Supplementary Data

Design and Synthesis of Novel Chloropyridazine Hybrids as Promising Anticancer Agents Acting by Apoptosis Induction and PARP-1 Inhibition Through Molecular Hybridization Strategy

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⊤**:** Equal contribution.

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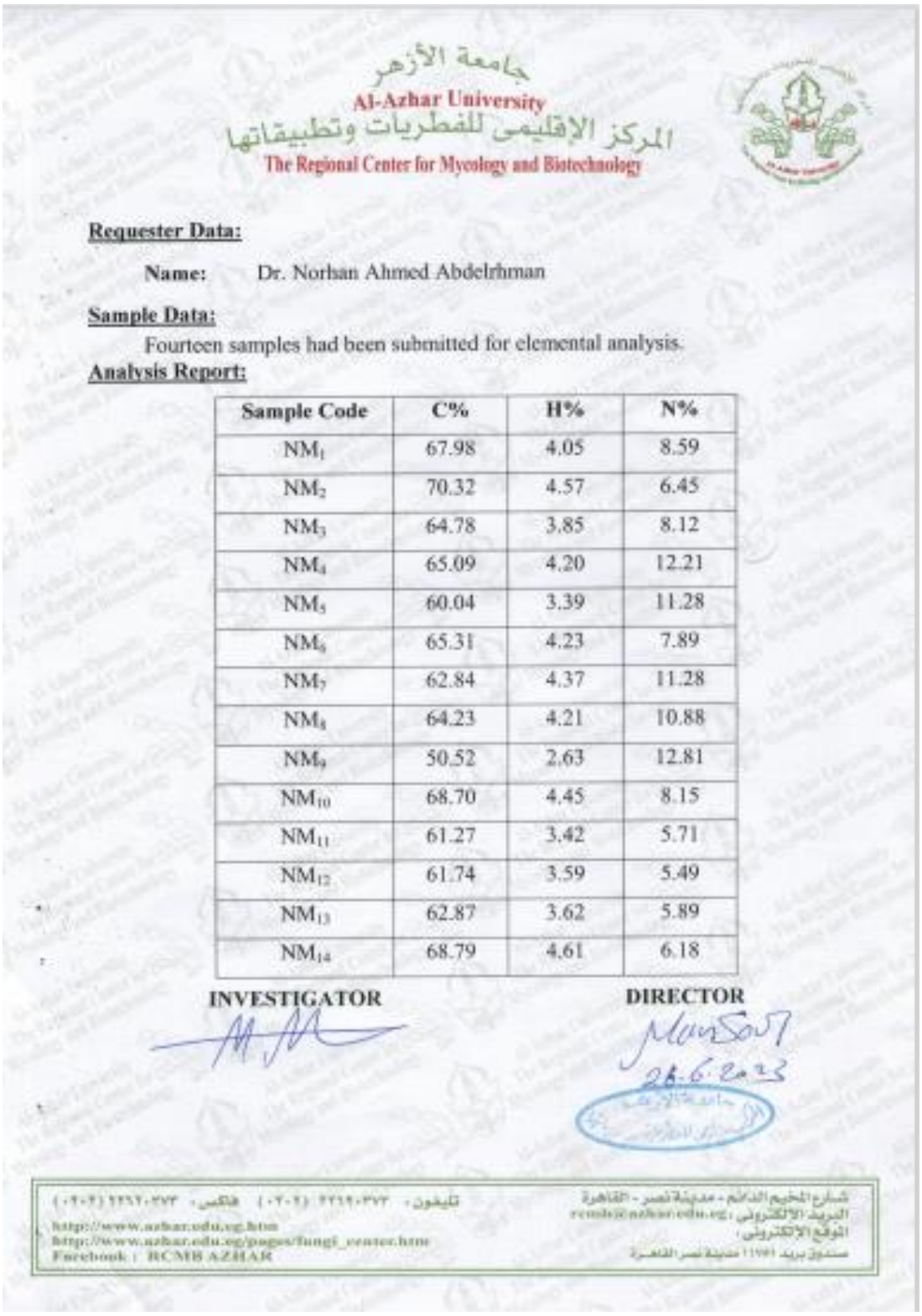
Yields and Elemental Analysis

Table S1. Yields and elemental analysis of target compounds (**3a-h, 4a-e,** and **5)**.

Old Code	New Code	%Yield	C%	H%	N%
NM1	**3a**	40	67.98	4.05	8.59
NM2	**4a**	40	70.32	4.57	6.45
NM3	**3f**	40	64.78	3.85	8.12
NM4	**3c**	45	65.09	4.20	12.21
NM5	**3h**	40	60.04	3.39	11.28
NM6	**3g**	50	65.31	4.23	7.89
NM7	**3e**	50	62.84	4.37	11.28
NM8	**3d**	50	64.23	4.21	10.88
NM9	**5**	45	50.52	2.63	12.81
NM10	**3b**	45	68.70	4.45	8.15
NM11	**4b**	45	61.27	3.42	5.71
NM12	**4d**	50	61.74	3.59	5.49
NM13	**4c**	45	62.87	3.62	5.89
NM14	**4e**	40	68.79	4.61	6.18



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المركز الإقليمى للفطريات وتطبيقاتها
The Regional Center for Mycology and Biotechnology

Requester Data:

Name: Dr. Norhan Ahmed Abdelrhman

Sample Data:

Fourteen samples had been submitted for elemental analysis.

Analysis Report:

Sample Code	C%	H%	N%
NM_1	67.98	4.05	8.59
NM_2	70.32	4.57	6.45
NM_3	64.78	3.85	8.12
NM_4	65.09	4.20	12.21
NM_5	60.04	3.39	11.28
NM_6	65.31	4.23	7.89
NM_7	62.84	4.37	11.28
NM_8	64.23	4.21	10.88
NM_9	50.52	2.63	12.81
NM_{10}	68.70	4.45	8.15
NM_{11}	61.27	3.42	5.71
NM_{12}	61.74	3.59	5.49
NM_{13}	62.87	3.62	5.89
NM_{14}	68.79	4.61	6.18

INVESTIGATOR

DIRECTOR

28.6.2023

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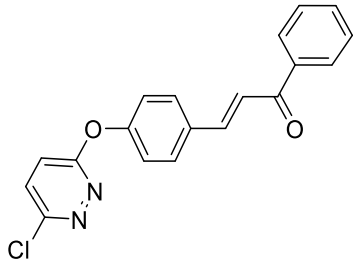
Figure S1. Elemental analysis of target compounds (**3a-h, 4a-e,** and **5)**.

^{1}H NMR, ^{13}C NMR, and Mass Spectra of Compounds (3a-h, 4a-e, and 5)

(E)-3-(4-((6-Chloropyridazin-3-yl)oxy)phenyl)-1-phenylprop-2-en-1-one (3a)



3a

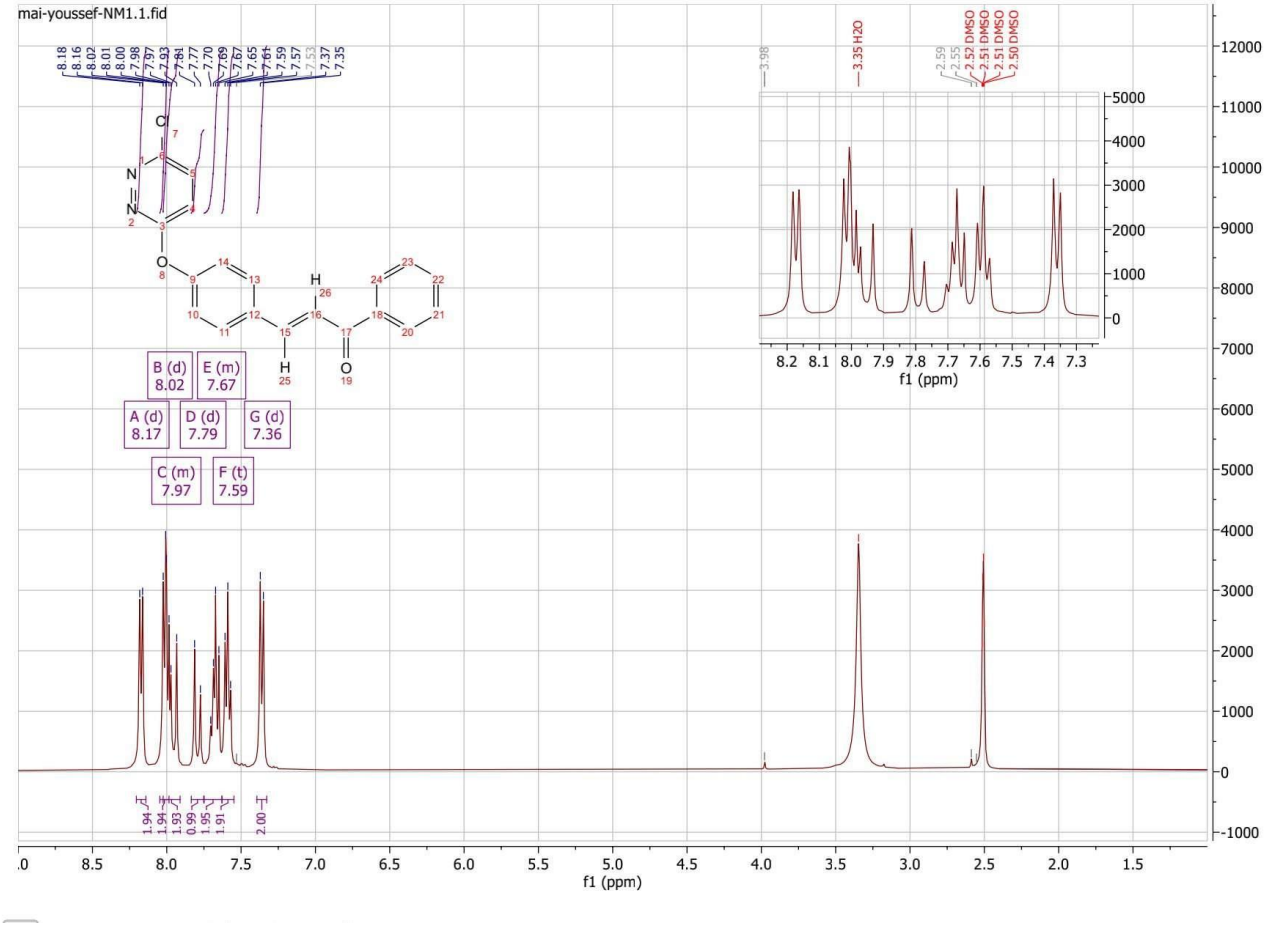


Figure S2. ^{1}H NMR (400 MHz, DMSO-d_6) spectrum of compound **3a**.

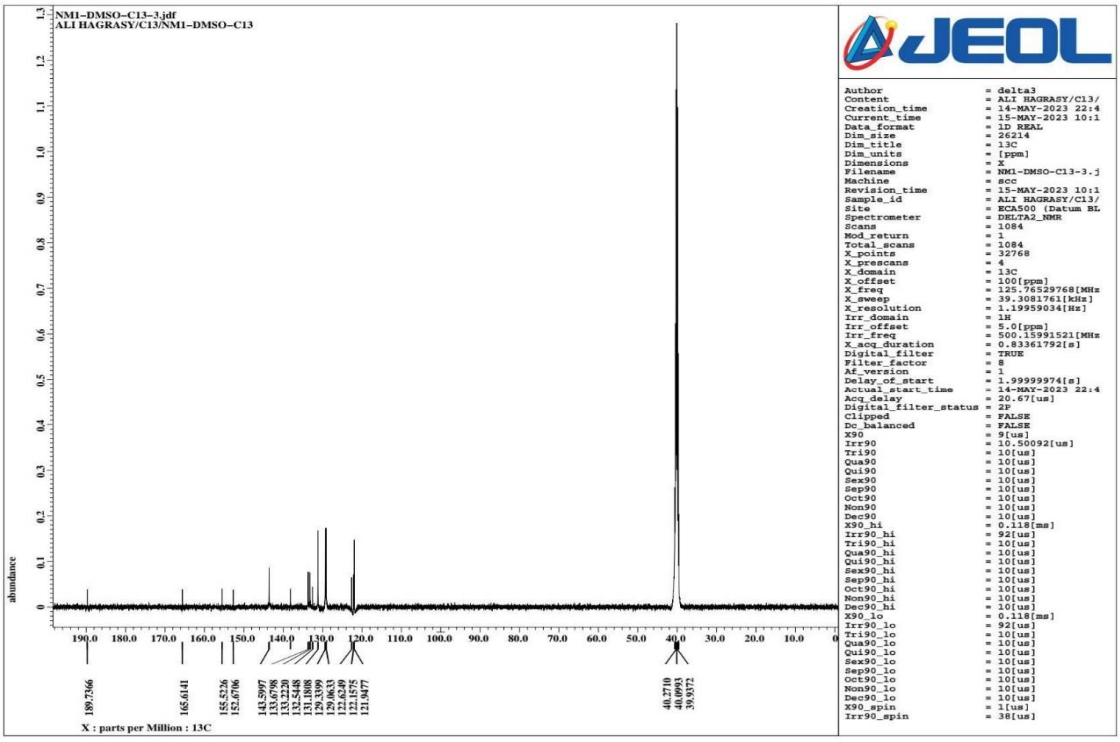


Figure S3. ^{13}C NMR (100 MHz, DMSO-d_6) spectrum of compound **3a**.

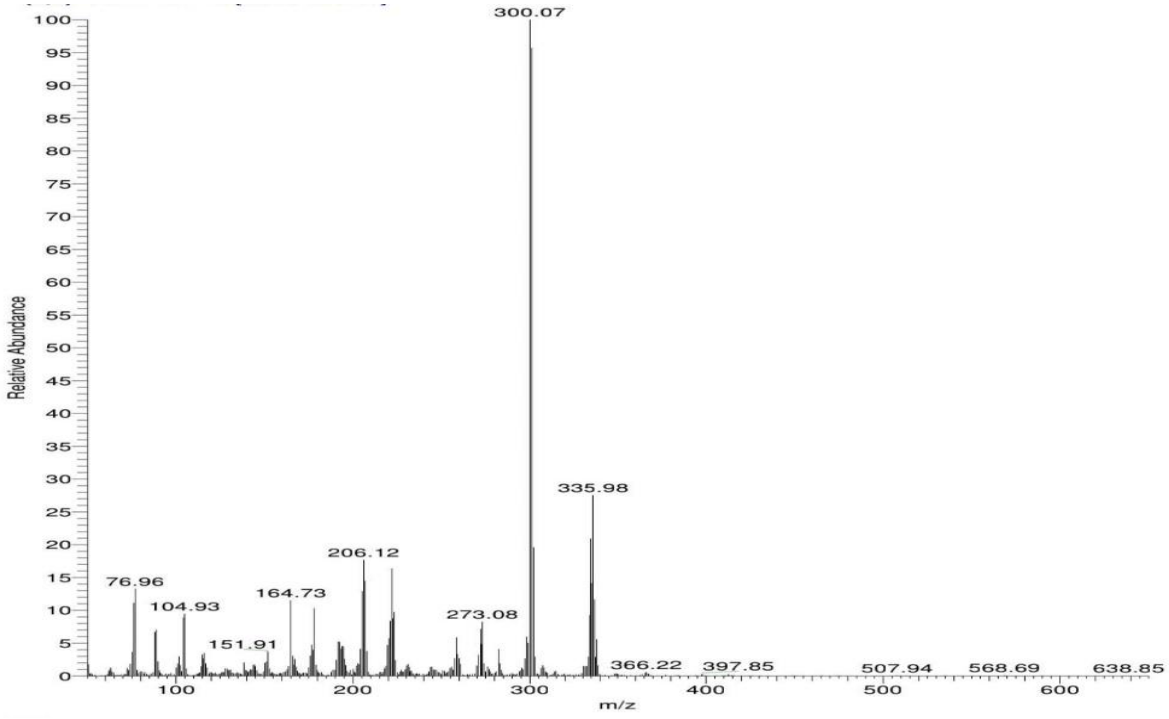
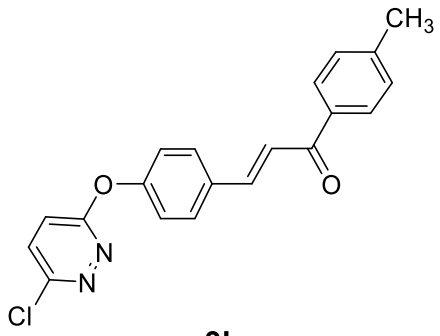


Figure S4. Mass spectrum of compound **3a**.

