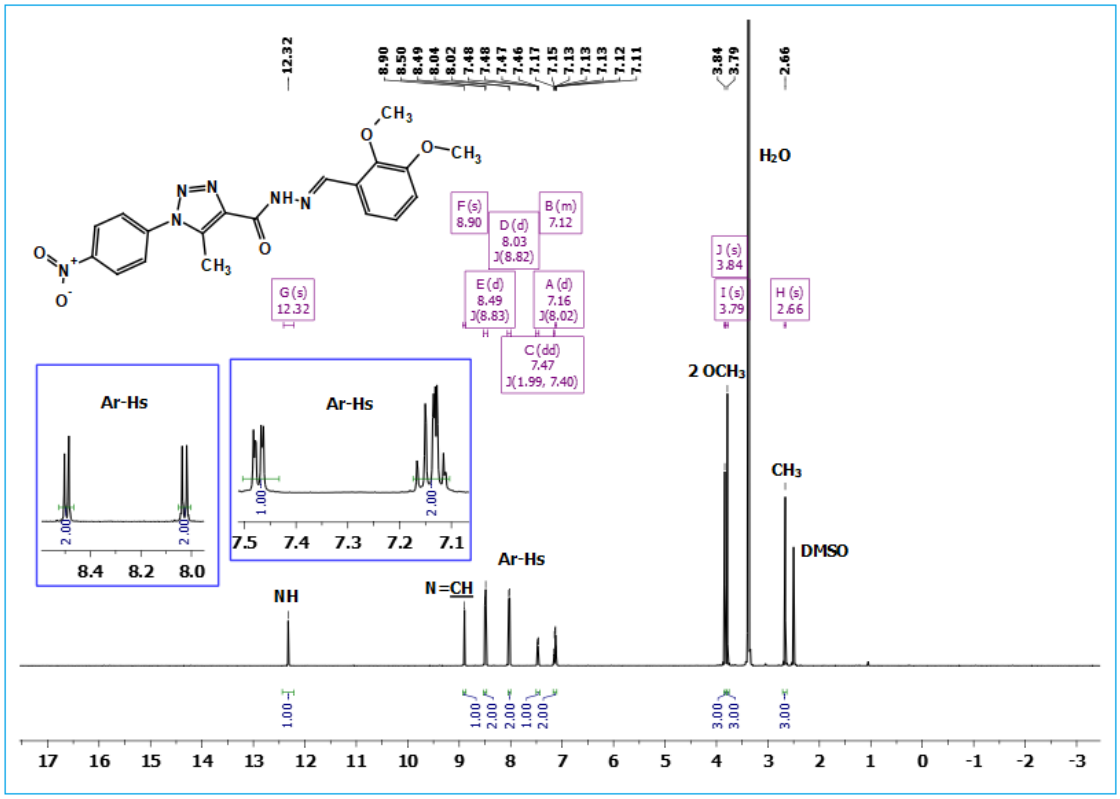


Figure S19. ^{1}H NMR spectrum (500 MHz, DMSO-d$_6$) for compound **5j**.

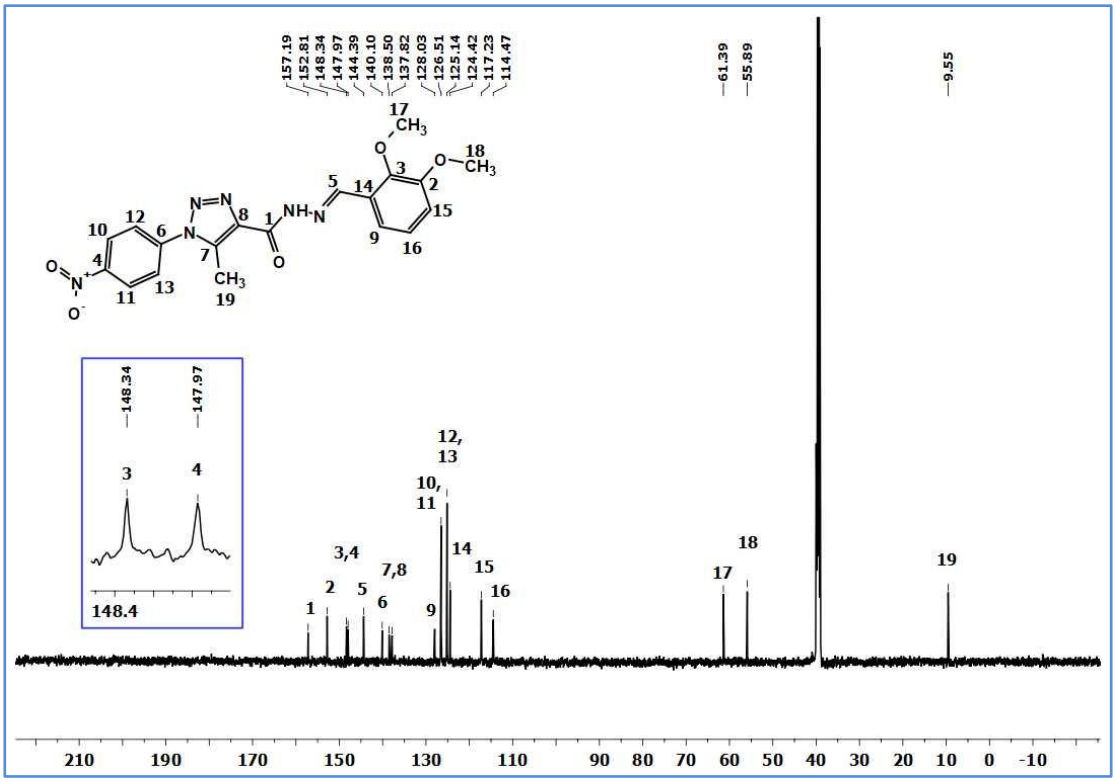


Figure S20. ^{13}C NMR spectrum (125 MHz, DMSO-d$_6$) for compound **5j**.

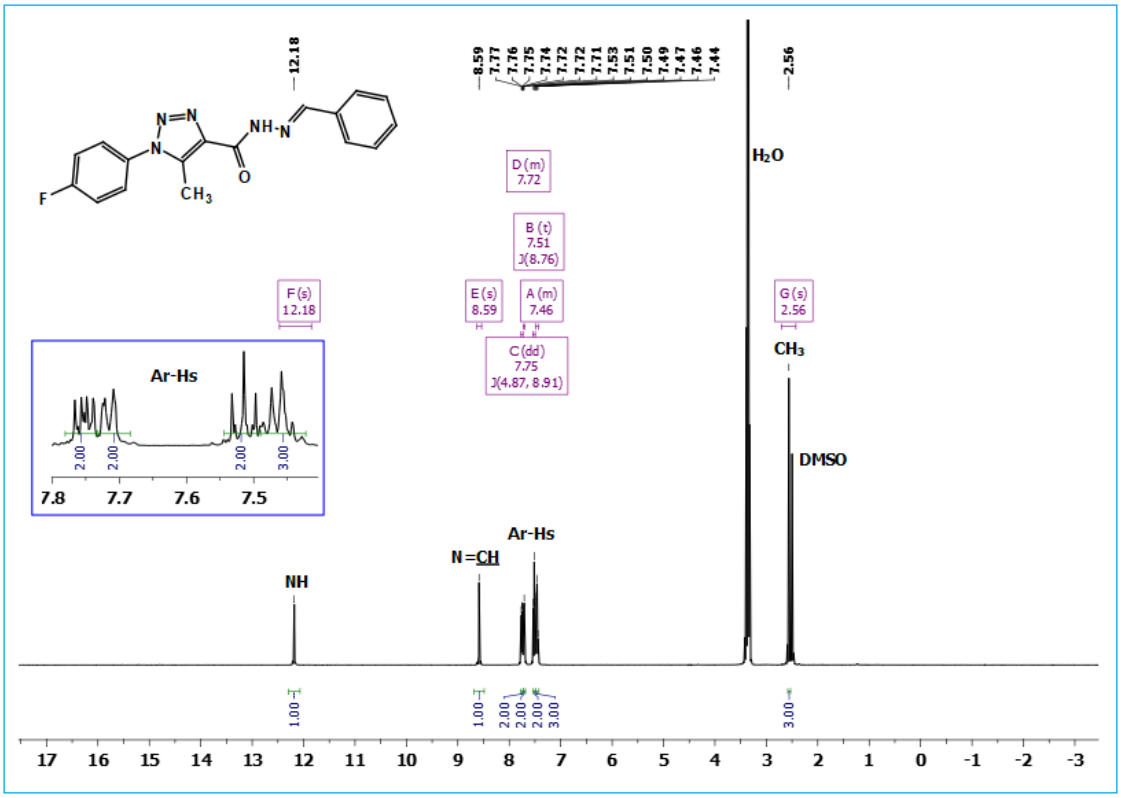


Figure S21. ^{1}H NMR spectrum (500 MHz, DMSO-d_6) for compound **5k**.

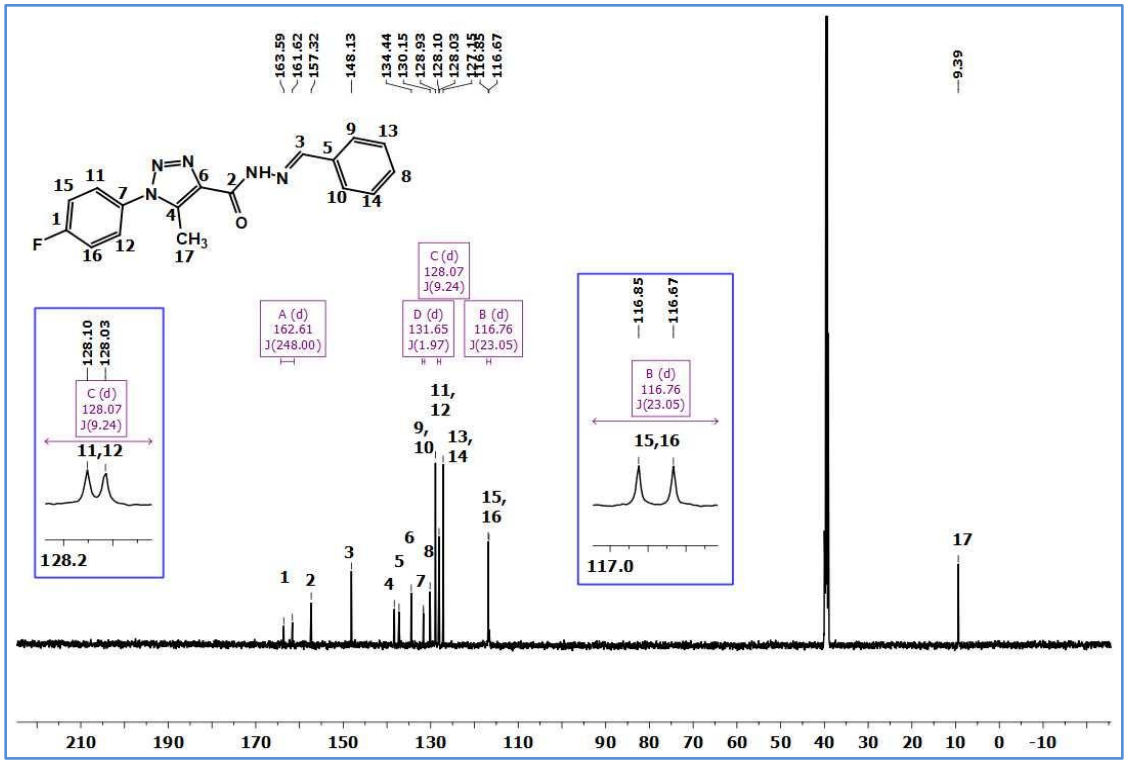


Figure S22. ^{13}C NMR spectrum (125 MHz, DMSO-d_6) for compound **5k**.

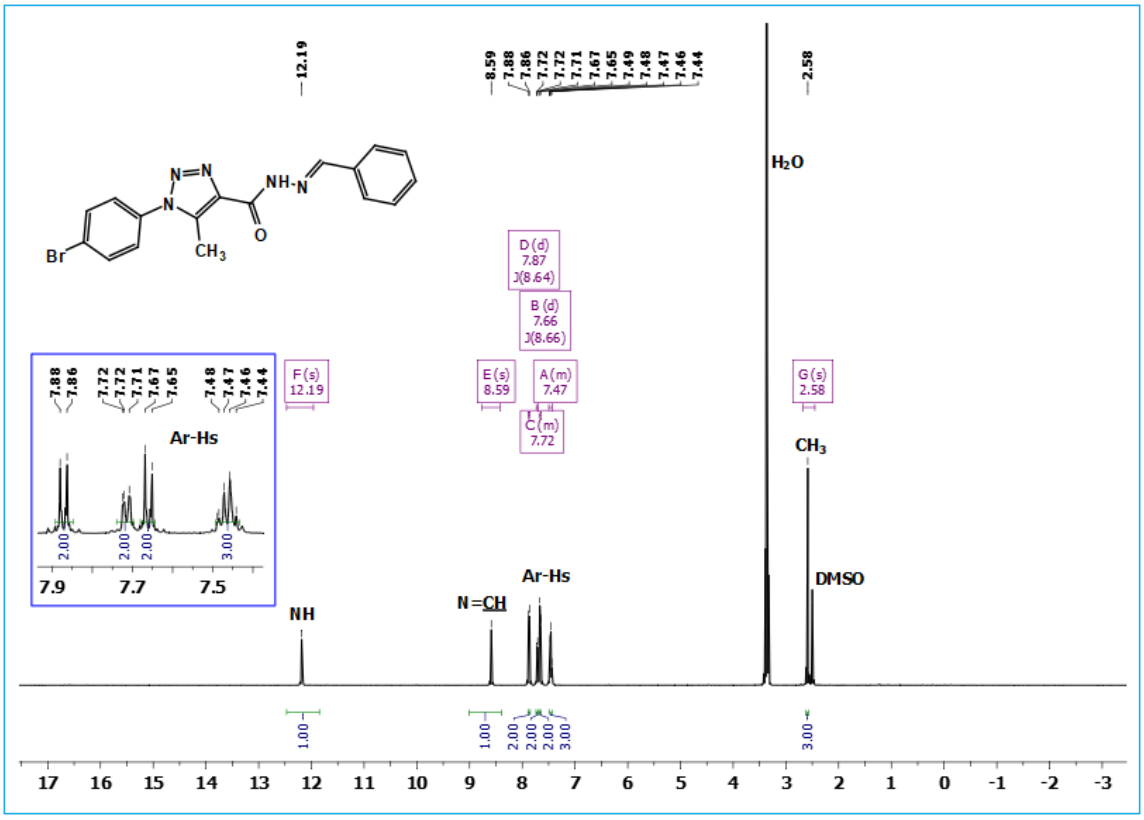


Figure S23. ^{1}H NMR spectrum (500 MHz, DMSO-d_6) for compound **5l**.

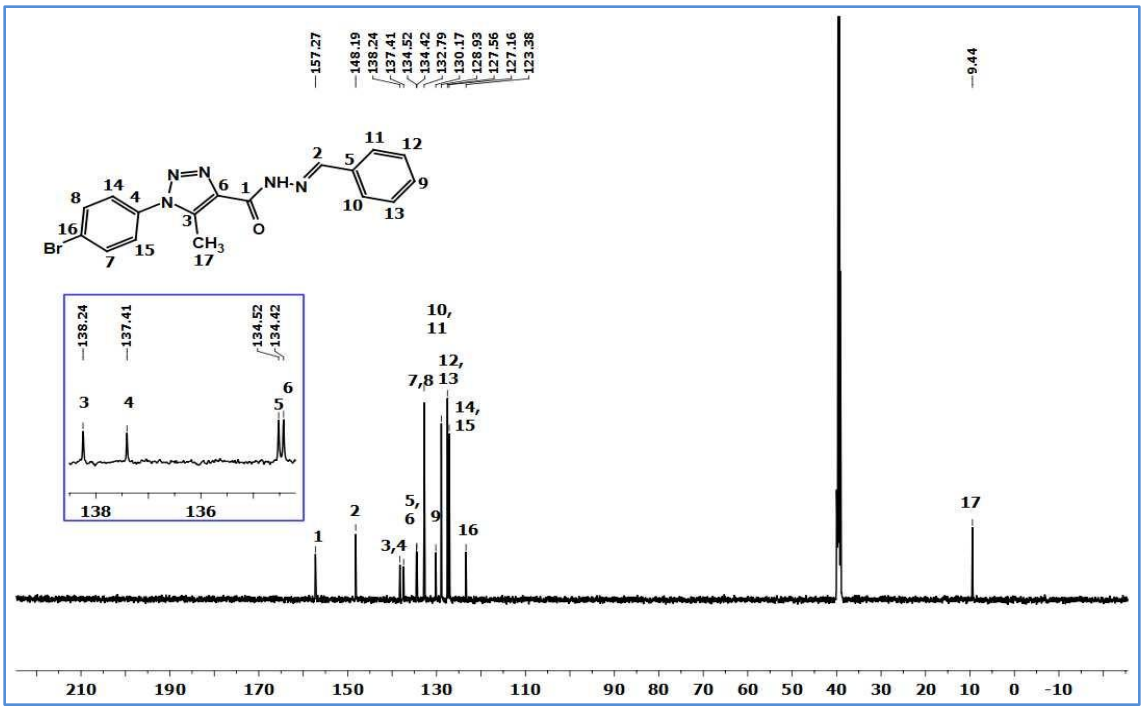


Figure S24. ^{13}C NMR spectrum (125 MHz, DMSO-d_6) for compound **5l**.