(E)-3-(4-((6-Chloropyridazin-3-yl)oxy)phenyl)-1-(p-tolyl)prop-2-en-1-one (3b)



3b

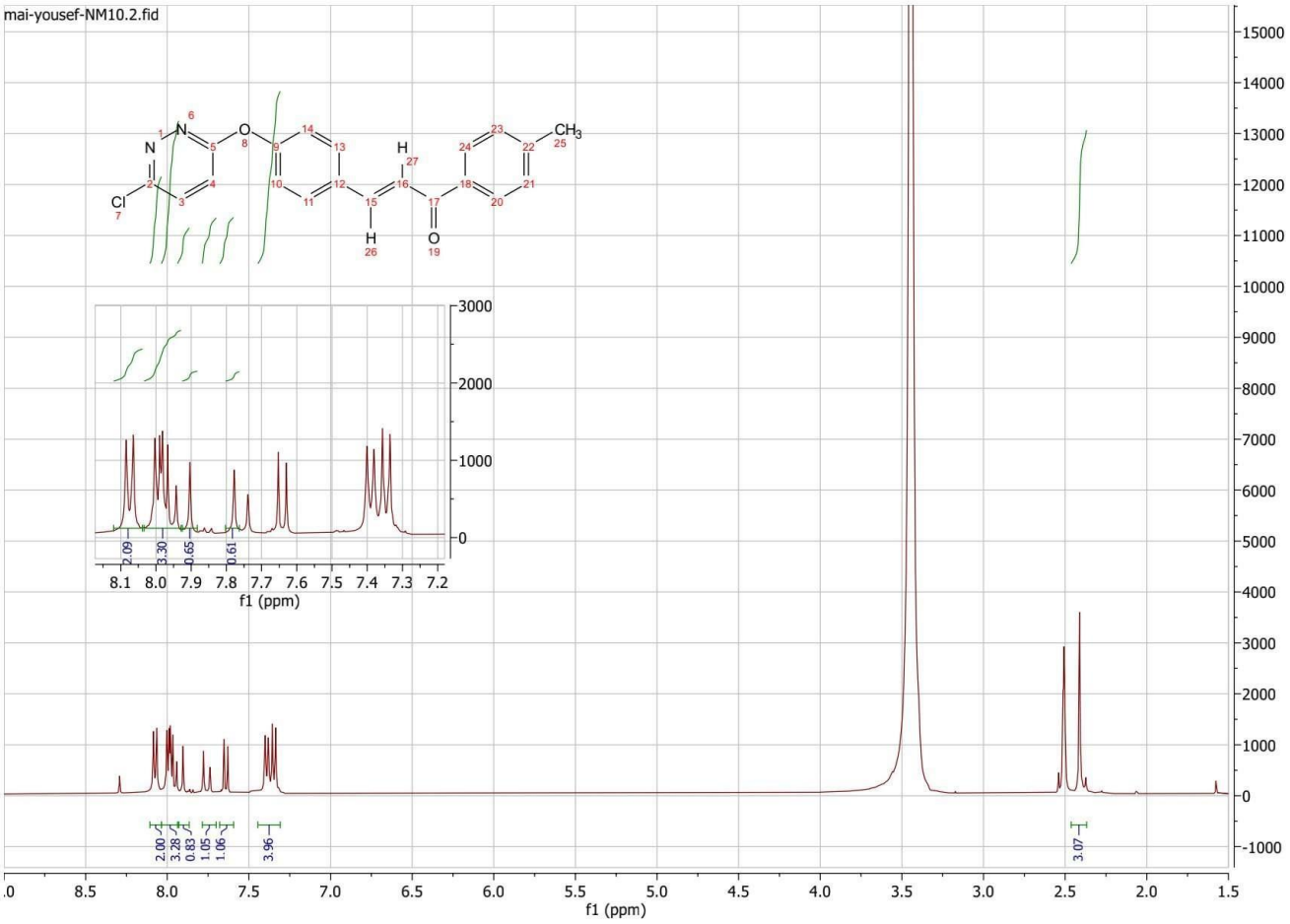


Figure S5. ^{1}H NMR (400 MHz, DMSO-d_6) spectrum of compound **3b**.

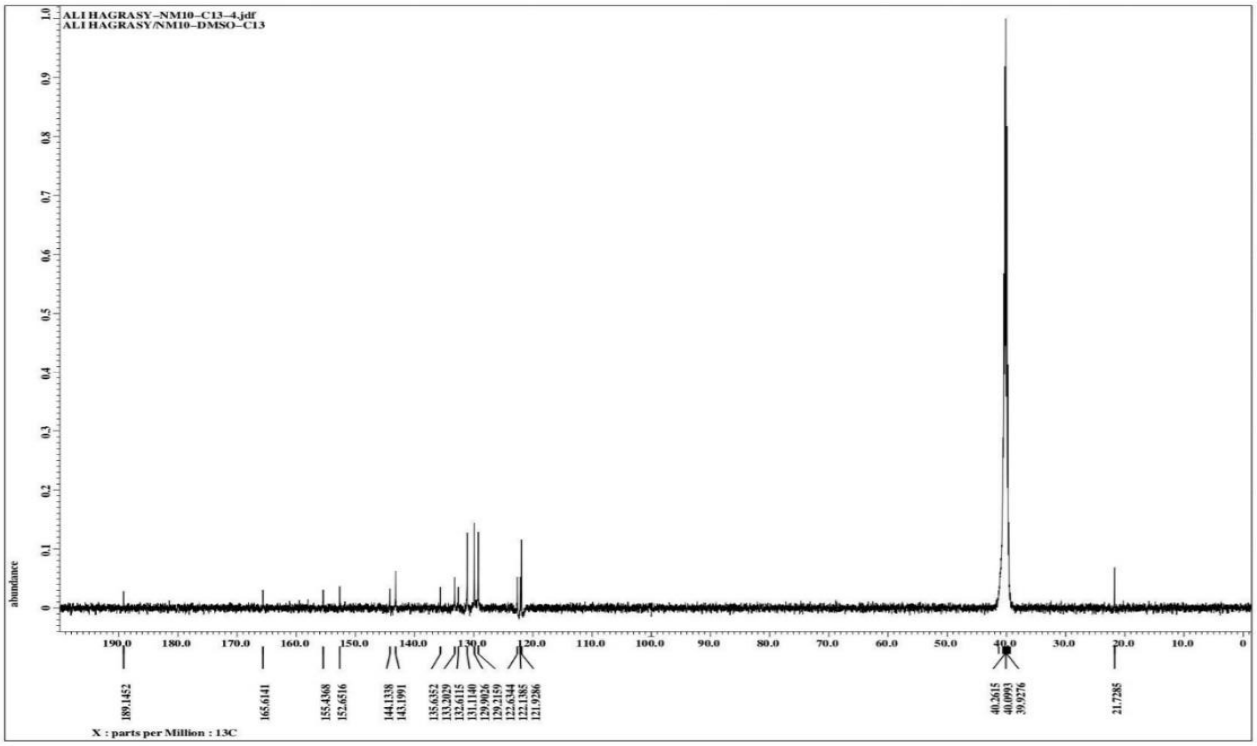


Figure S6. ^{13}C NMR (100 MHz, DMSO-d_6) spectrum of compound **3b**.

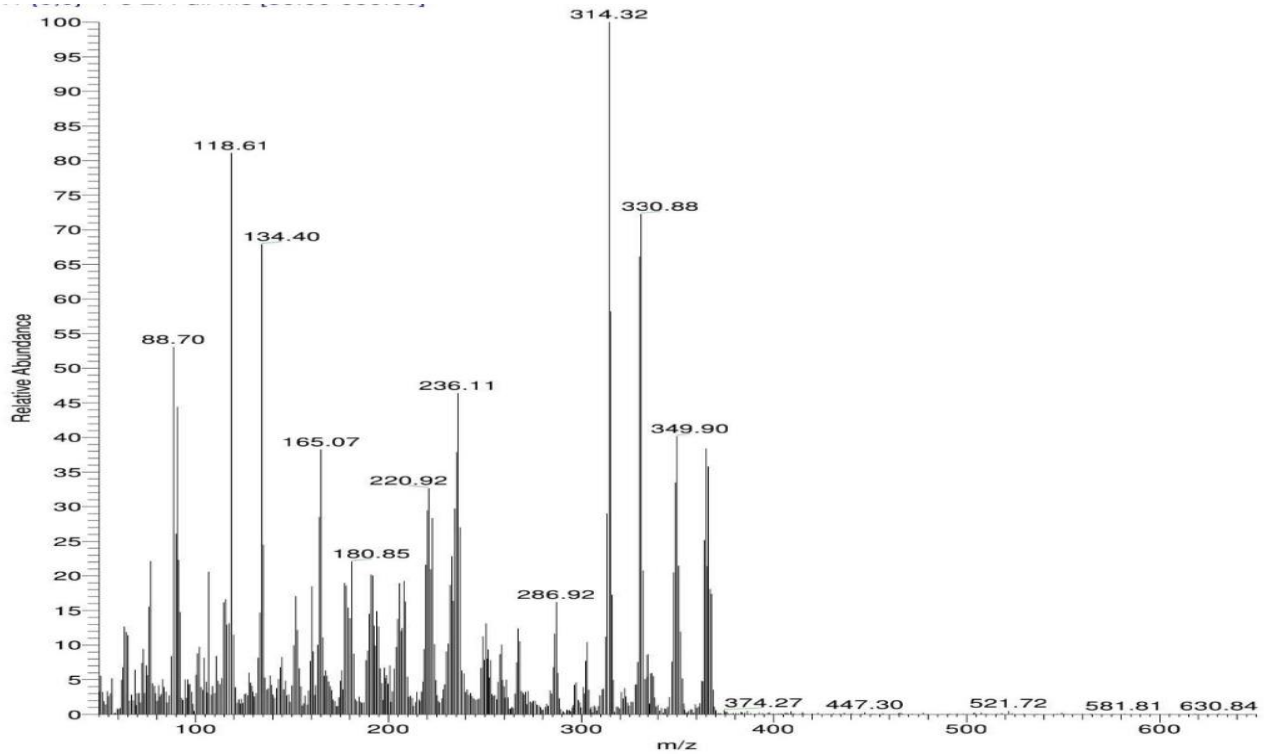
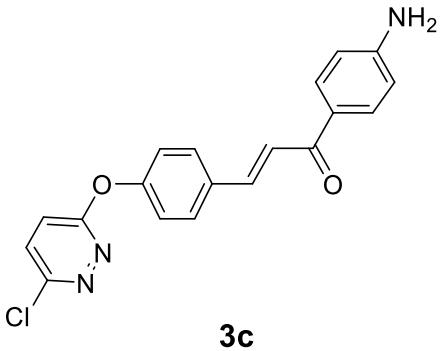


Figure S7. Mass spectrum of compound **3b**.

(E)-1-(4-Aminophenyl)-3-(4-((6-chloropyridazin-3-yl)oxy)phenyl)prop-2-en-1-one (3c)



3c

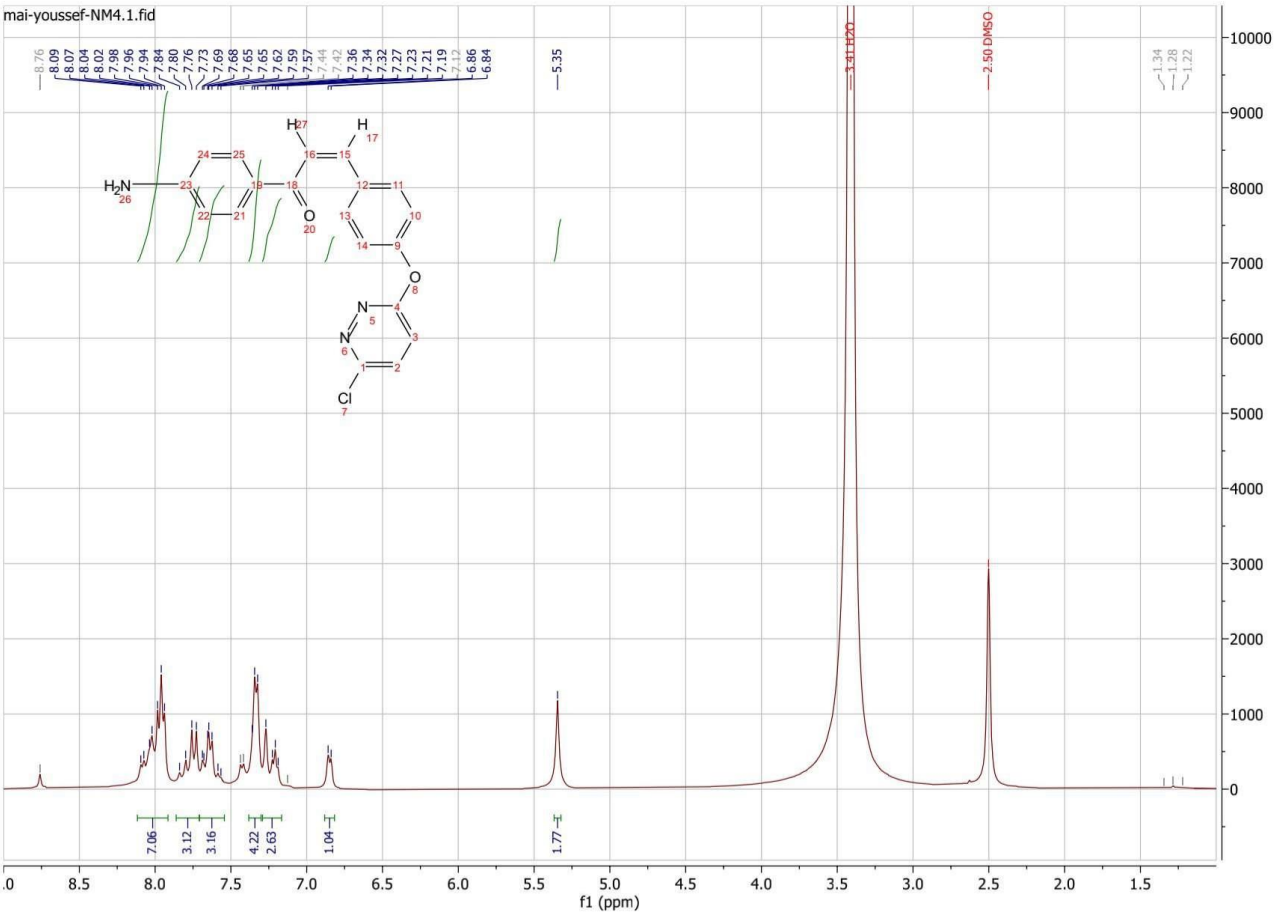


Figure S8. ^{1}H NMR (400 MHz, DMSO-d_6) spectrum of compound **3c**.

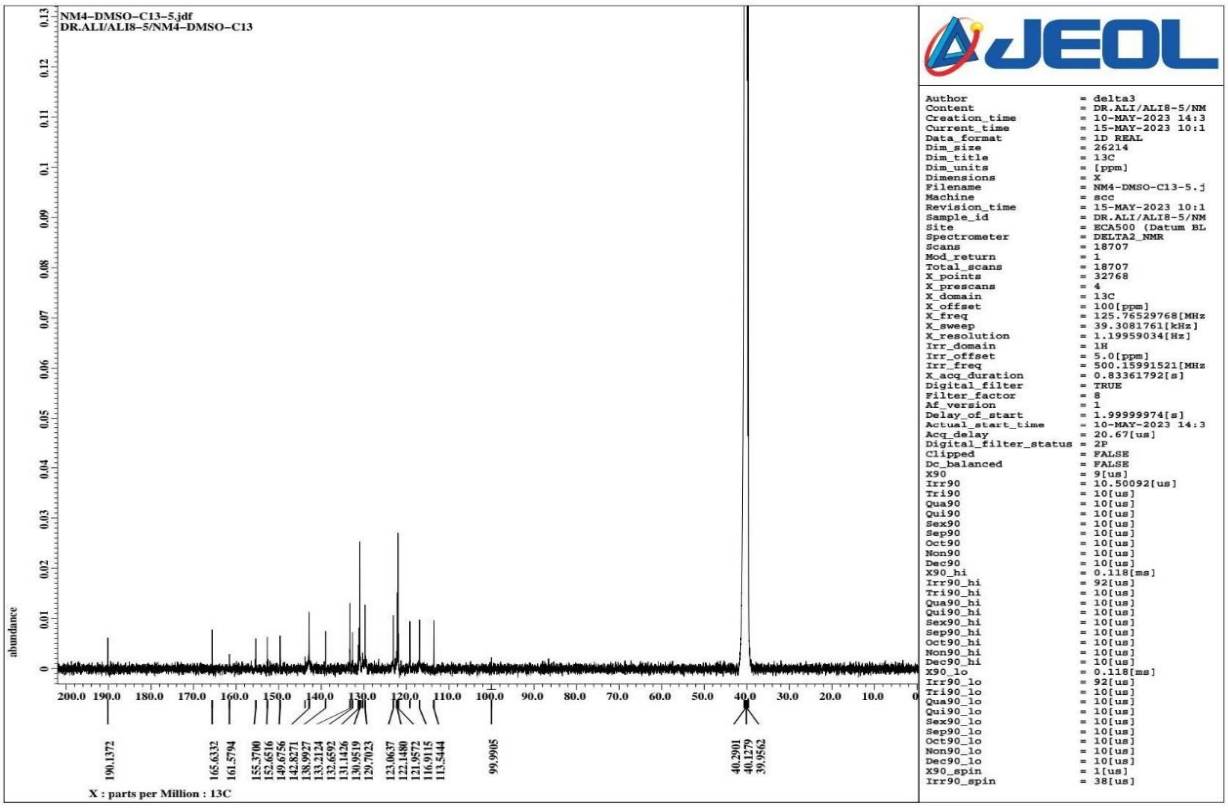


Figure S9. ^{13}C NMR (100 MHz, DMSO-d_6) spectrum of compound **3c**.

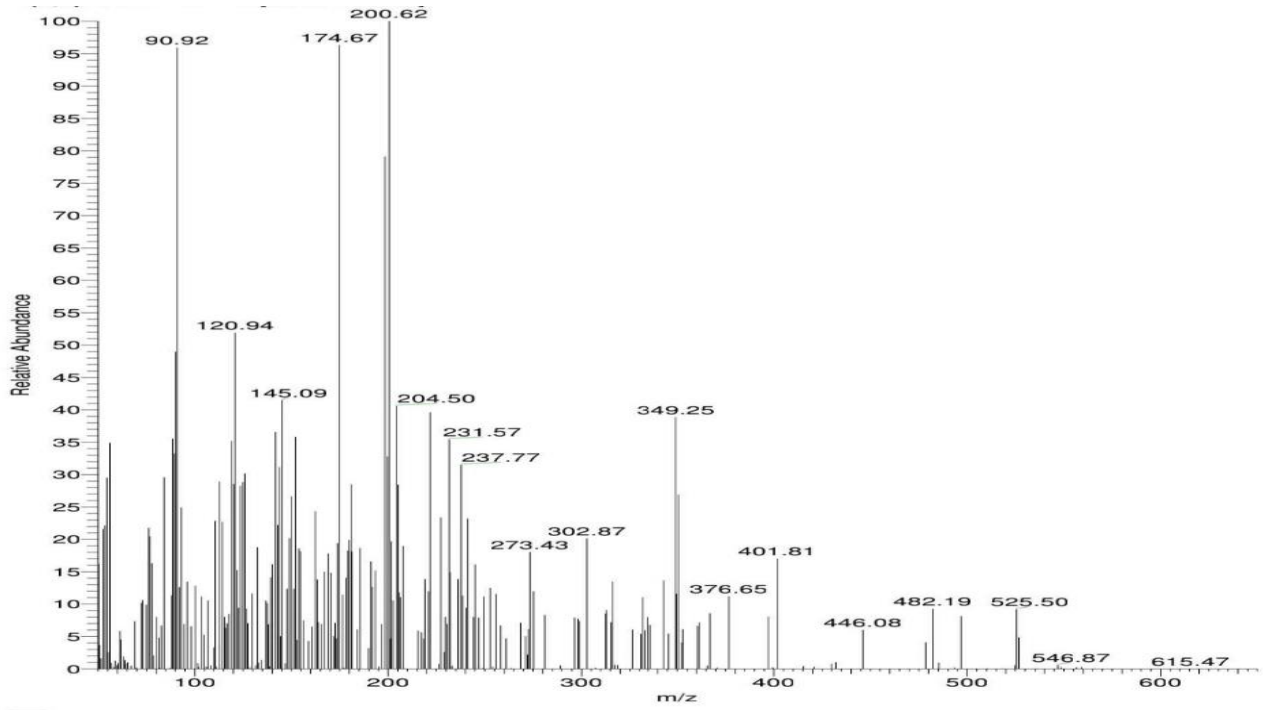
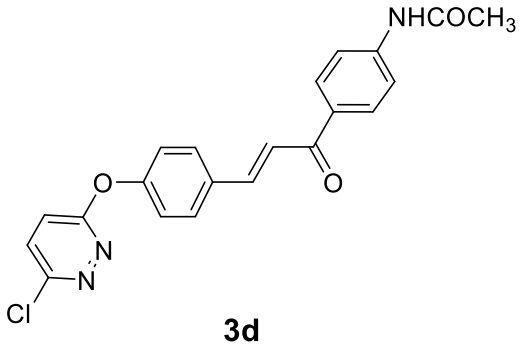


Figure S10. Mass spectrum of compound **3c**.

(E)-3-(4-((6-Chloropyridazin-3-yl)oxy)phenyl)-1-(4-(acetamido)phenyl)prop-2-en-1-one (3d)



3d

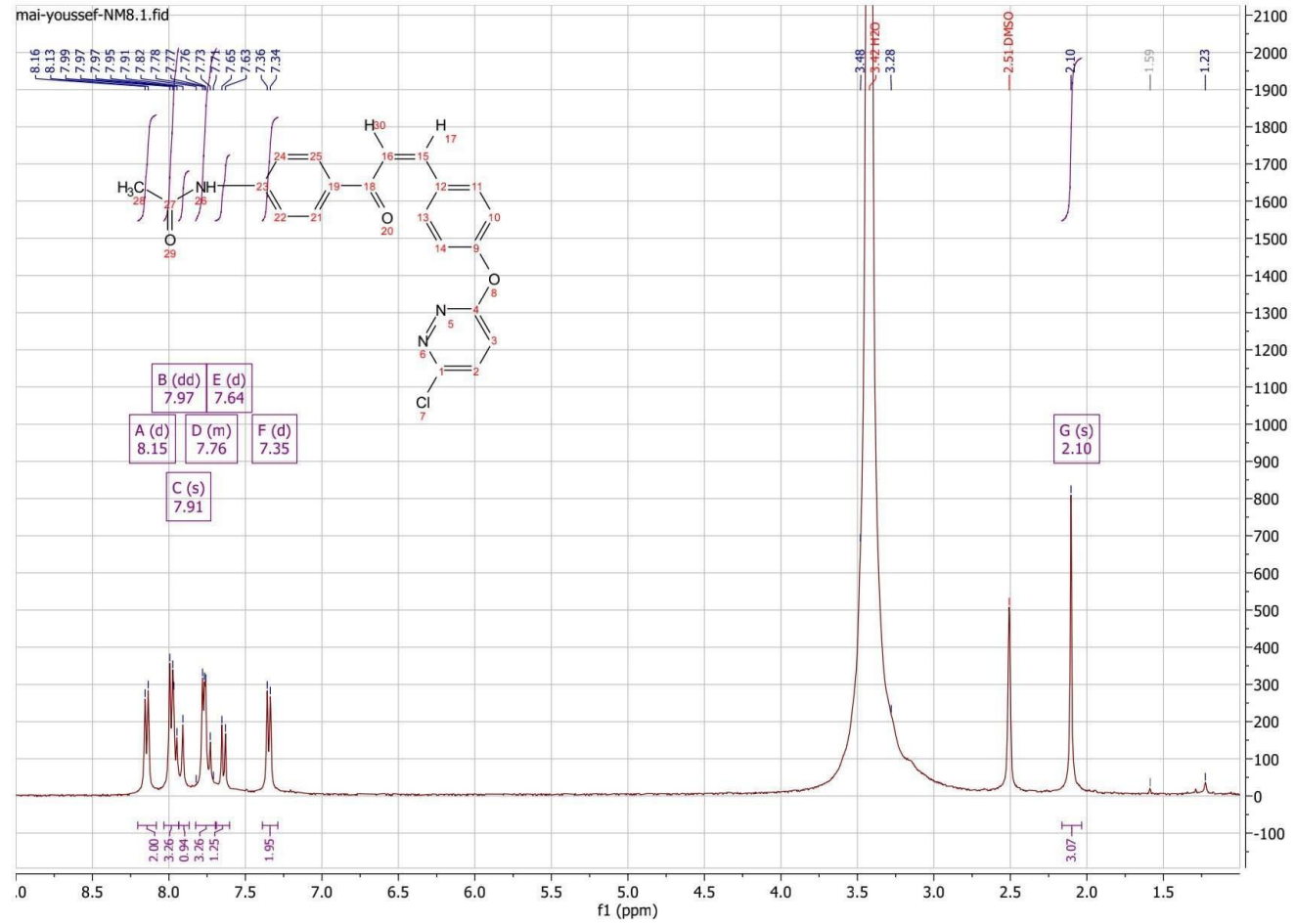


Figure S11. ^{1}H NMR (400 MHz, DMSO-d_6) spectrum of compound **3d**.

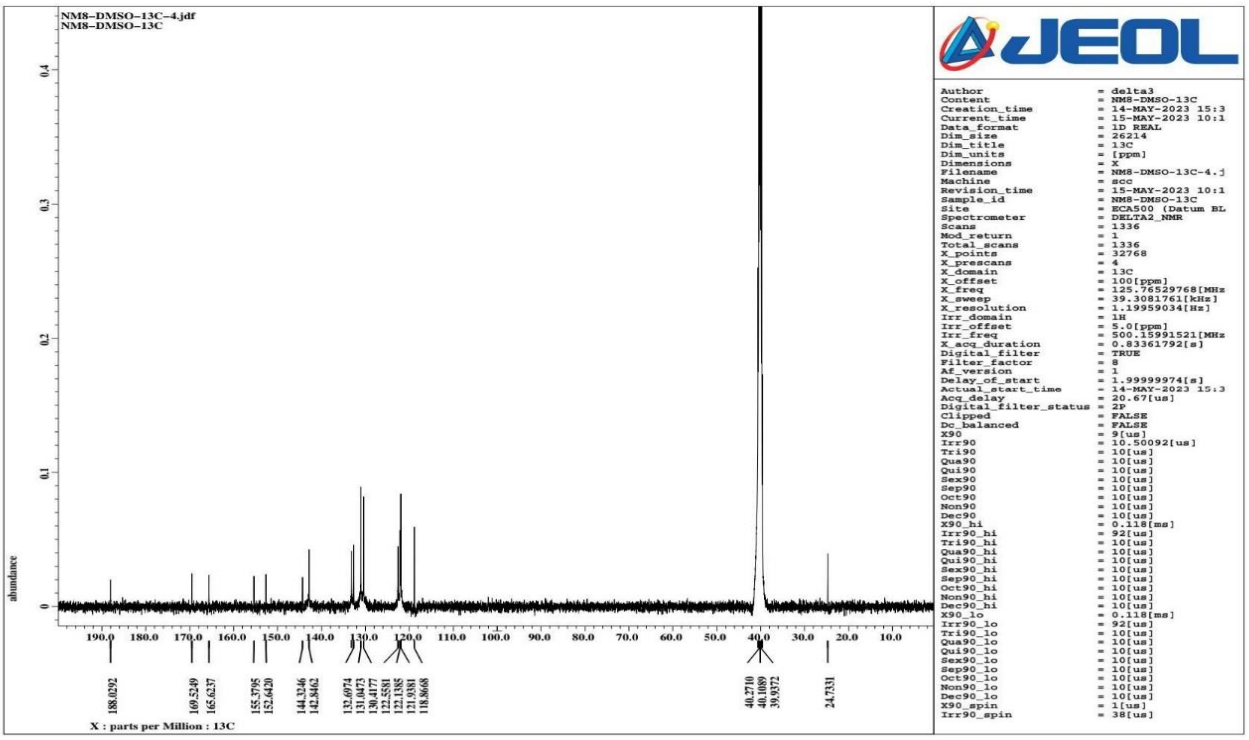


Figure S12. ^{13}C NMR (100 MHz, DMSO-d_6) spectrum of compound **3d**.

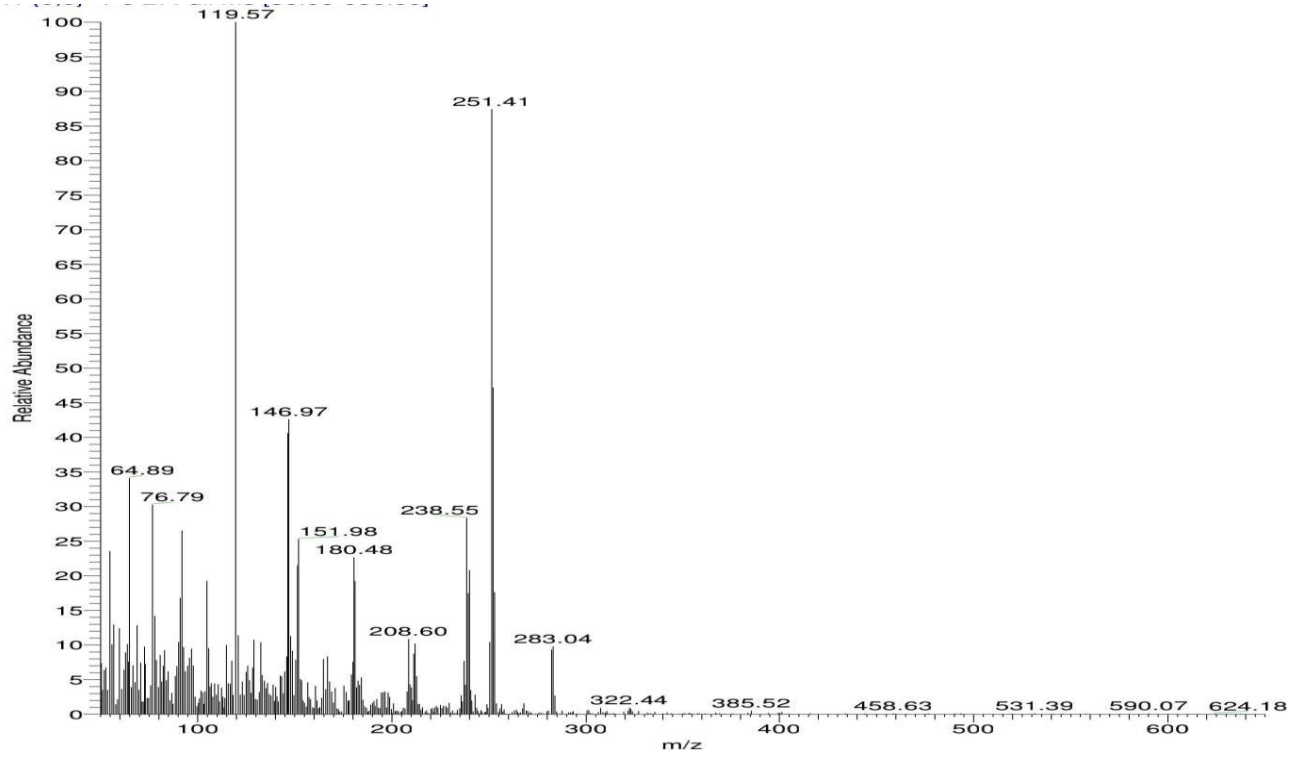
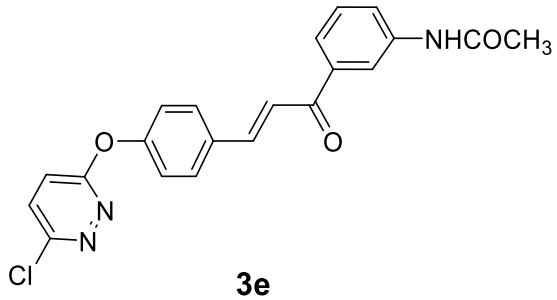


Figure S13. Mass spectrum of compound **3d**.

(E)-N-(3-(3-(4-((6-Chloropyridazin-3-yl)oxy)phenyl)acryloyl)phenyl)acetamide (3e)



3e

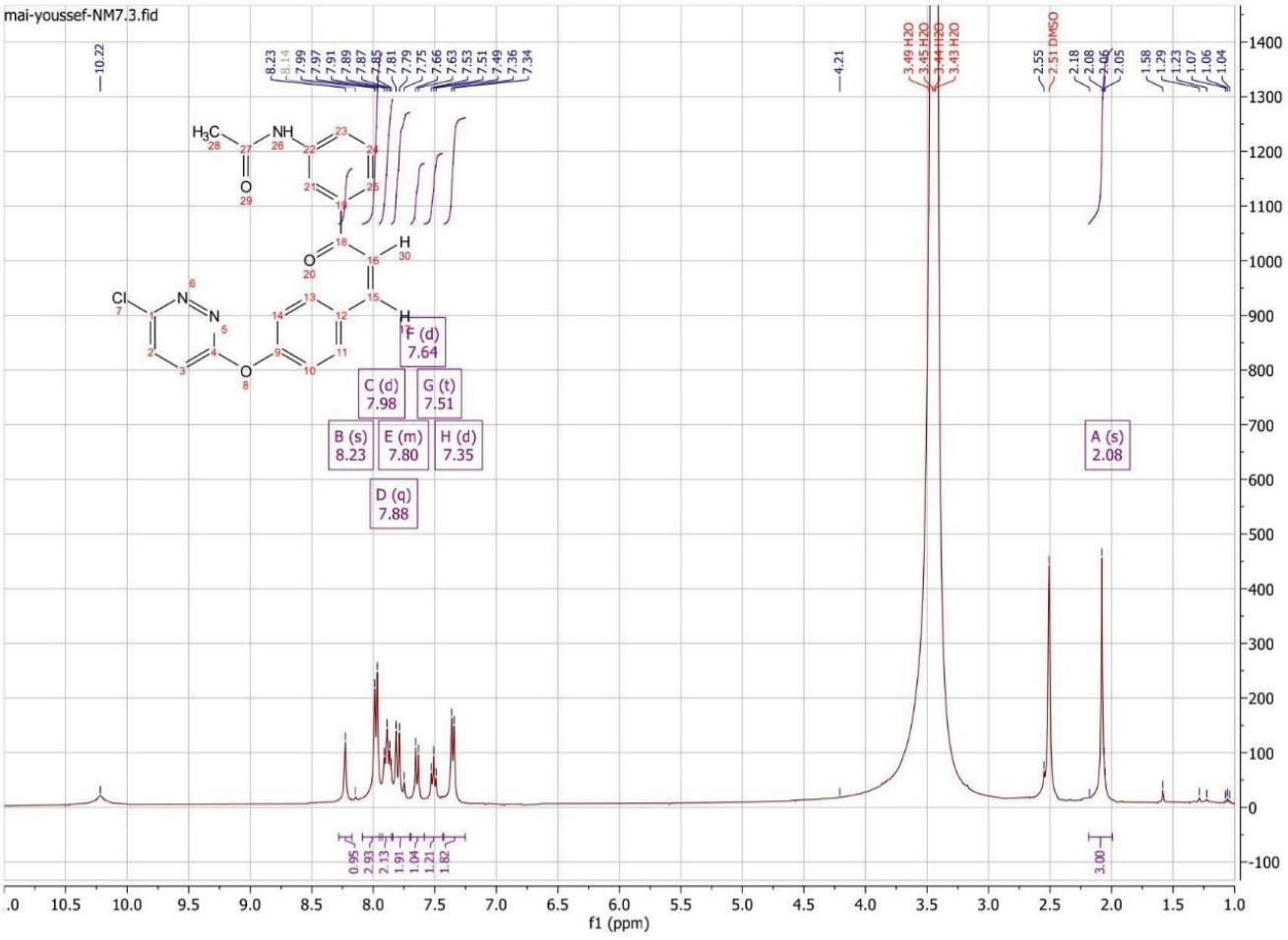


Figure S14. ^{1}H NMR (400 MHz, DMSO-d_6) spectrum of compound **3e**.