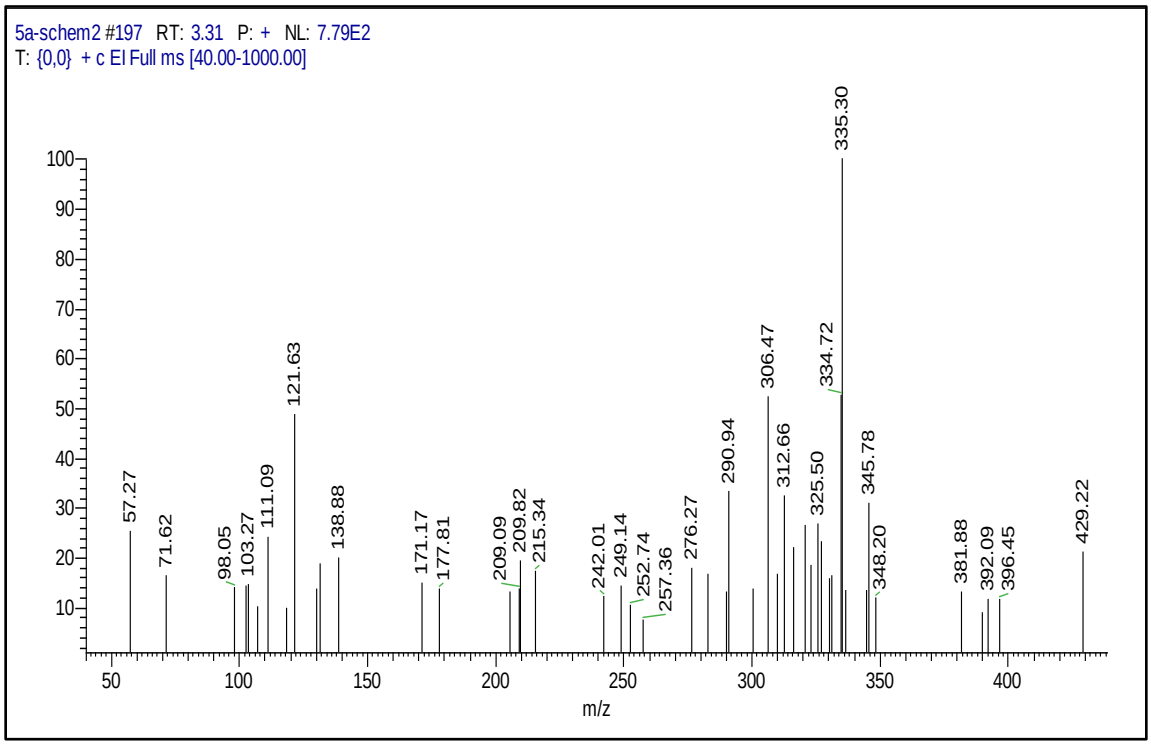


Figure S25. Mass spectrum for compound **5a**.

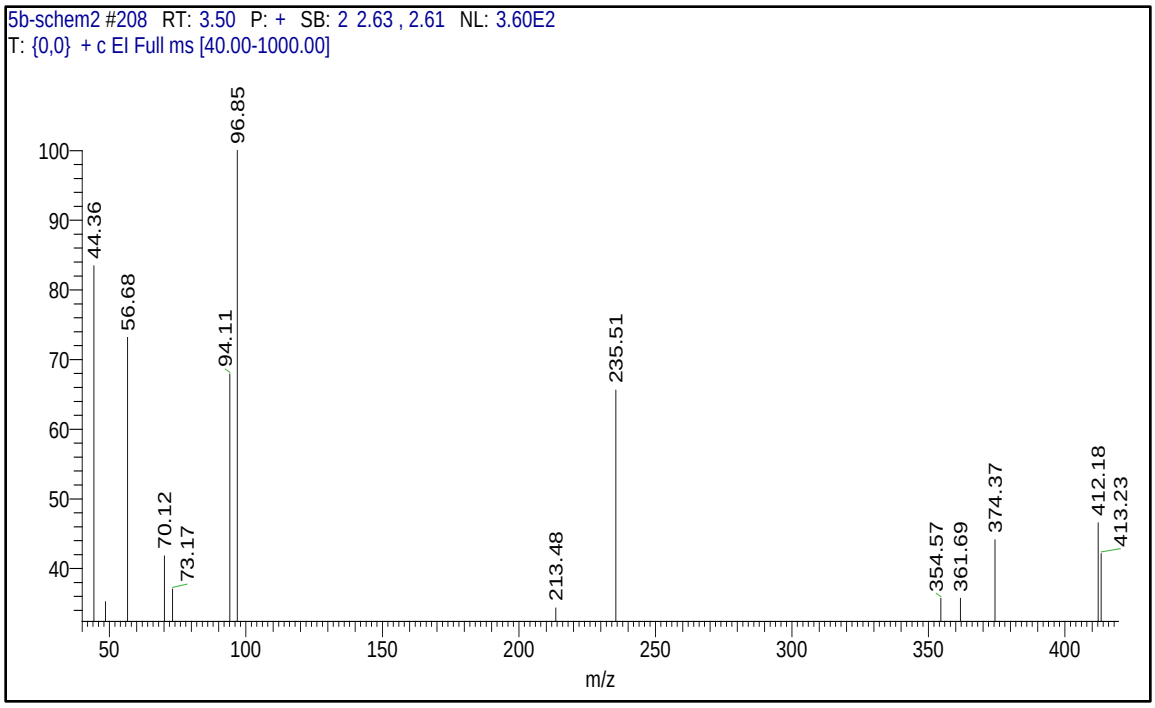


Figure S26. Mass spectrum for compound **5b**.

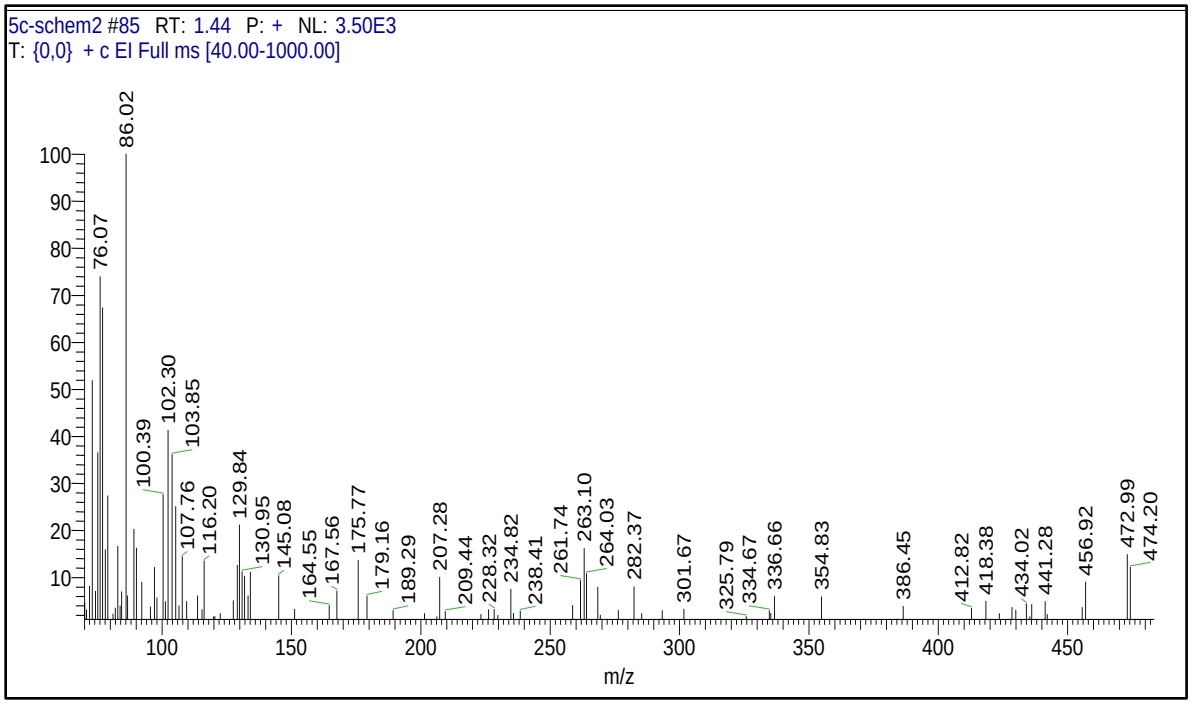


Figure S27. Mass spectrum for compound **5c**.

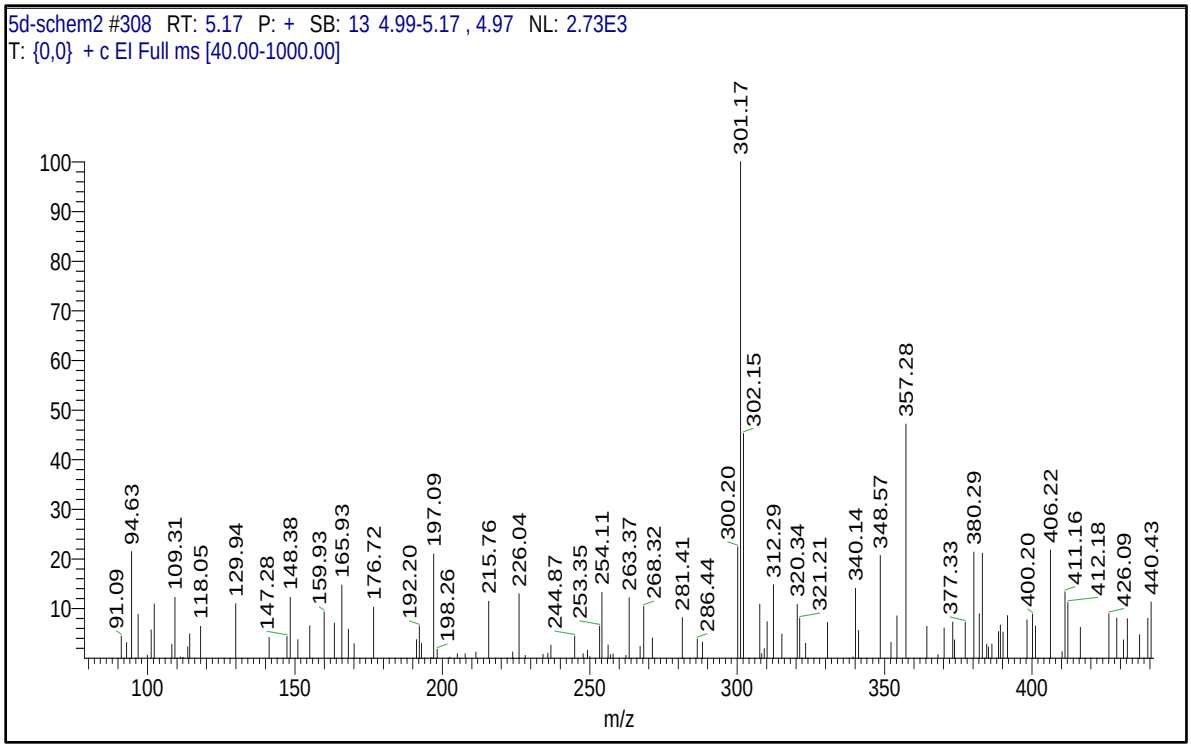


Figure S28. Mass spectrum for compound **5d**.