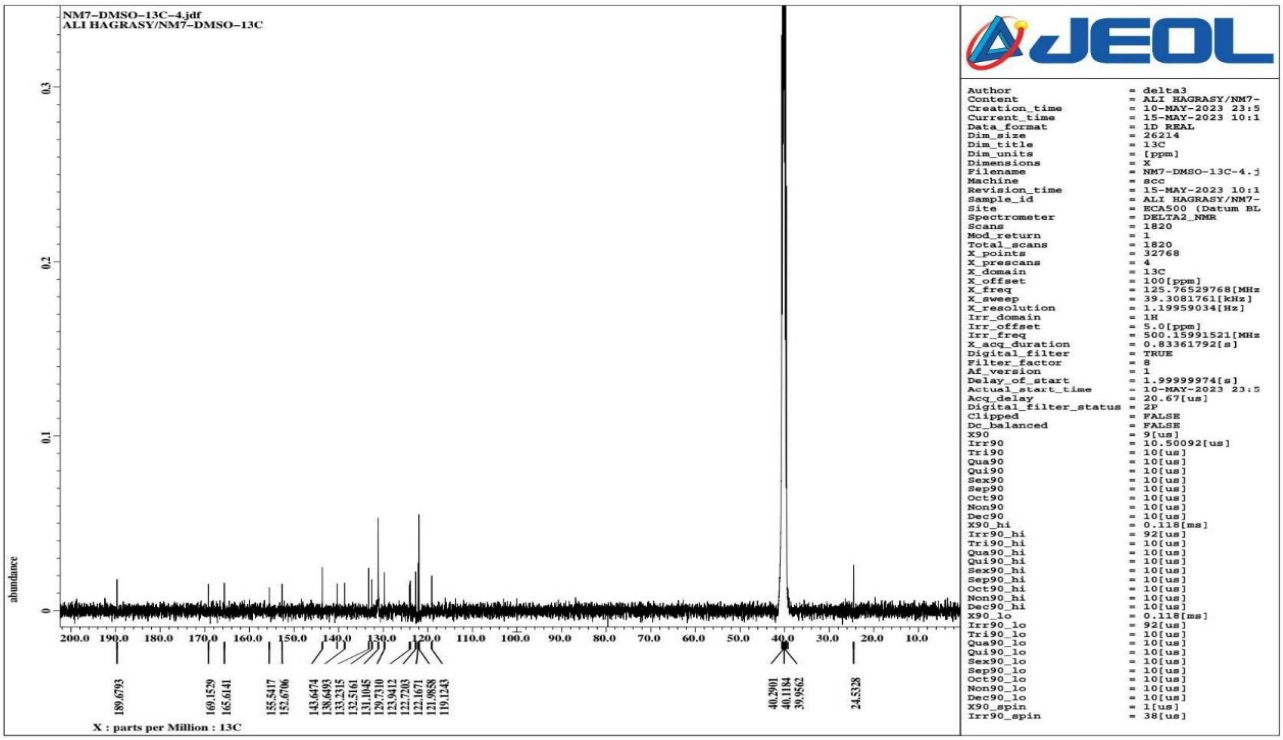


Figure S15. ^{13}C NMR (100 MHz, DMSO-d_6) spectrum of compound **3e**.

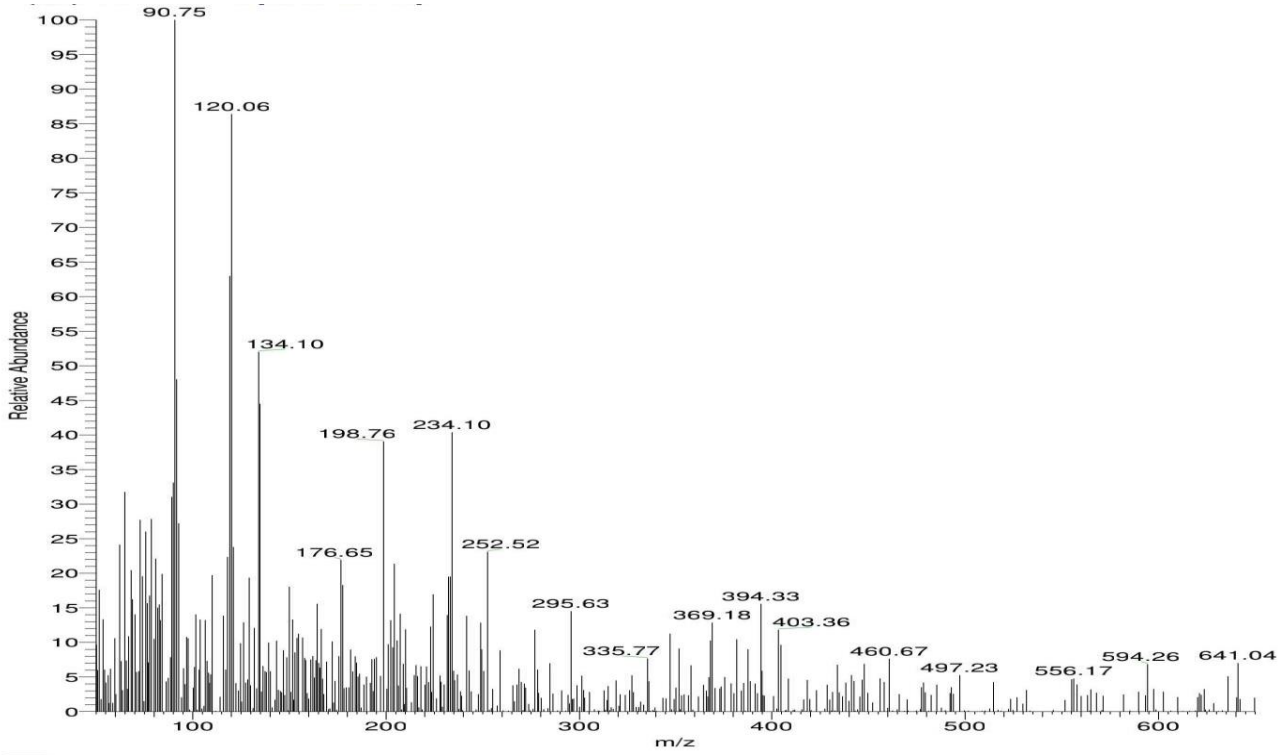
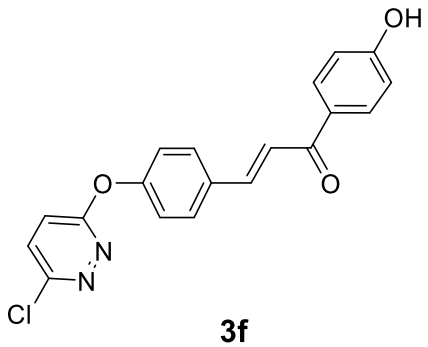


Figure S16. Mass spectrum of compound **3e**.

(E)-3-(4-((6-Chloropyridazin-3-yl)oxy)phenyl)-1-(4-hydroxyphenyl)prop-2-en-1-one (3f)



3f

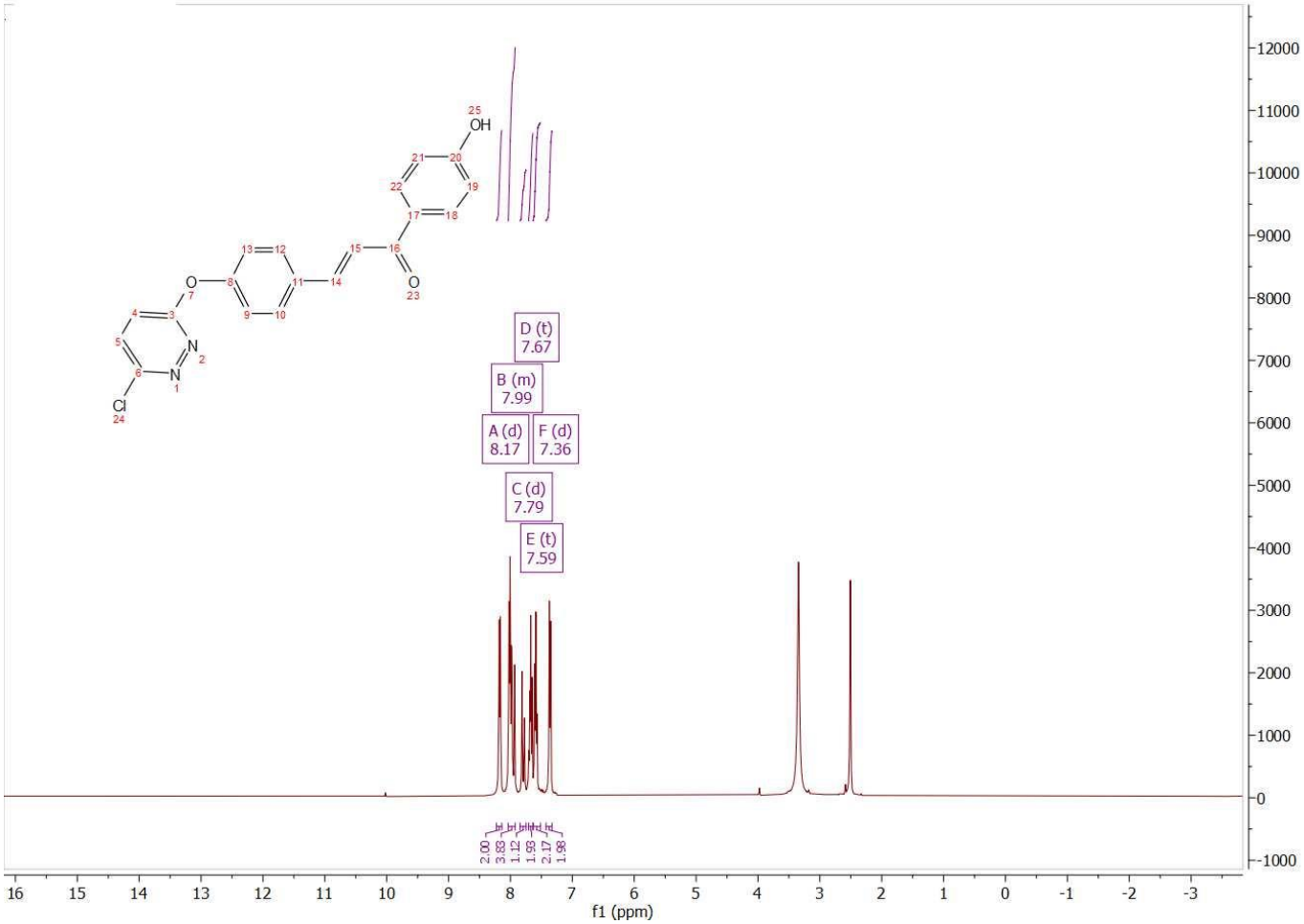


Figure S17. ^{1}H NMR (400 MHz, DMSO-d_6) spectrum of compound **3f**.

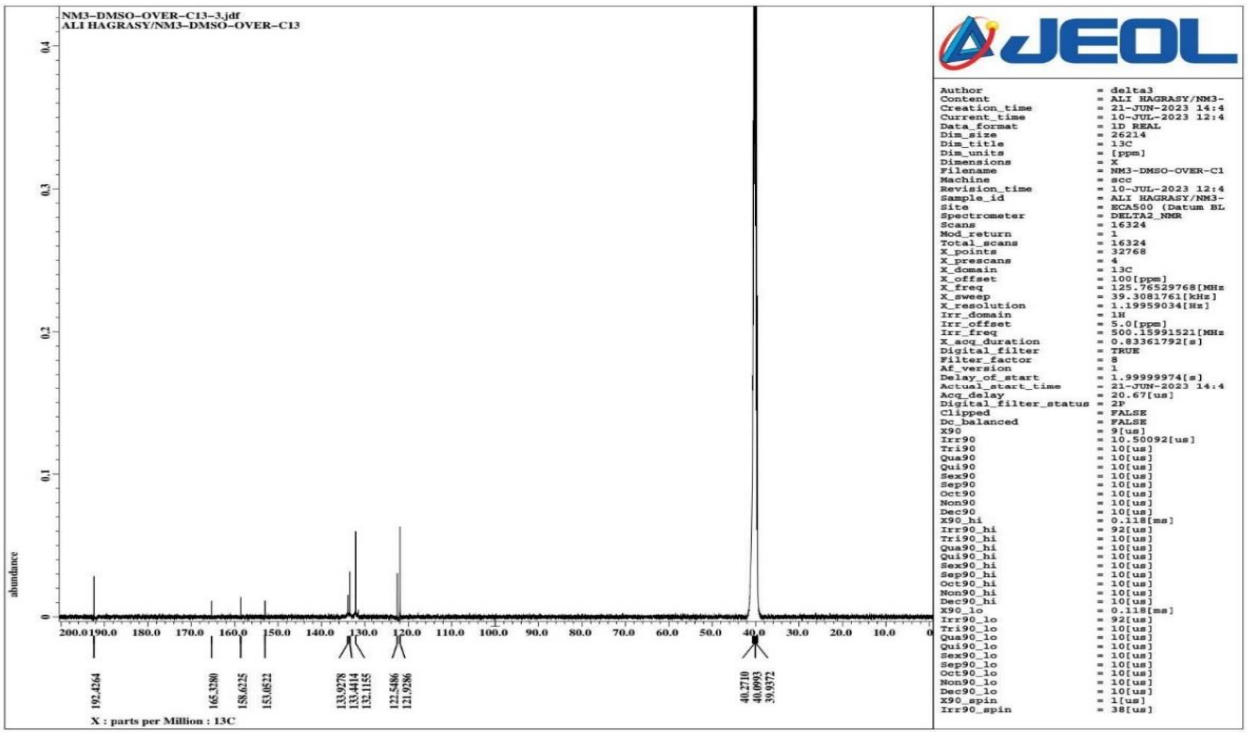


Figure S18. ^{13}C NMR (100 MHz, DMSO-d_6) spectrum of compound **3f**.

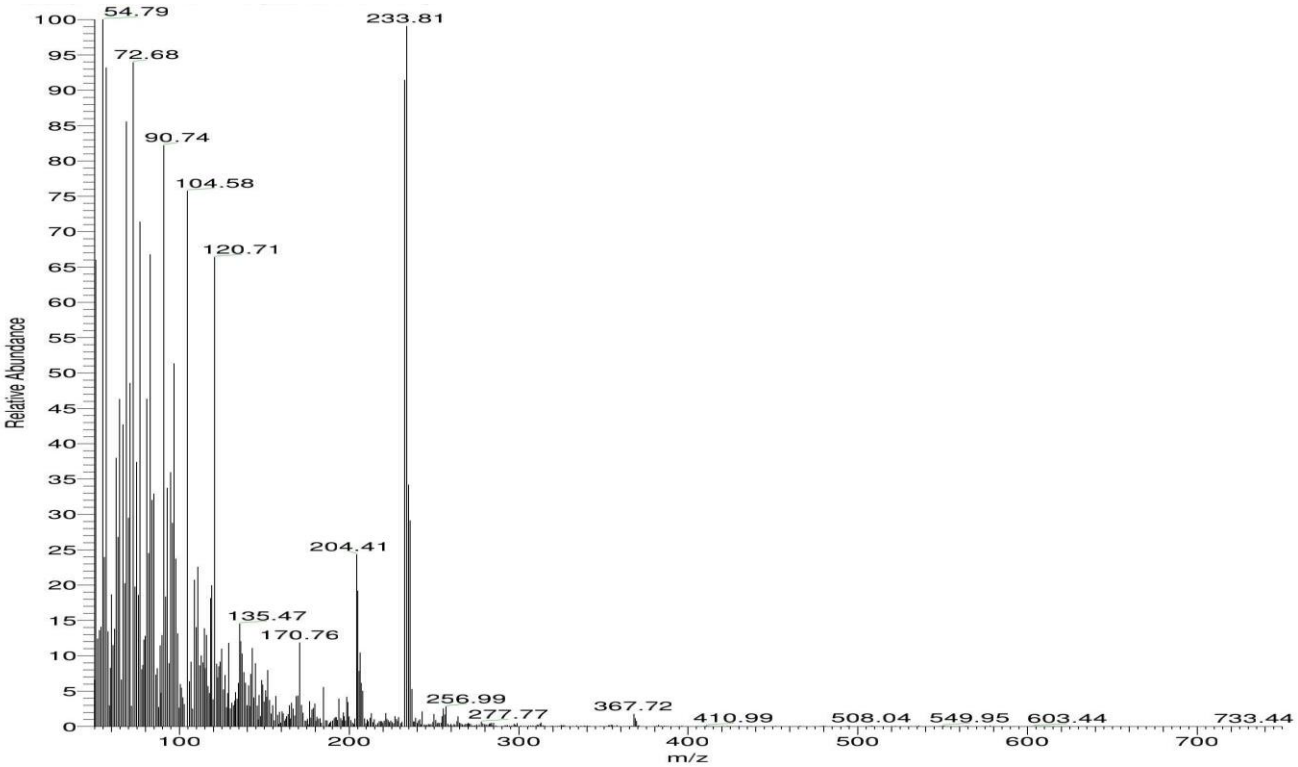
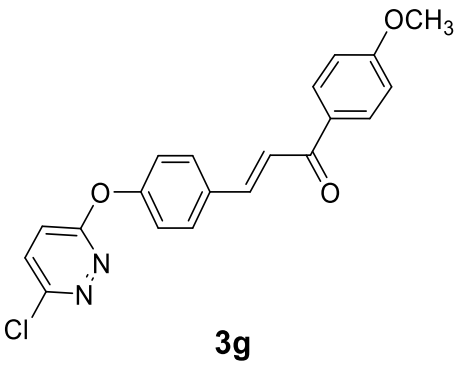


Figure S19. Mass spectrum of compound **3f**.