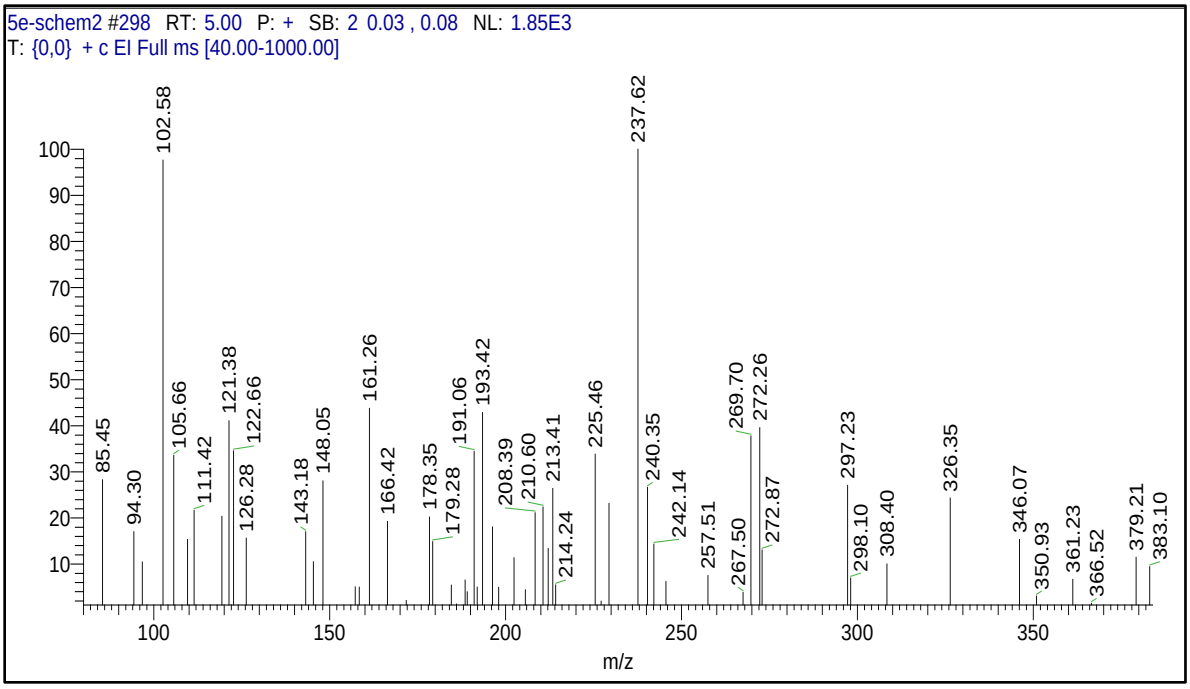


Figure S29. Mass spectrum for compound **5e**.

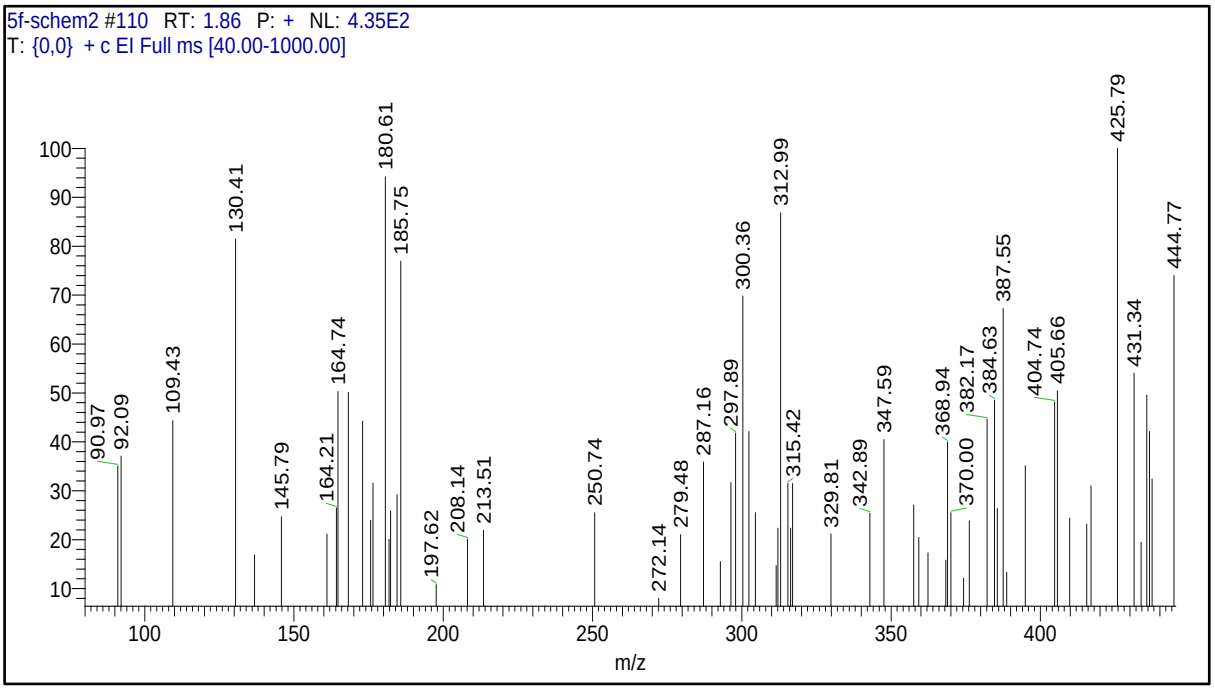


Figure S30. Mass spectrum for compound **5f**.

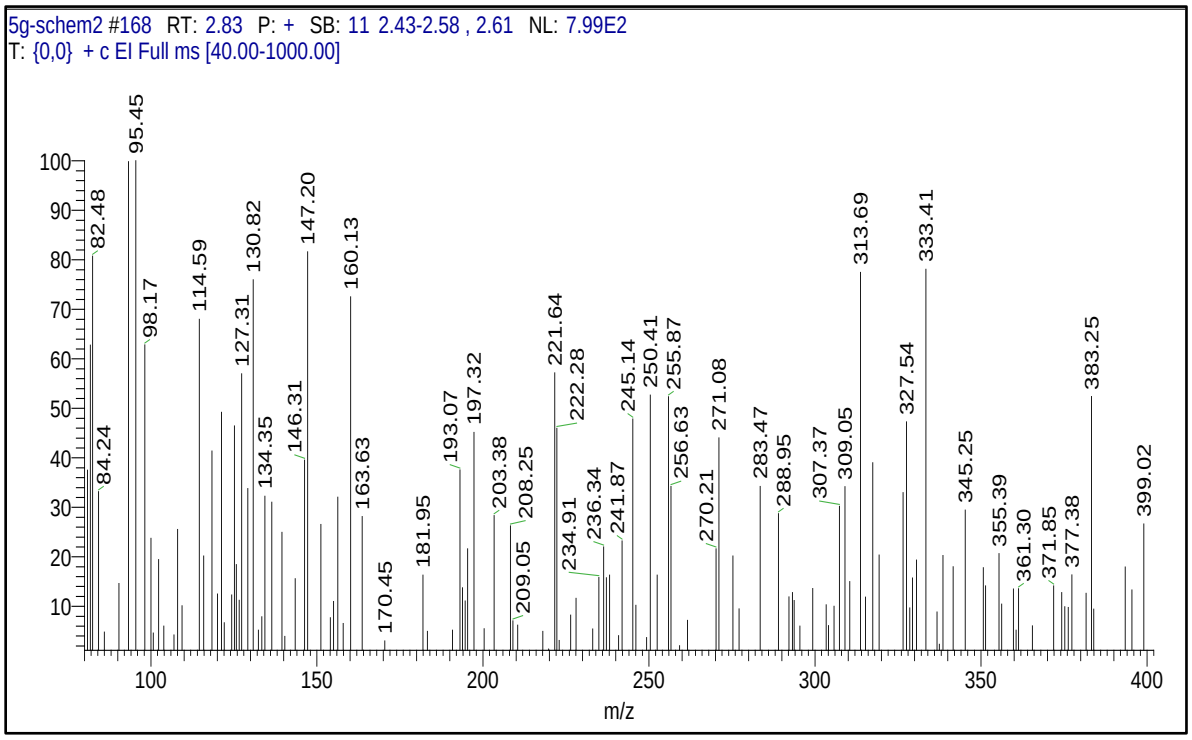


Figure S31. Mass spectrum for compound **5g**.