(E)-3-(4-((6-Chloropyridazin-3-yl)oxy)phenyl)-1-(4-methoxyphenyl)prop-2-en-1-one (3g)



3g

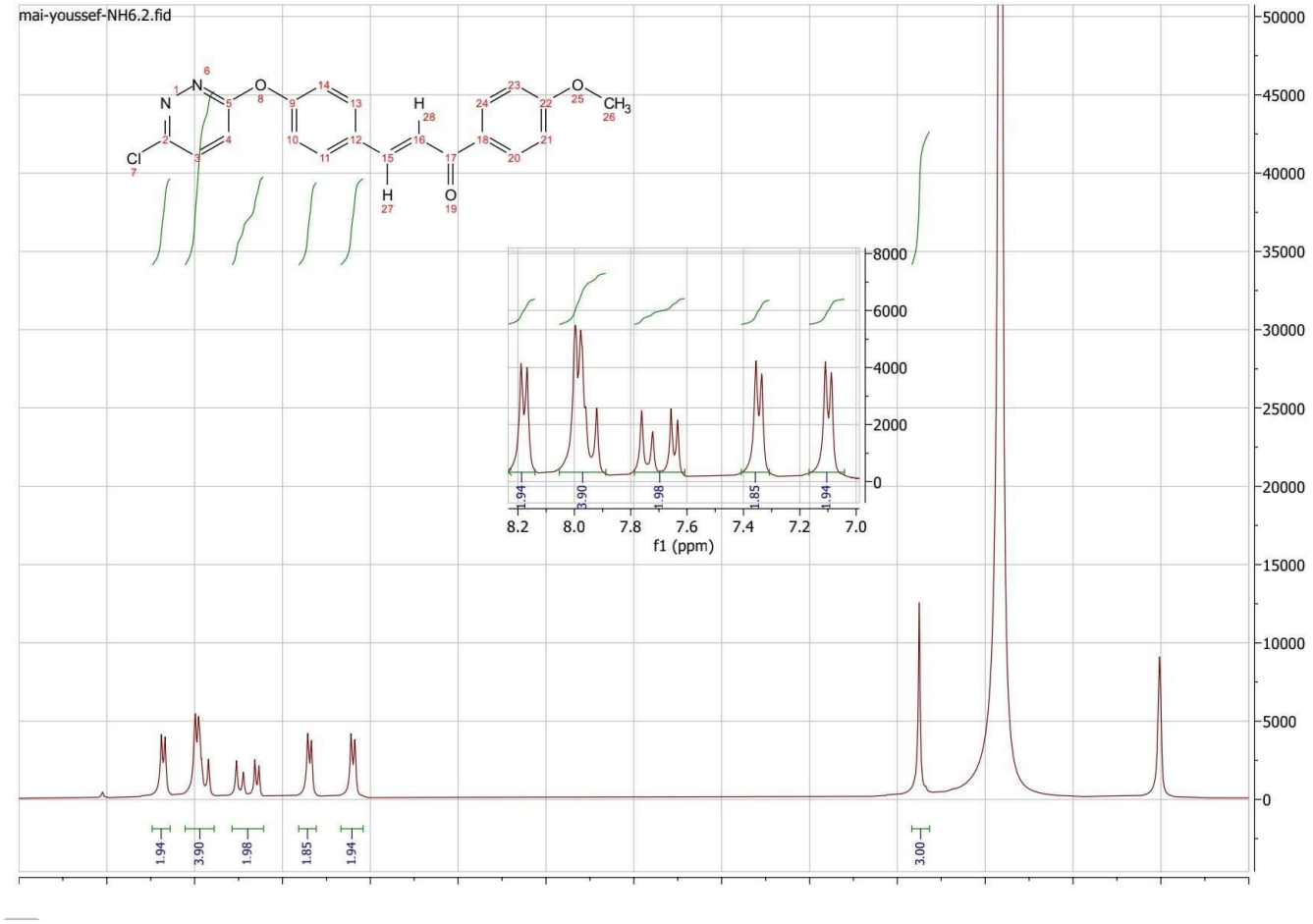


Figure S20. ^{1}H NMR (400 MHz, DMSO-d_6) spectrum of compound **3g**.

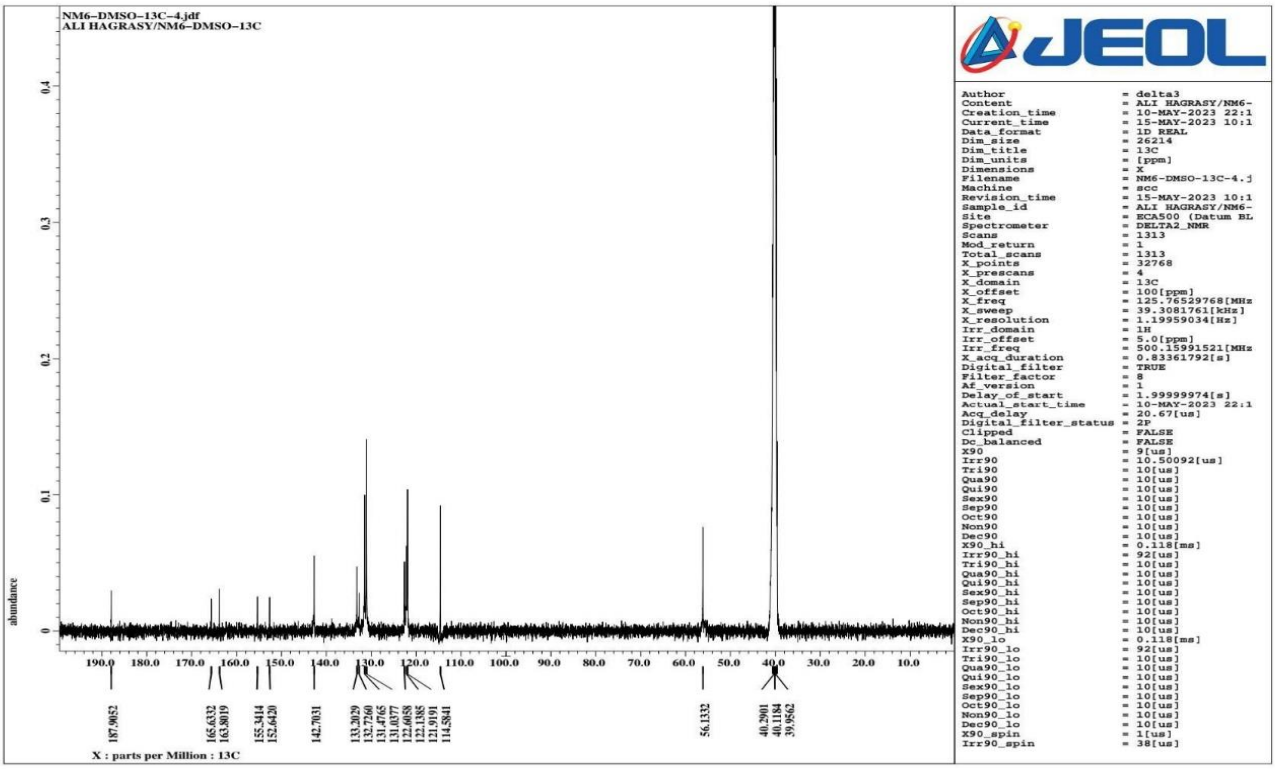


Figure S21. ^{13}C NMR (100 MHz, DMSO-d_6) spectrum of compound **3g**.

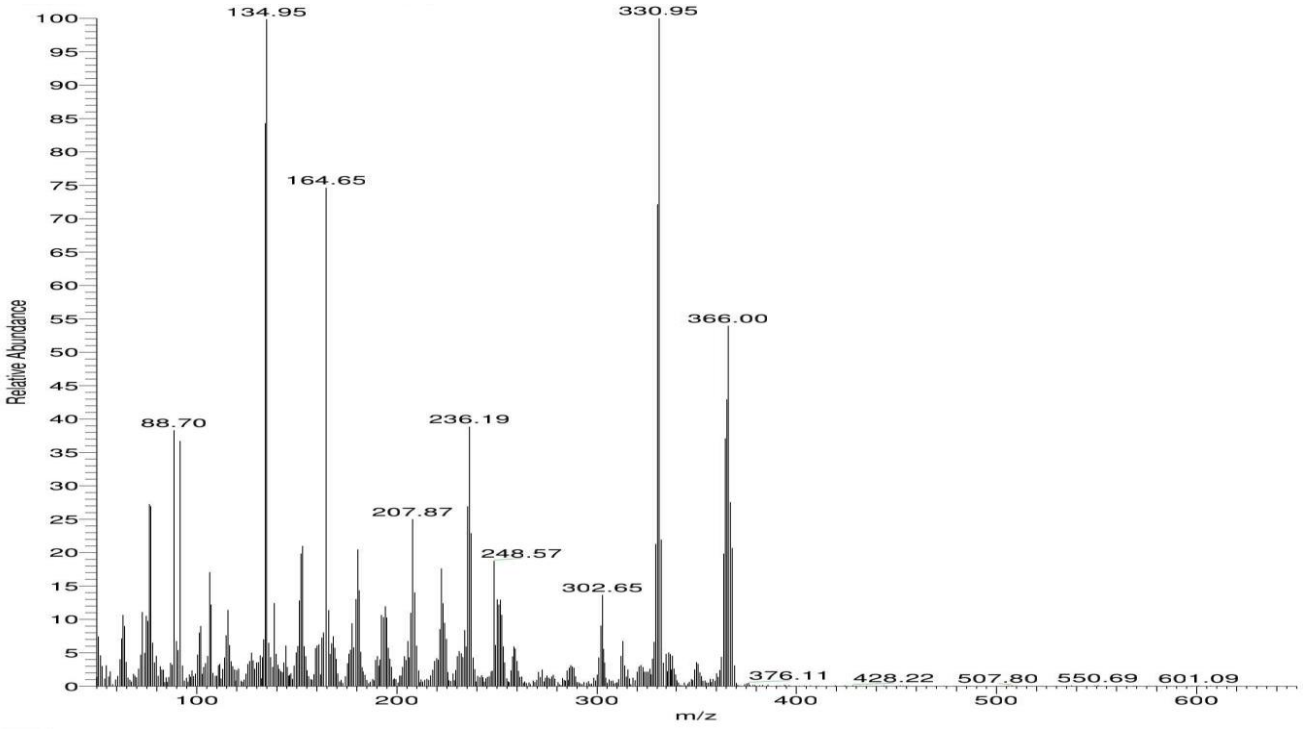
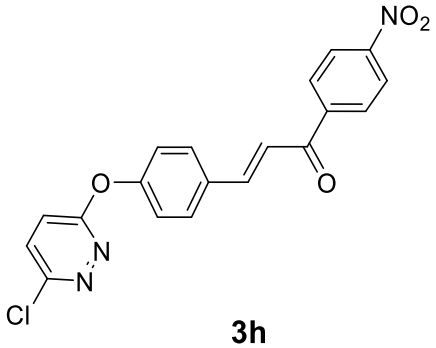


Figure S22. Mass spectrum of compound **3g**.

(E)-3-(4-((6-Chloropyridazin-3-yl)oxy)phenyl)-1-(4-nitrophenyl)prop-2-en-1-one (3h)



3h

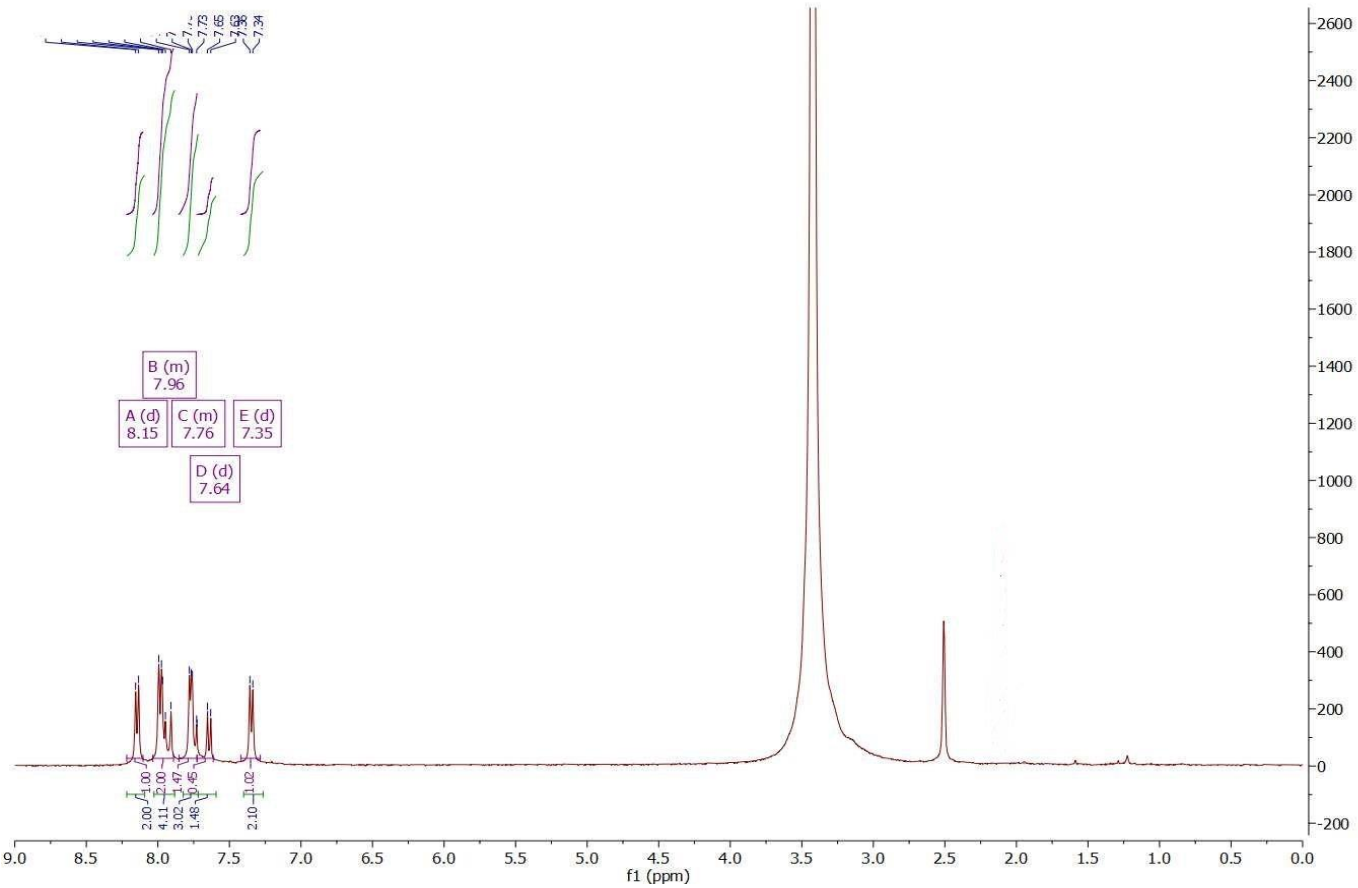


Figure S23. ^{1}H NMR (400 MHz, DMSO-d_6) spectrum of compound **3h**.