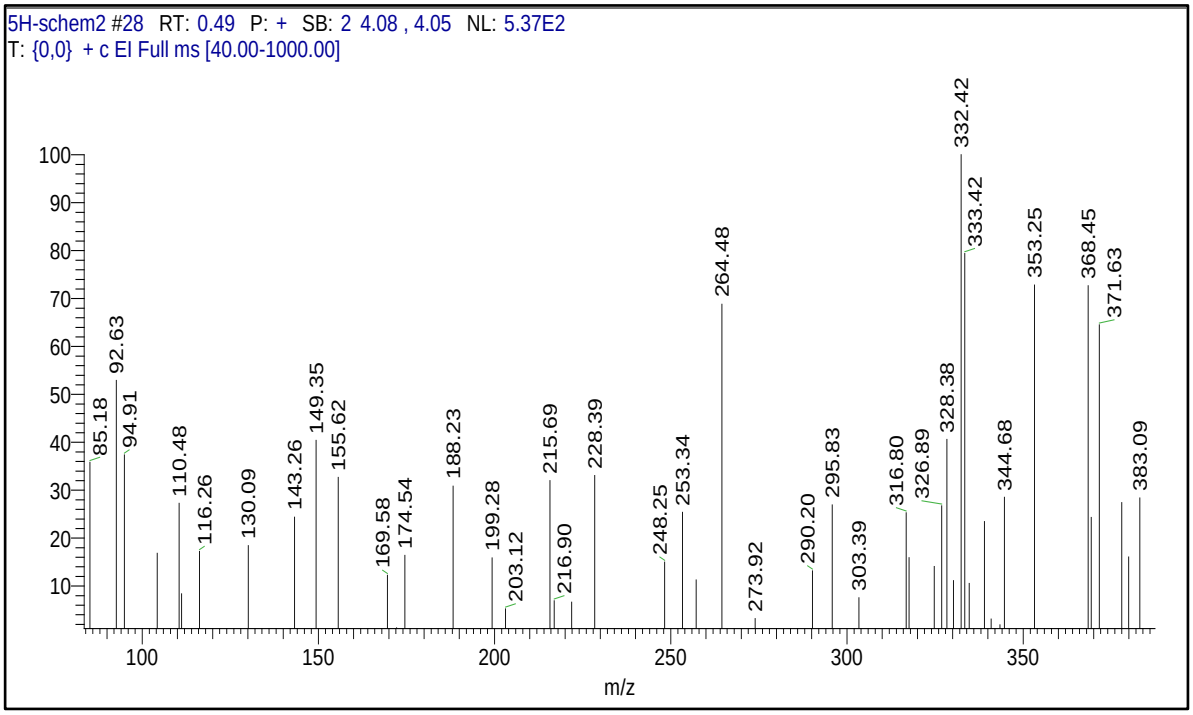


Figure S32. Mass spectrum for compound **5h**.

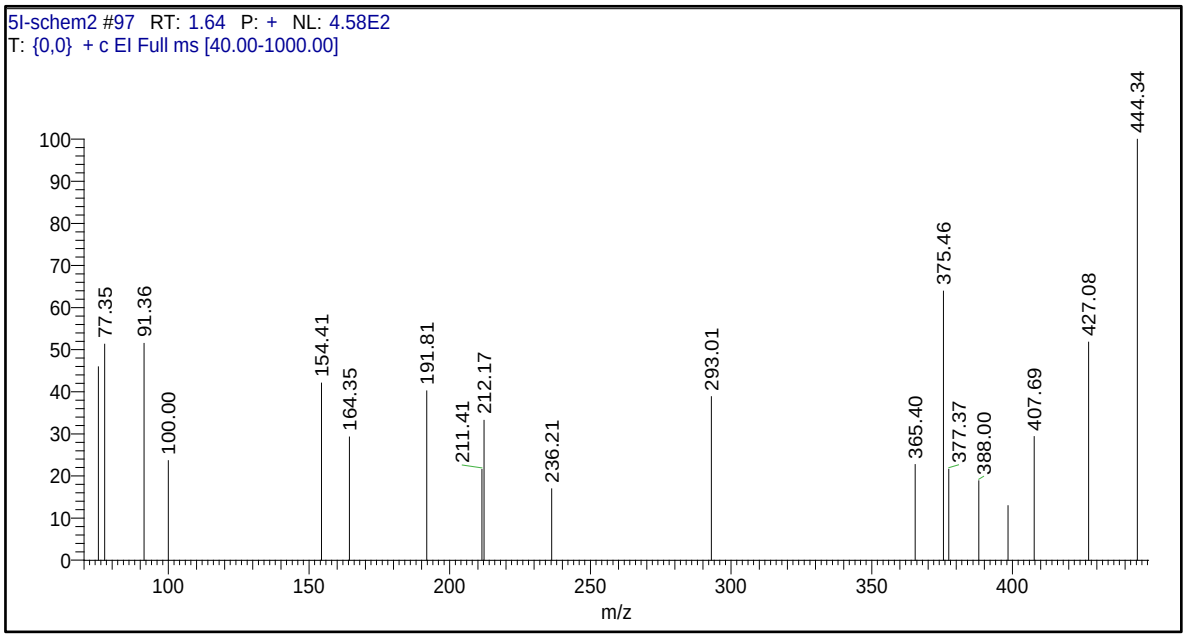


Figure S33. Mass spectrum for compound **5i**.

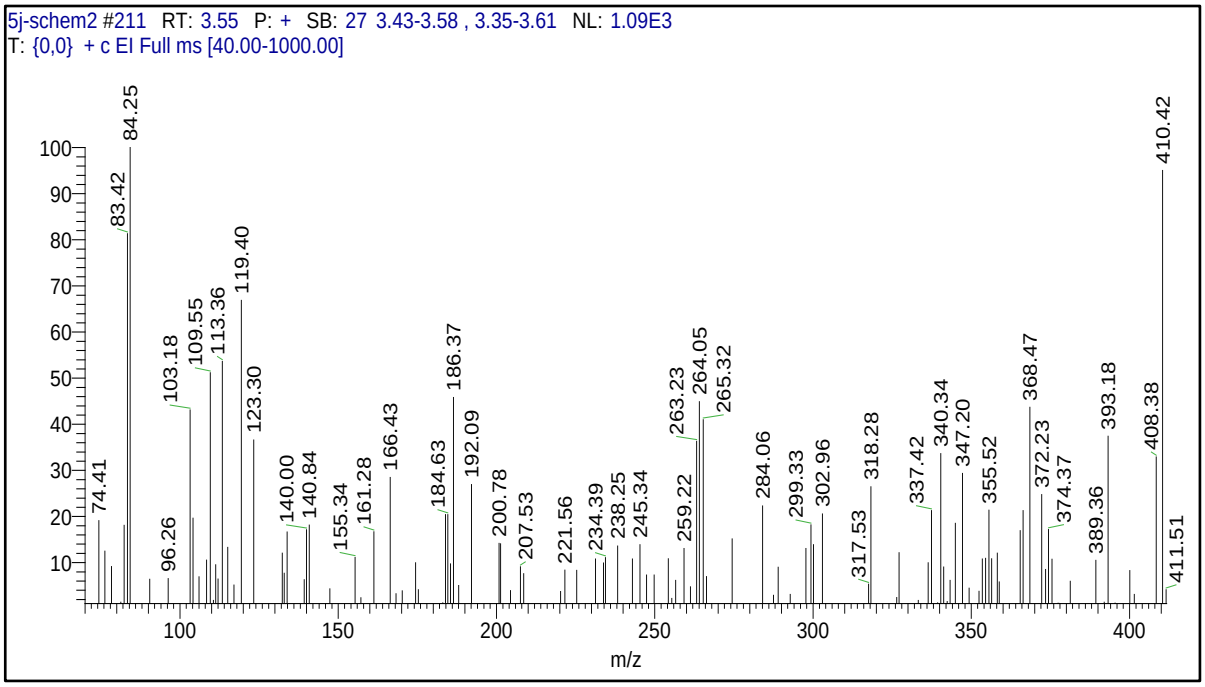


Figure S34. Mass spectrum for compound **5j**.

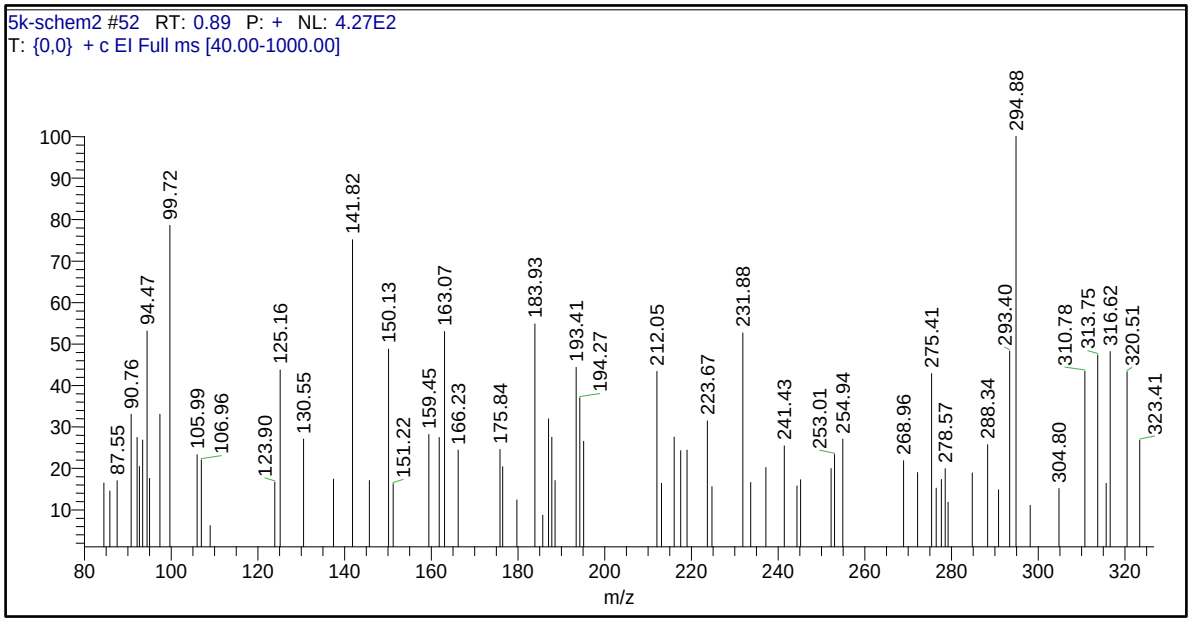


Figure S35. Mass spectrum for compound **5k**.

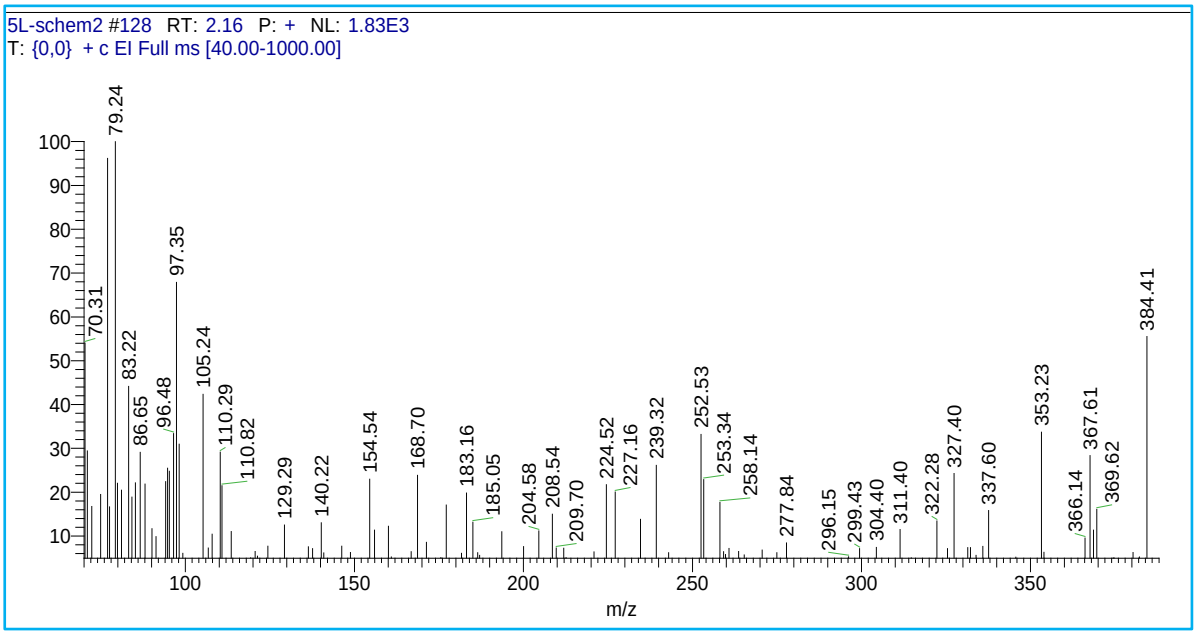
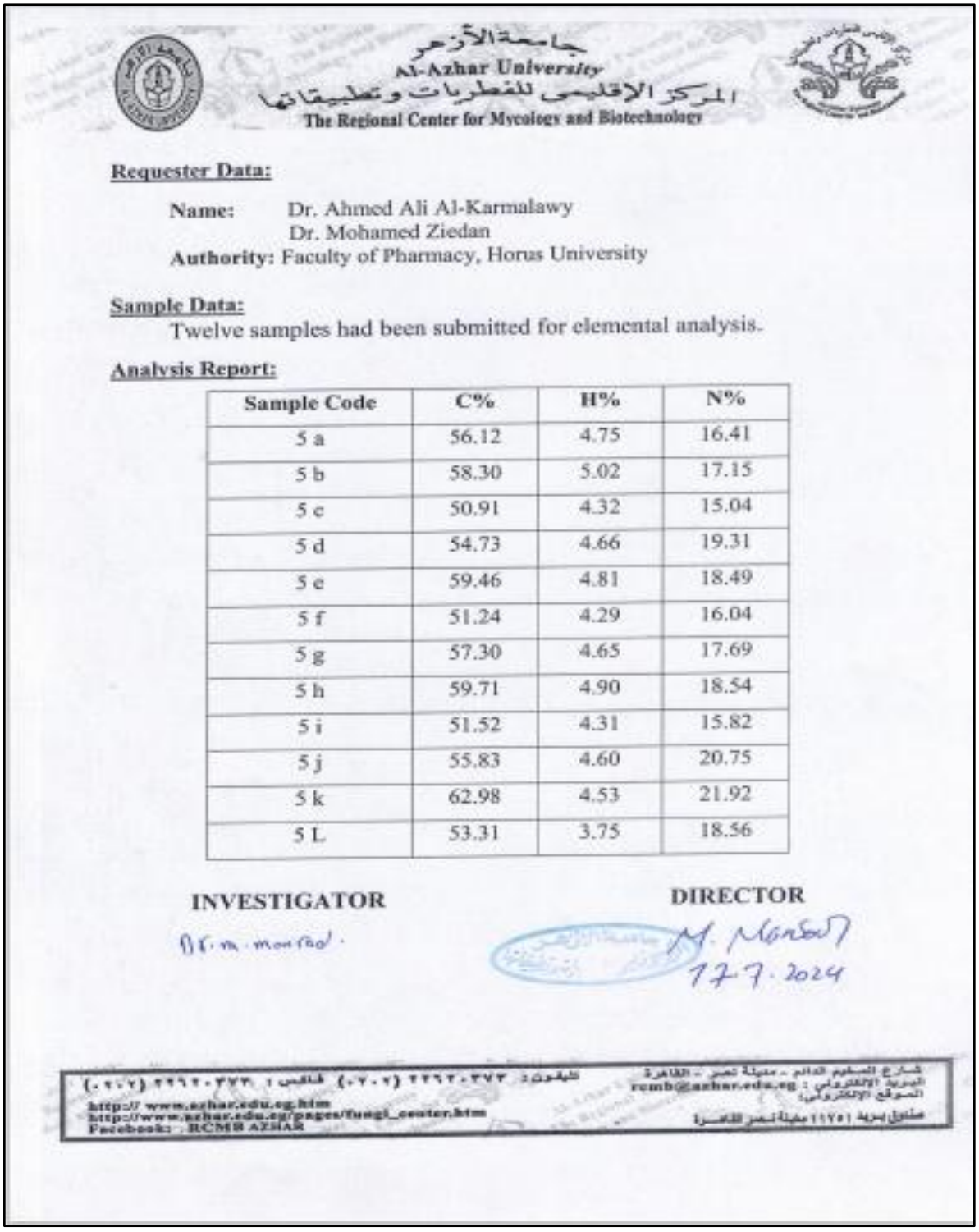


Figure S36. Mass spectrum for compound **5l**.



جامعة الأزهر
Al-Azhar University
المركز الإقليمي للفطريات وتطبيقاتها
The Regional Center for Mycology and Biotechnology

Requester Data:

Name: Dr. Ahmed Ali Al-Karmalawy
Dr. Mohamed Ziedan
Authority: Faculty of Pharmacy, Horus University

Sample Data:

Twelve samples had been submitted for elemental analysis.

Analysis Report:

Sample Code	C%	H%	N%
5 a	56.12	4.75	16.41
5 b	58.30	5.02	17.15
5 c	50.91	4.32	15.04
5 d	54.73	4.66	19.31
5 e	59.46	4.81	18.49
5 f	51.24	4.29	16.04
5 g	57.30	4.65	17.69
5 h	59.71	4.90	18.54
5 i	51.52	4.31	15.82
5 j	55.83	4.60	20.75
5 k	62.98	4.53	21.92
5 L	53.31	3.75	18.56

INVESTIGATOR

DIRECTOR

17.7.2024

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http:// www.azhar.edu.eg.htm
http://www.azhar.edu.eg/pages/fungi_center.htm
Facebook: RCMB AZHAR

Table S1. Elemental analysis of targets (**5a-l**).