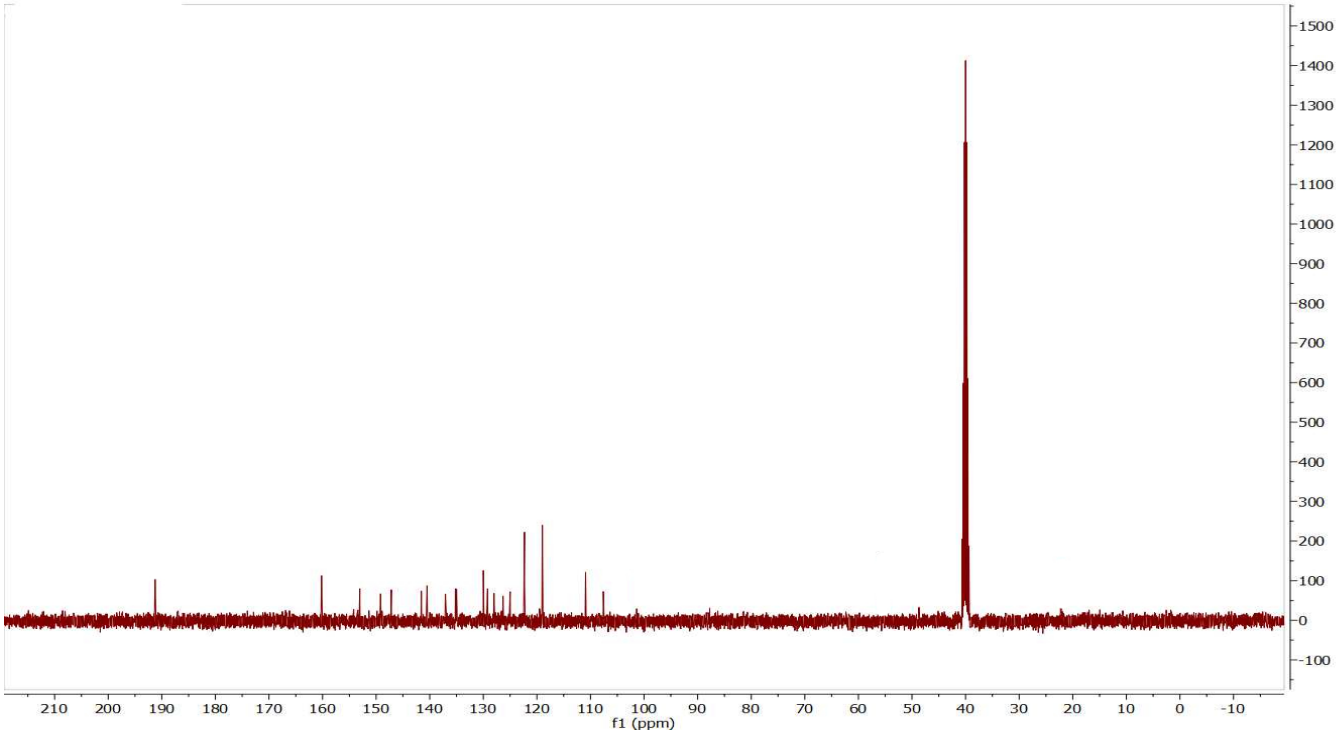


Figure S24. ^{13}C NMR (100 MHz, DMSO-d_6) spectrum of compound **3h**.

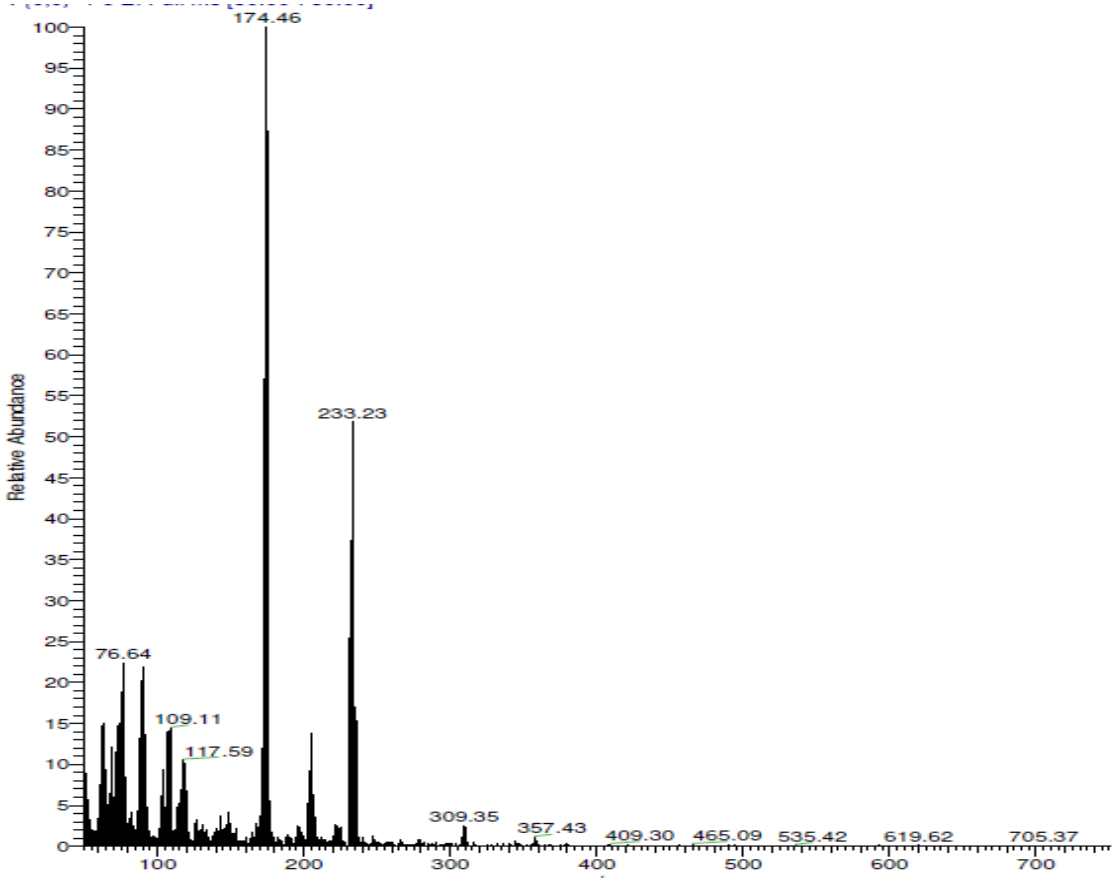
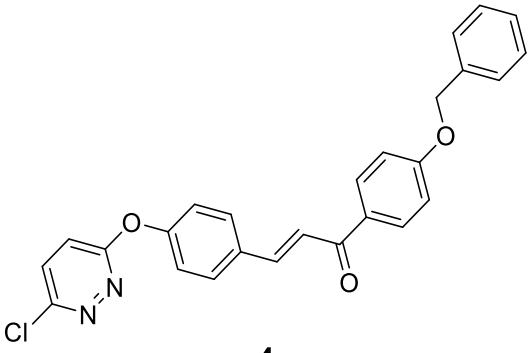


Figure S25. Mass spectrum of compound **3h**.

(E)-1-(4-(Benzyloxy)phenyl)-3-(4-((6-chloropyridazin-3-yl)oxy)phenyl)prop-2-en-1-one (4a)



4a

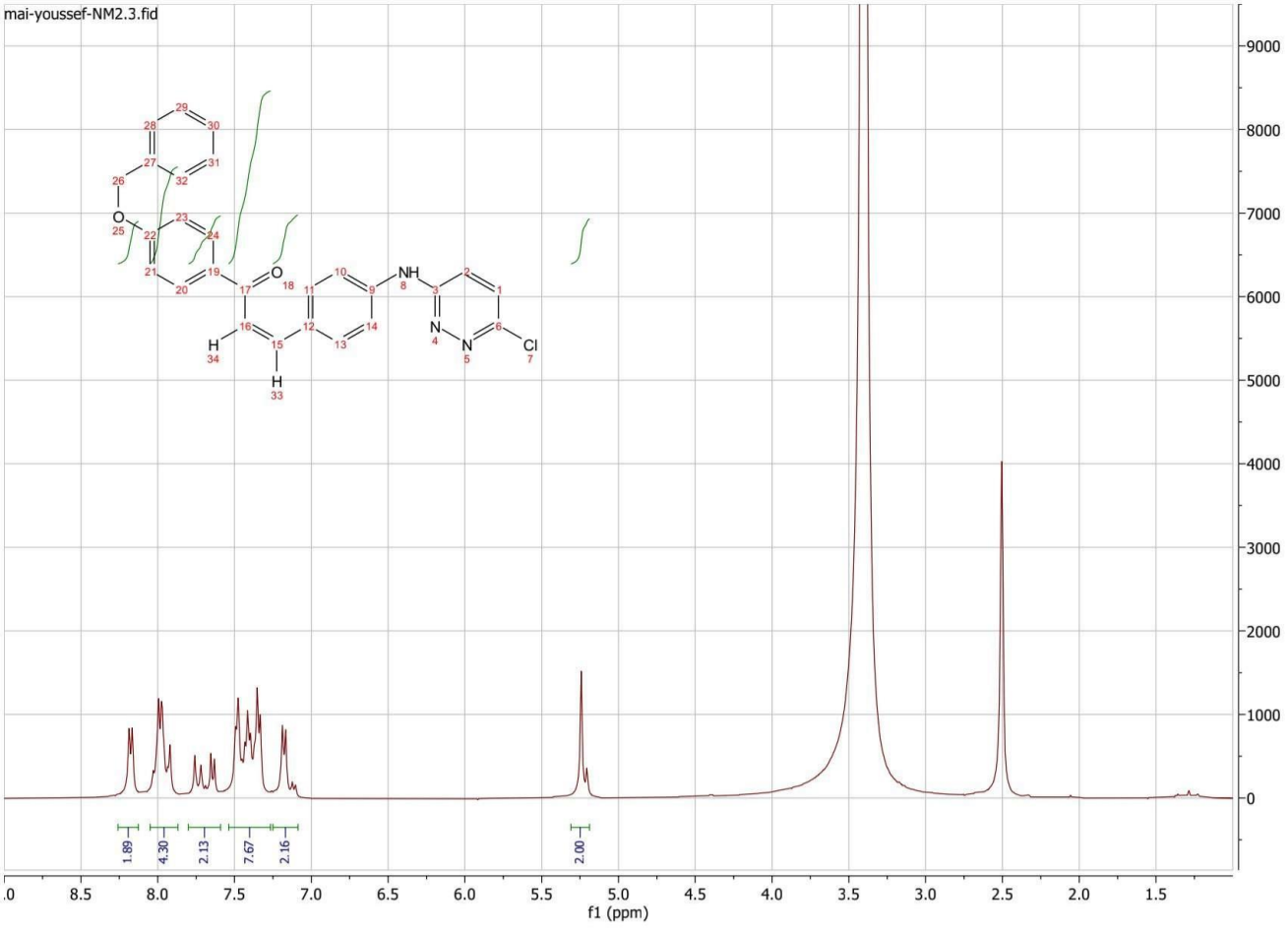


Figure S26. ^{1}H NMR (400 MHz, DMSO-d_6) spectrum of compound **4a**.

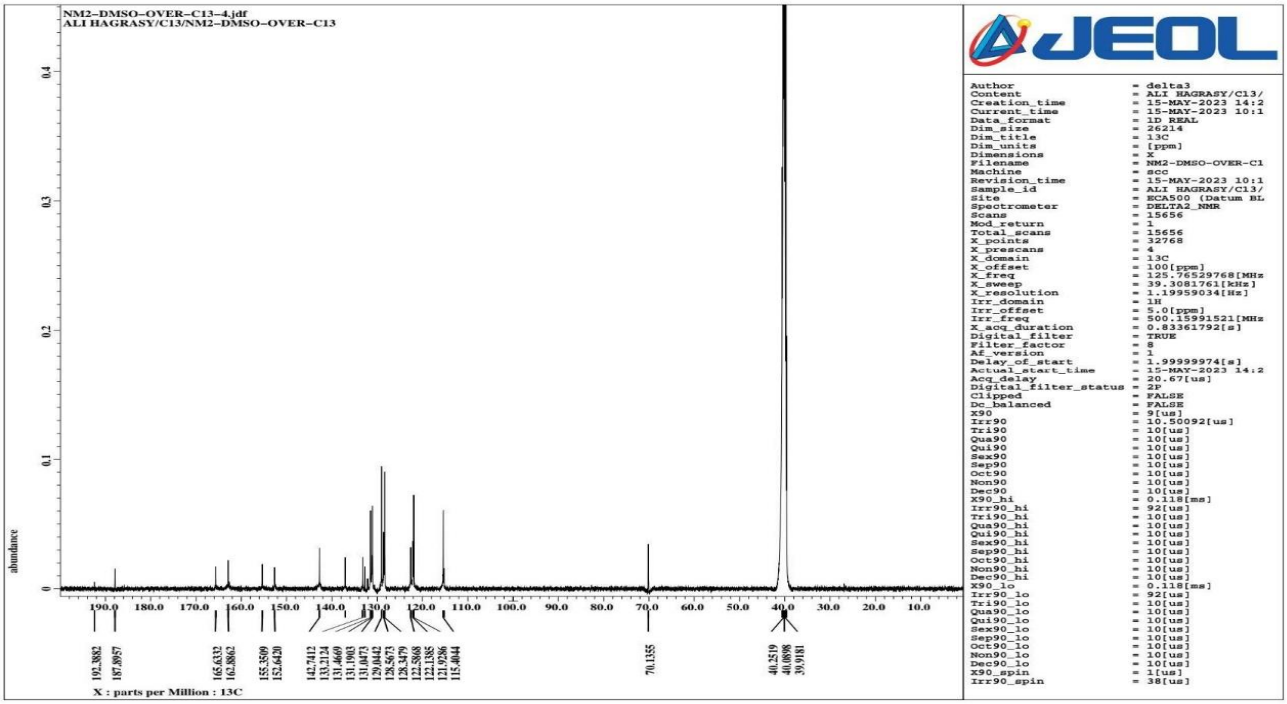


Figure S27. ^{13}C NMR (100 MHz, DMSO-d_6) spectrum of compound **4a**.

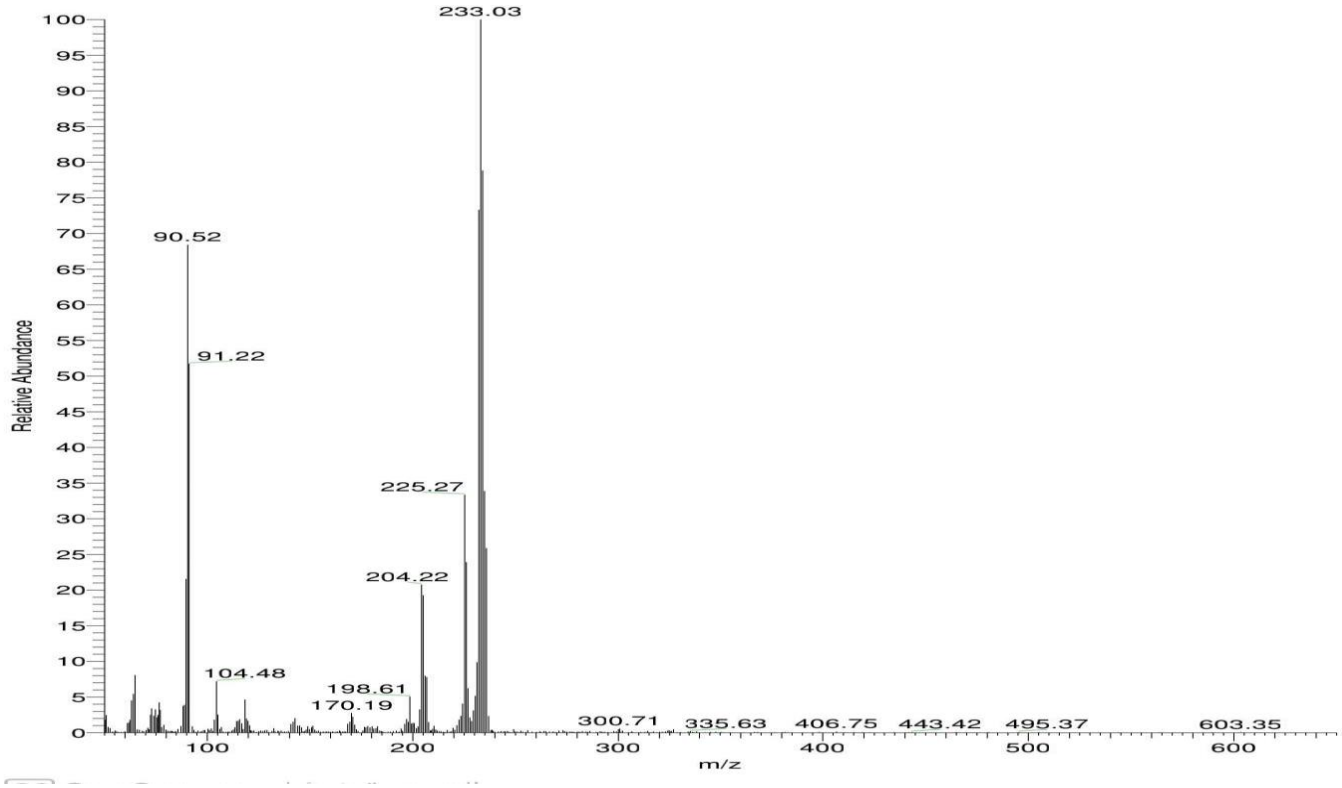
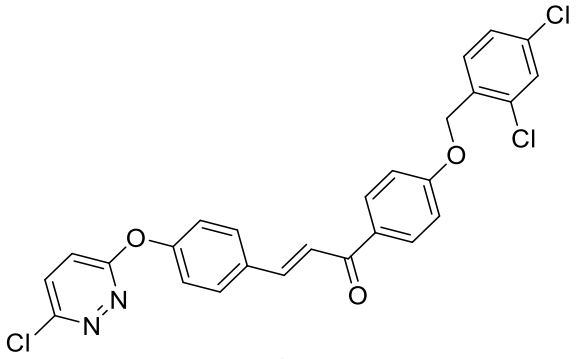


Figure S28. Mass spectrum of compound **4a**.