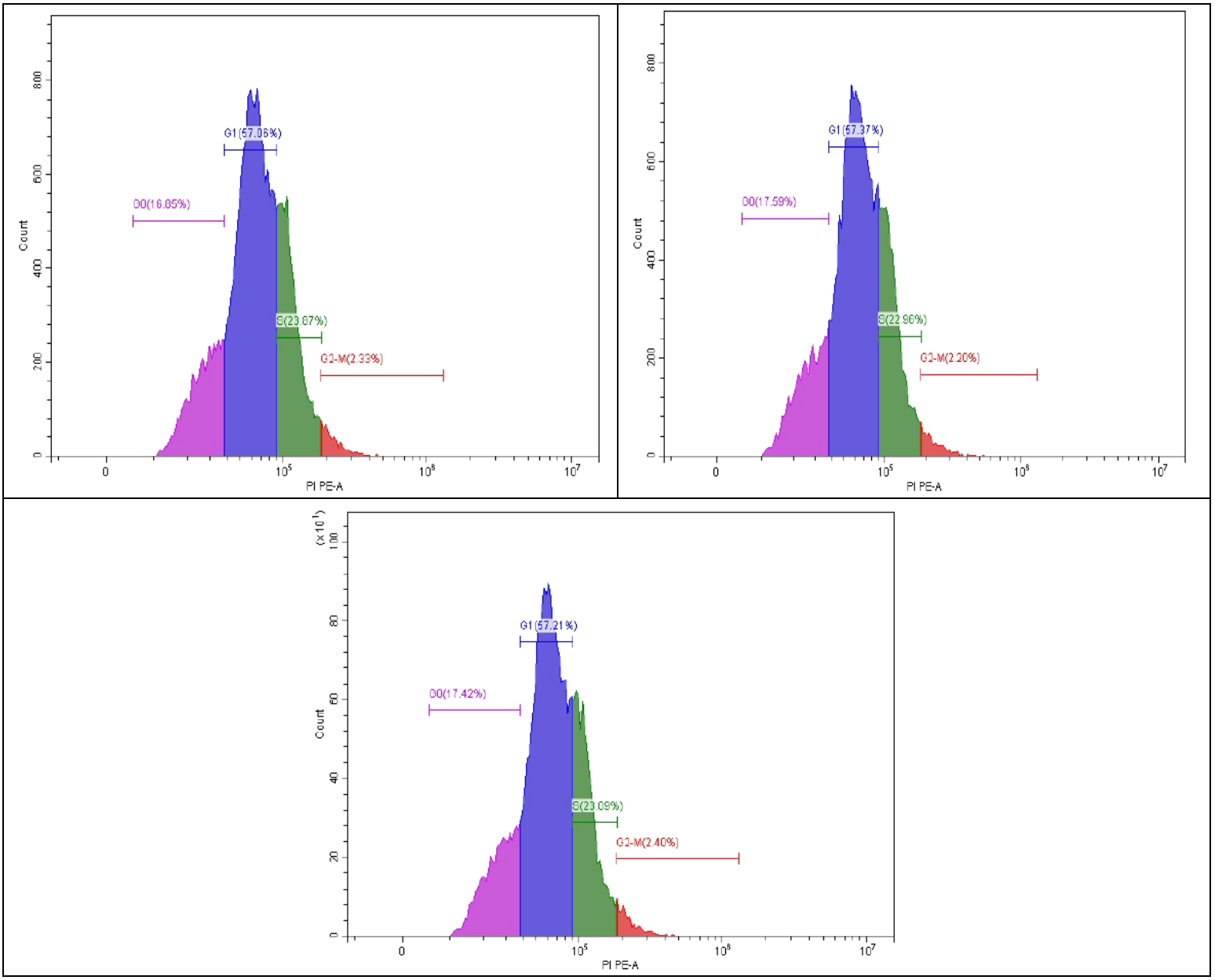

Figure S37. the triplicate experiments of cell cycle analysis histogram of untreated control A375 cancer cell line.

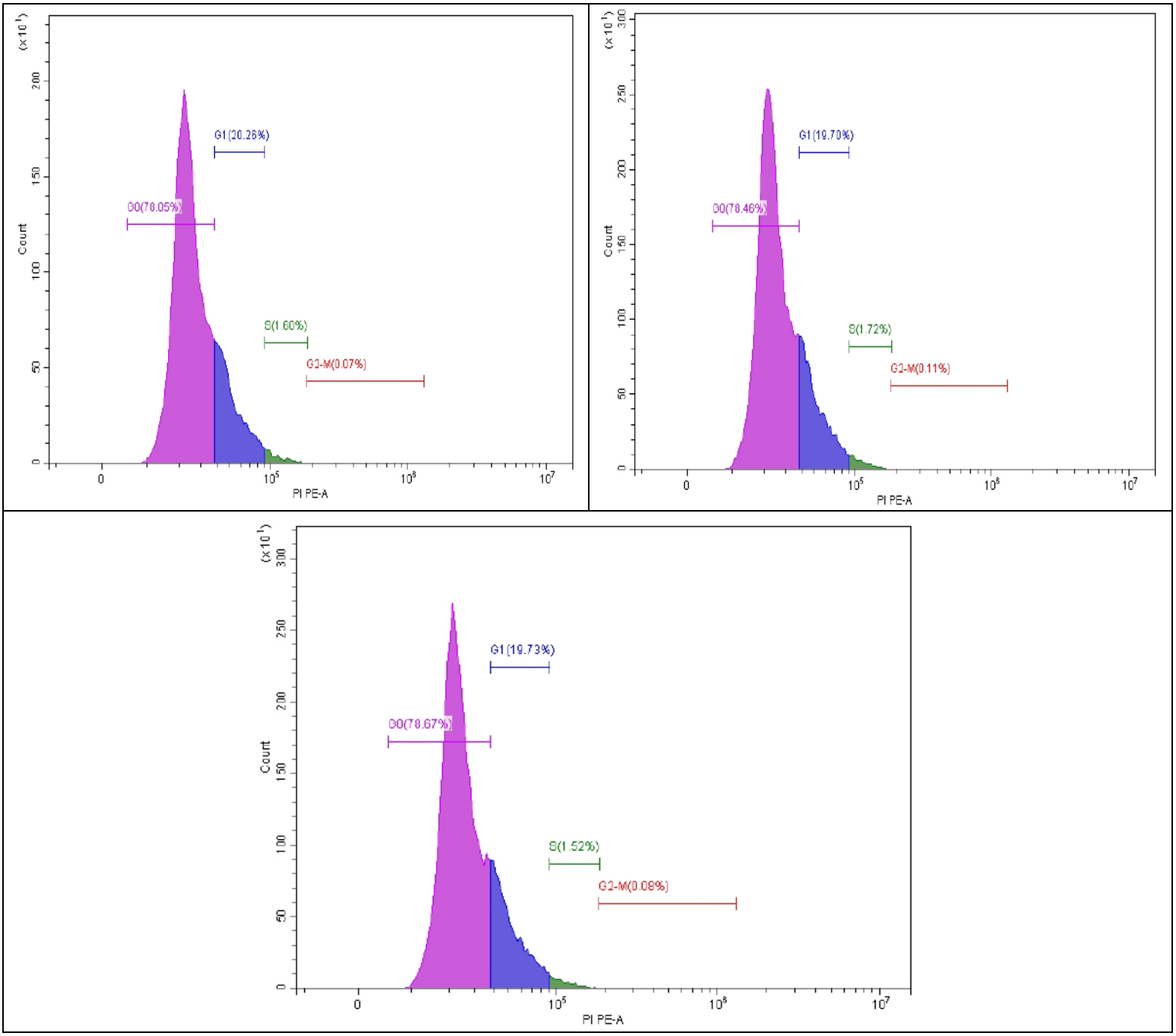

Figure S38. the triplicate experiments of cell cycle analysis histogram of compound **5g**-treated control A375 cancer cell line.