(E)-3-(4-((6-Chloropyridazin-3-yl)oxy)phenyl)-1-(4-((2,4-dichlorobenzyl)oxy)phenyl)prop-2-en-1-one (4b)



4b

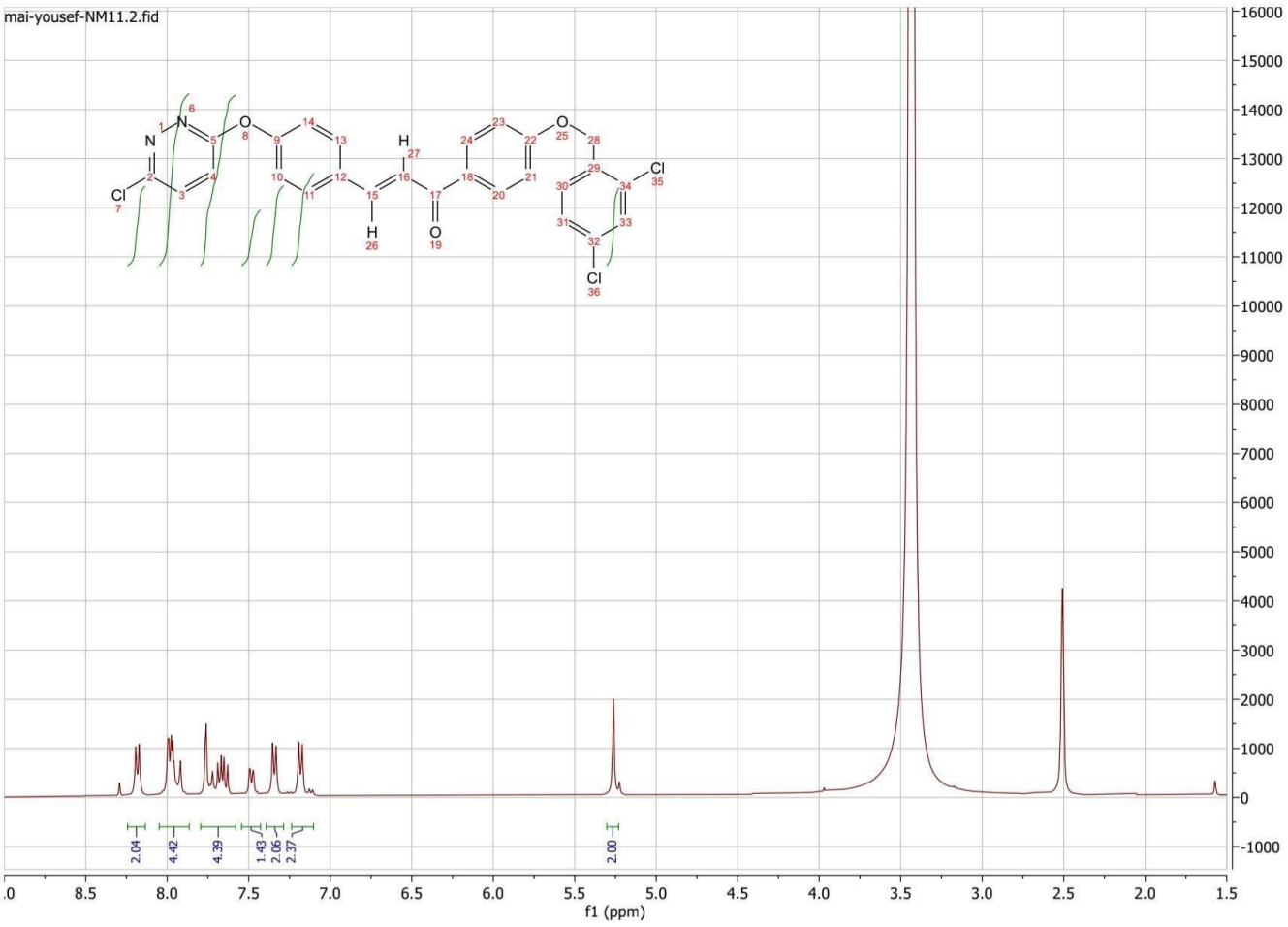


Figure S29. ^{1}H NMR (400 MHz, DMSO-d_6) spectrum of compound **4b**.

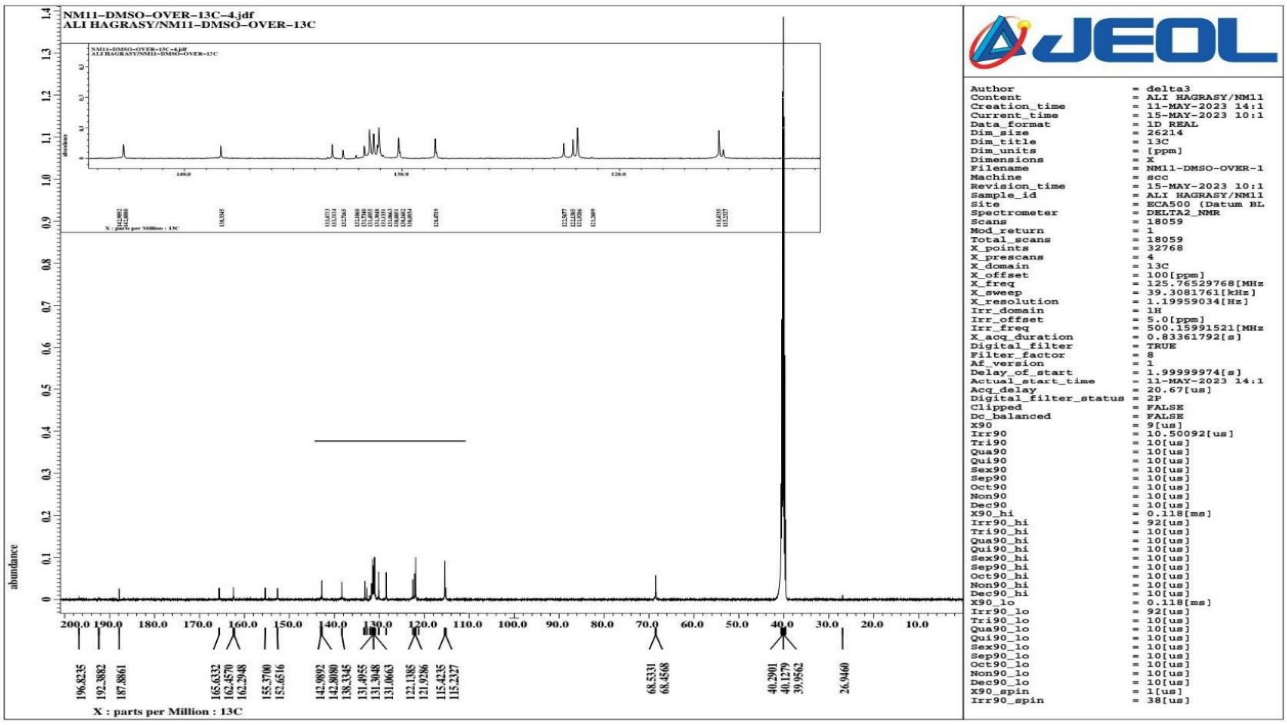


Figure S30. ^{13}C NMR (100 MHz, DMSO-d_6) spectrum of compound **4b**.

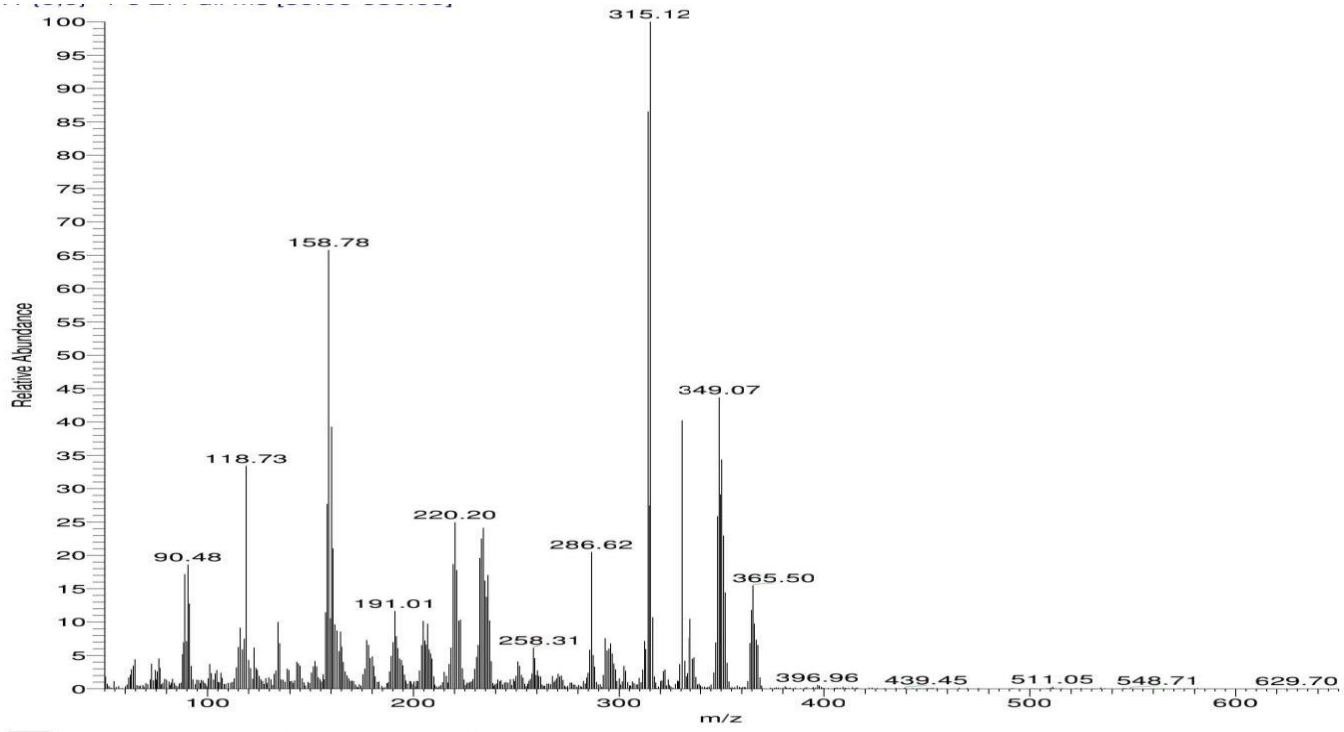
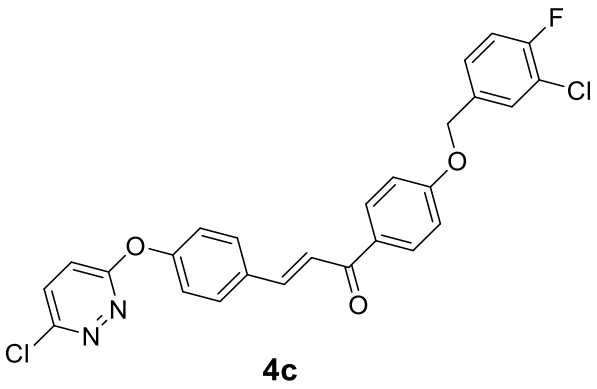


Figure S31. Mass spectrum of compound **4b**.

(E)-1-(4-((3-Chloro-4-fluorobenzyl)oxy)phenyl)-3-(4-((6-chloropyridazin-3-yl)oxy)phenyl)prop-2-en-1-one (4c)



4c

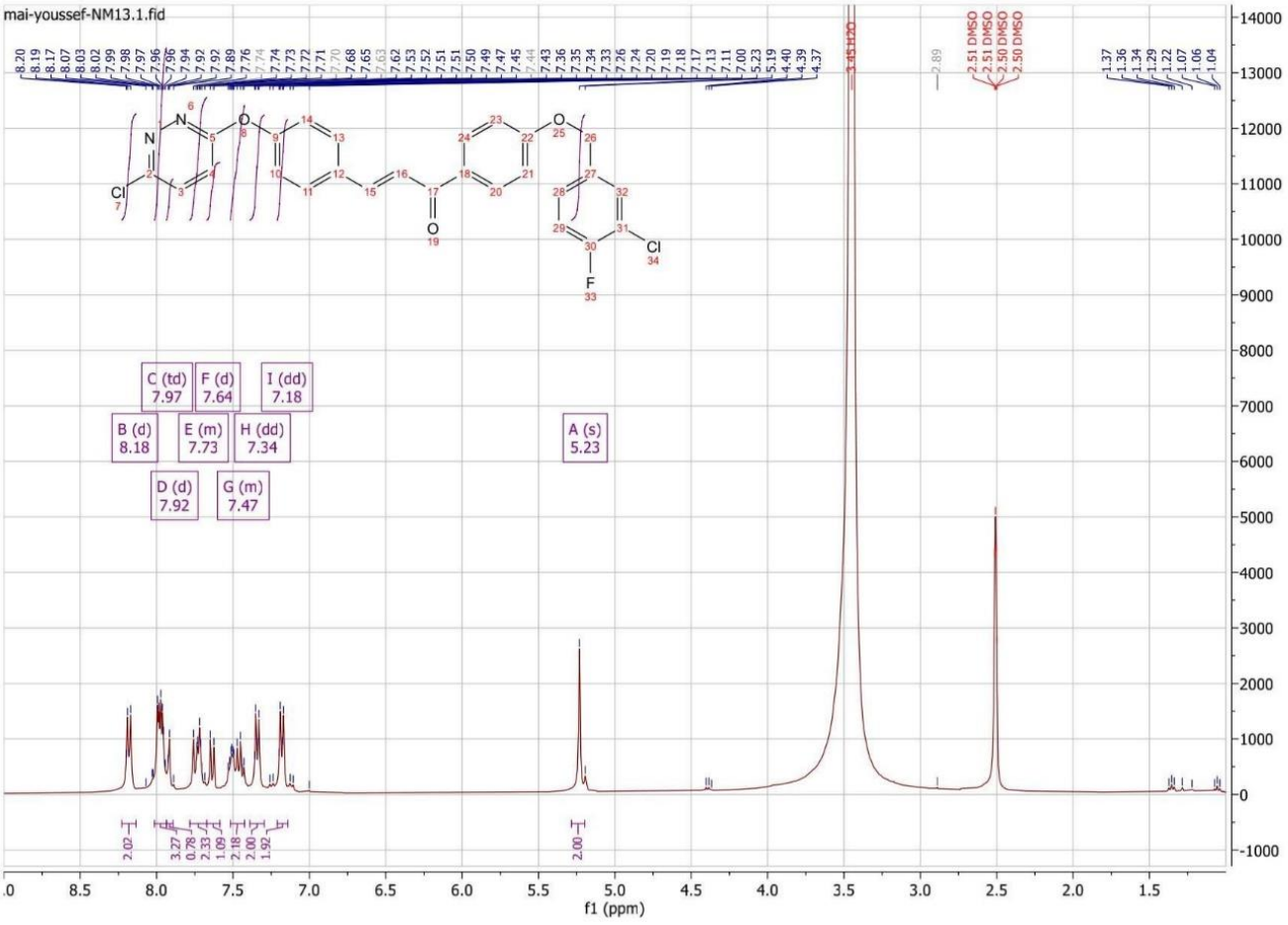


Figure S32. ^{1}H NMR (400 MHz, DMSO-d_6) spectrum of compound **4c**.