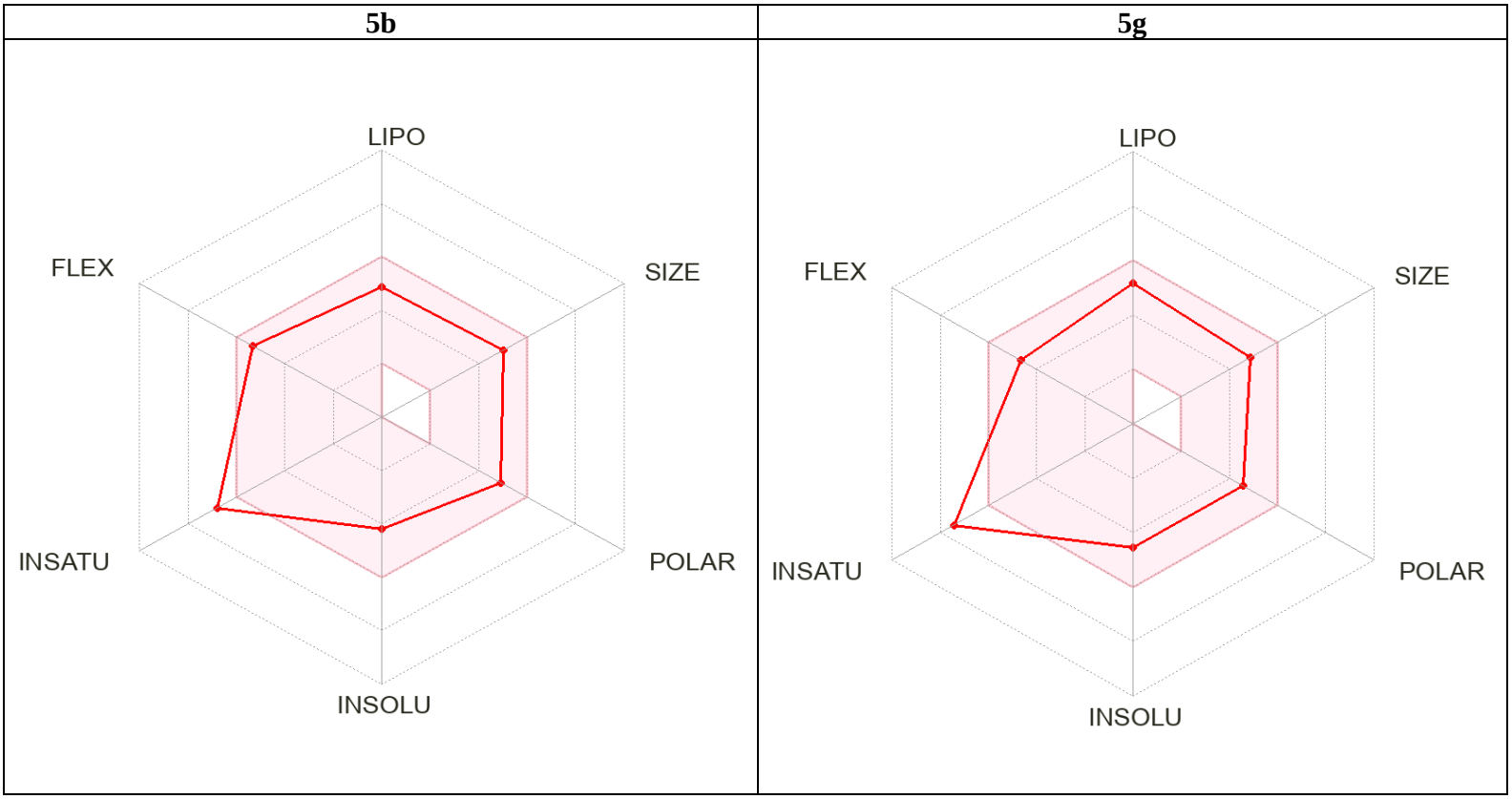

Figure S39. Radar bioavailability for studied compounds in which the area in pink displays specific property optimal range. (LIPO = lipophilicity expressed as XLOGP3 (range from -0.7 to 5.0). SIZE= size expressed as molecular weight (range from 150 g/mol to 500 g/mol). POLAR = polarity expressed as TPSA (topological polar surface area) (range from 20 $Å^2$ to 130 $Å^2$). INSOLU = water insolubility by log S (ESOL) (range from -6 to 0). INSATU = insaturation expressed as for each carbons fraction in sp3 hybridization (range from 0.25 to 1). FLEX = flexibility, expressed as number of rotatable bonds (range from 0 to 9).

Materials and Methods

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

The antitumor activities of compounds (**5a-l**) 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 **5a-l** 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. Cytotoxic inhibitory concentration 50 (IC_{50}) evaluation against HNO97, HCT_{116}, A375, and HEPG2 cancer cell lines

The antitumor activities of compounds (**5a-l**) against HNO97, HCT116, A375, and HEPG2 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 **5a-l** 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. EGFR inhibitory assay (ELISA Kit assay)

This ELISA kit uses the Sandwich-ELISA principle. The micro-ELISA plate provided in this kit has been pre-coated with an antibody specific to Human EGFR. Samples (or Standards) (**5b**, **5f**, **5g**, and **5j**) are added to the micro-ELISA plate wells and combined with the specific antibody. Then a biotinylated detection antibody specific for Human EGFR and Avidin-Horseradish Peroxidase (HRP) conjugate are added successively to each micro plate well and incubated. Free components are washed away. The substrate solution is added to each well. Only those wells that contain Human EGFR, biotinylated detection antibody and Avidin-HRP conjugate will appear blue in color. The enzyme-substrate reaction is terminated by the addition of stop solution and the color turns yellow. The optical density (OD) is measured spectrophotometrically at a wavelength of 450 ± 2 nm. The OD value is proportional to the concentration of Human EGFR. You can calculate the concentration of Human EGFR in the samples by comparing the OD of the samples to the standard curve.

Assay procedure

1. Determine wells for diluted standard, blank and sample. Add 100 μL each dilution of standard, blank and sample into the appropriate wells (It is recommended that all samples and standards be assayed in duplicate. It is recommended to determine the dilution ratio of samples through preliminary experiments or technical support recommendations). Cover the plate with the sealer provided in the kit. Incubate for 90 min at 37 ℃. Note: solutions should be added to the bottom of the micro-ELISA plate well, avoid touching the inside wall and causing foaming as much as possible.

2. Decant the liquid from each well, do not wash. Immediately add 100 μL of Biotinylated Detection Ab working solution to each well. Cover the plate with a new sealer. Incubate for 1 h at 37 ℃.

3. Decant the solution from each well add 350 μL of wash buffer to each well. Soak for 1 min and aspirate or decant the solution from each well and pat it dry against clean absorbent paper. Repeat this wash step 3 times.

Note: a microplate washer can be used in this step and other wash steps. Make the tested strips in use immediately after the wash step. Do not allow wells to be dry.

4. Add 100 μL of HRP Conjugate working solution to each well. Cover the plate with a new sealer. Incubate for 30 min at 37 ℃.

5. Decant the solution from each well, repeat the wash process for 5 times as conducted in step 3.
6. Add 90 μL of Substrate Reagent to each well. Cover the plate with a new sealer. Incubate for about 15 min at 37 °C. Protect the plate from light.
Note: the reaction time can be shortened or extended according to the actual color change, but not more than 30 min. Preheat the Microplate Reader for about 15 min before OD measurement.
7. Add 50 μL of Stop Solution to each well.
Note: adding the stop solution should be done in the same order as the substrate solution.
8. Determine the optical density (OD value) of each well at once with a micro-plate reader set to 450 nm.

SI4. Telomerase inhibitory assay (ELISA Kit assay)

This ELISA kit uses the Sandwich-ELISA principle. The micro-ELISA plate provided in this kit has been pre-coated with an antibody specific to Human TE. Samples (or Standards) (**5b**, **5f**, **5g**, and **5j**) are added to the micro-ELISA plate wells and combined with the specific antibody. Then a biotinylated detection antibody specific for Human TE and Avidin-Horseradish Peroxidase (HRP) conjugate are added successively to each micro plate well and incubated. Free components are washed away. The substrate solution is added to each well. Only those wells that contain Human TE, biotinylated detection antibody and Avidin-HRP conjugate will appear blue in color. The enzyme-substrate reaction is terminated by the addition of stop solution and the color turns yellow. The optical density (OD) is measured spectrophotometrically at a wavelength of 450 ± 2 nm. The OD value is proportional to the concentration of Human TE. You can calculate the concentration of Human TE in the samples by comparing the OD of the samples to the standard curve.

Assay procedure

1. Determine wells for diluted standard, blank and sample. Add 100 μL each dilution of standard, blank and sample into the appropriate wells (It is recommended that all samples and standards be assayed in duplicate. It is recommended to determine the dilution ratio of samples through preliminary experiments or technical support recommendations). Cover the plate with the sealer provided in the kit. Incubate for 90 min at 37 ℃.
Note: solutions should be added to the bottom of the micro-ELISA plate well, avoid touching the inside wall and causing foaming as much as possible.

2. Decant the liquid from each well, do not wash. Immediately add 100 μL of Biotinylated Detection Ab working solution to each well. Cover the plate with a new sealer. Incubate for 1 h at 37 °C.

3. Decant the solution from each well add 350 μL of wash buffer to each well. Soak for 1 min and aspirate or decant the solution from each well and pat it dry against clean absorbent paper. Repeat this wash step 3 times.

Note: a microplate washer can be used in this step and other wash steps. Make the tested strips in use immediately after the wash step. Do not allow wells to be dry.

4. Add 100 μL of HRP Conjugate working solution to each well. Cover the plate with a new sealer. Incubate for 30 min at 37 °C.

5. Decant the solution from each well, repeat the wash process for 5 times as conducted in step 3.

6. Add 90 μL of Substrate Reagent to each well. Cover the plate with a new sealer. Incubate for about 15 min at 37 °C. Protect the plate from light.

Note: the reaction time can be shortened or extended according to the actual color change, but not more than 30 min. Preheat the Microplate Reader for about 15 min before OD measurement.

7. Add 50 μL of Stop Solution to each well. Note: adding the stop solution should be done in the same order as the substrate solution. 8. Determine the optical density (OD value) of each well at once with a micro-plate reader set to 450 nm.

SI5. Assay of the apoptosis-related proteins (Enzyme-linked Immunosorbent assay)

The microplate provided in this kit has been pre-coated with an antibody specific to Caspases 3, 8, and 9, CDK-2, CDK-4, and CDK-6. Standards or samples are then added to the appropriate microplate wells with a biotin-conjugated antibody specific to Caspases 3, 8, and 9, CDK-2, CDK-4, and CDK-6. 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, 8, and 9, CDK-2, CDK-4, and CDK-6, 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, 8, and 9, CDK-2, CDK-4, and CDK-6 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, 8, and 9, CDK-2, CDK-4, and CDK-6 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.

Assay procedure

1. Determine wells for diluted standard, blank, and sample (**5g**). 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 auto washer, 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 ^{o}C.
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 ^{o}C (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.

SI6. Cell cycle analysis

Human melanoma cancer cells (A375) were grown in six-well plates (each one contains 2x 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 (**5g**), 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

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