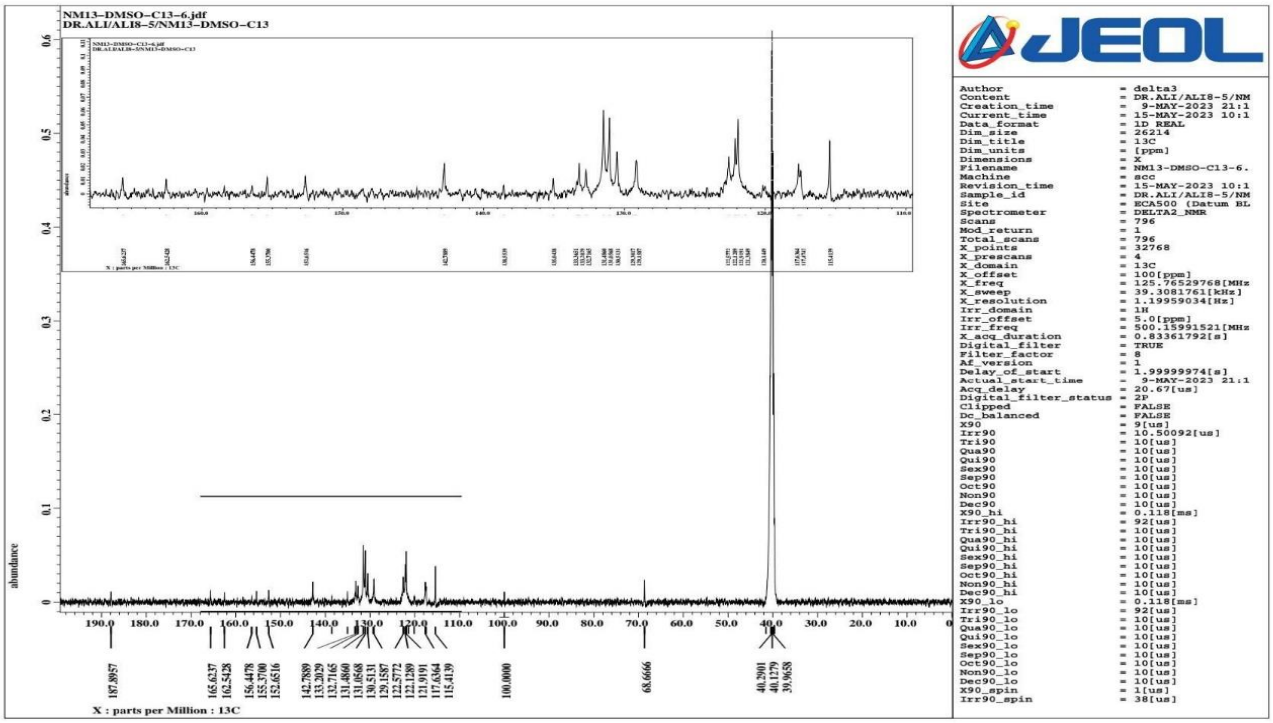


Figure S33. ^{13}C NMR (100 MHz, DMSO-d_6) spectrum of compound **4c**.

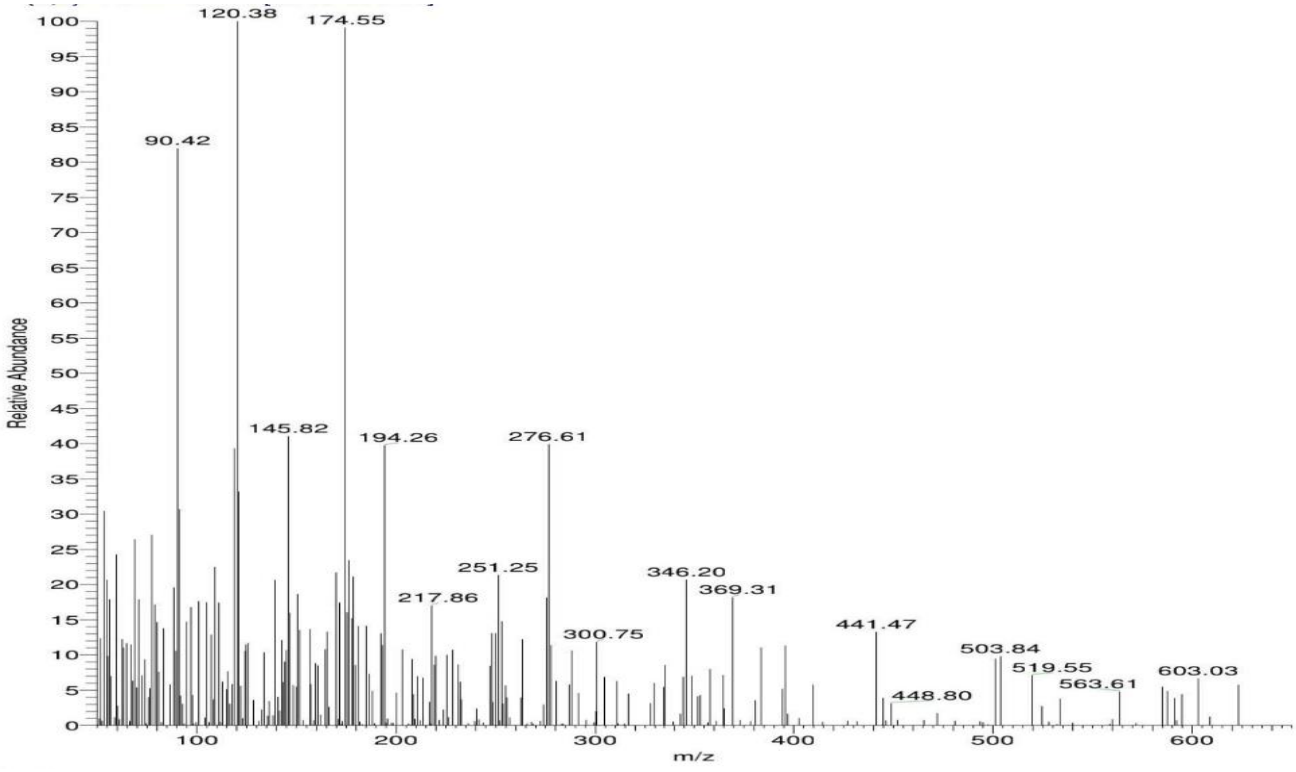
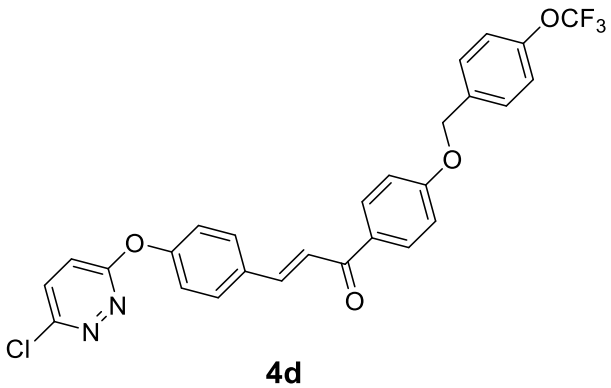


Figure S34. Mass spectrum of compound **4c**.

(E)-3-(4-((6-Chloropyridazin-3-yl)oxy)phenyl)-1-(4-((4-(trifluoromethoxy)benzyl)oxy)phenyl)prop-2-en-1-one (4d)



4d

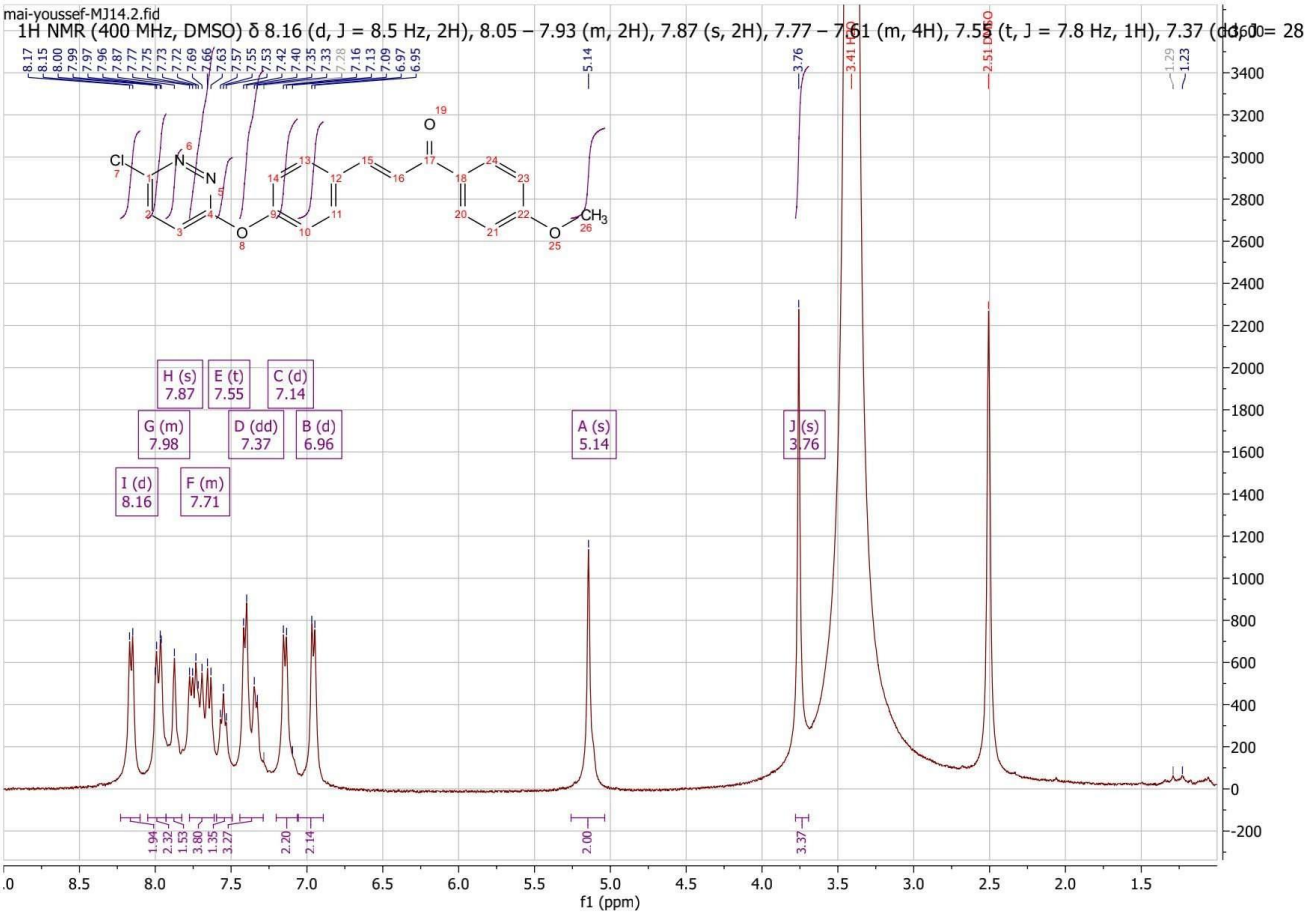


Figure S35. ^{1}H NMR (400 MHz, DMSO-d_6) spectrum of compound **4d**.

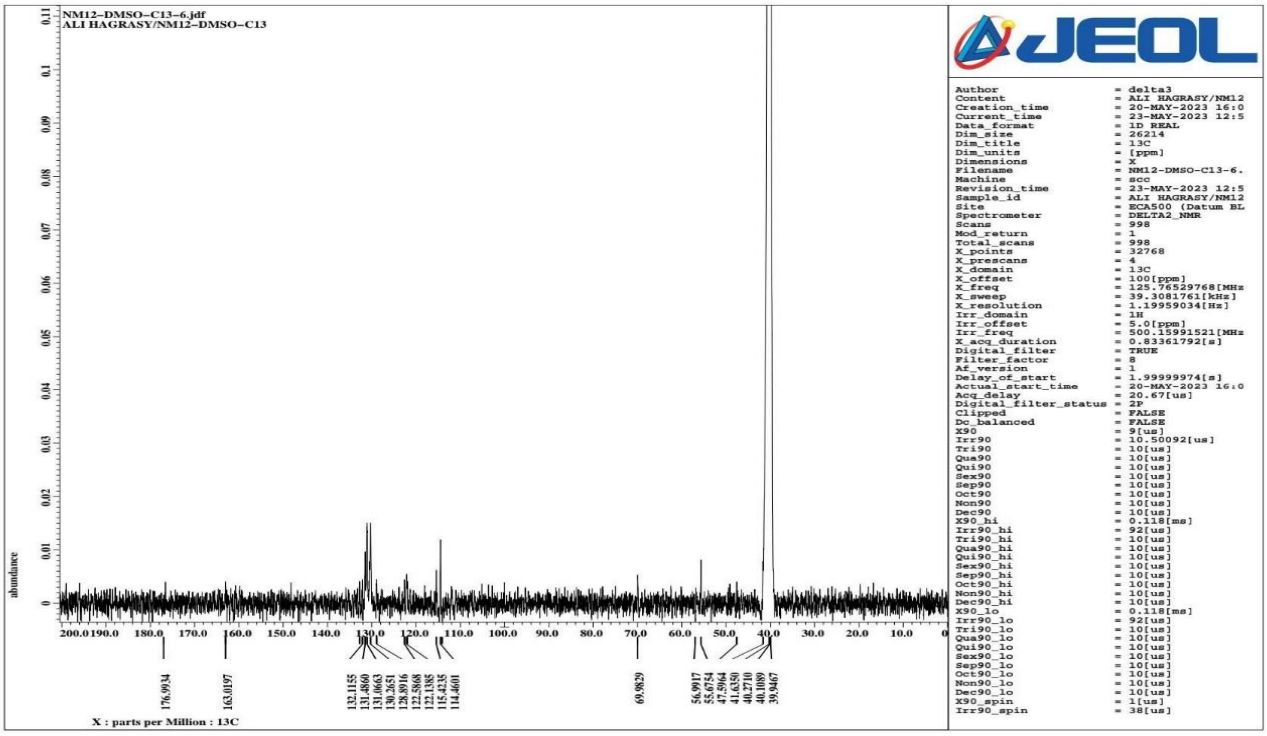


Figure S36. ^{13}C NMR (100 MHz, DMSO-d_6) spectrum of compound **4d**.

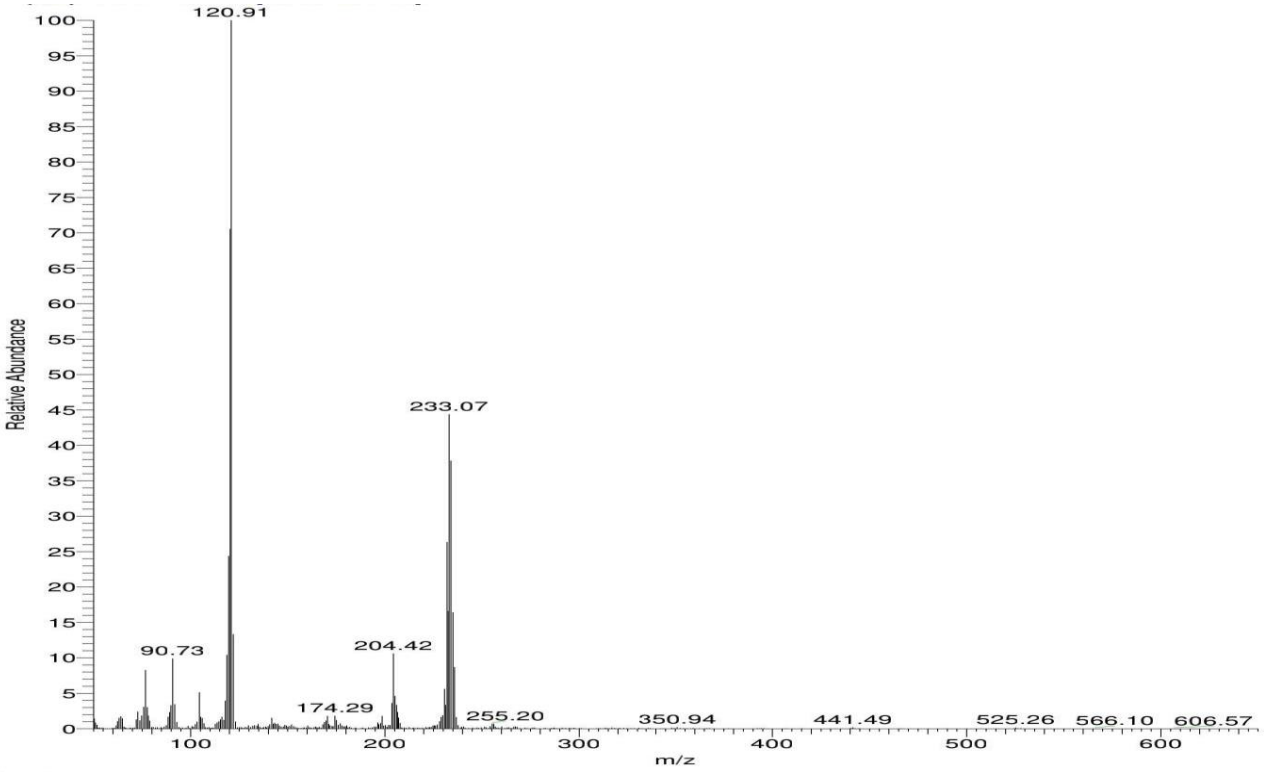
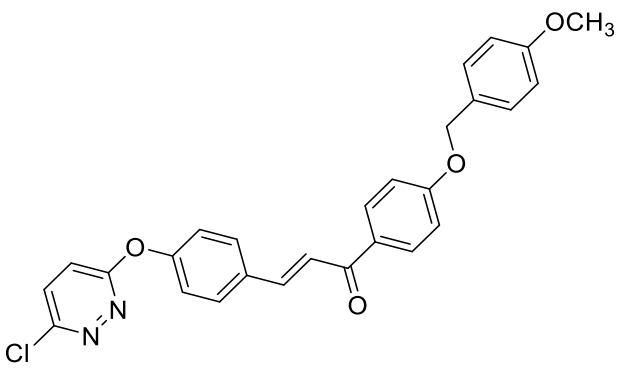


Figure S37. Mass spectrum of compound **4d**.

(E)-3-(4-((6-Chloropyridazin-3-yl)oxy)phenyl)-1-(4-((4-methoxybenzyl)oxy)phenyl)prop-2-en-1-one (4e)



4e

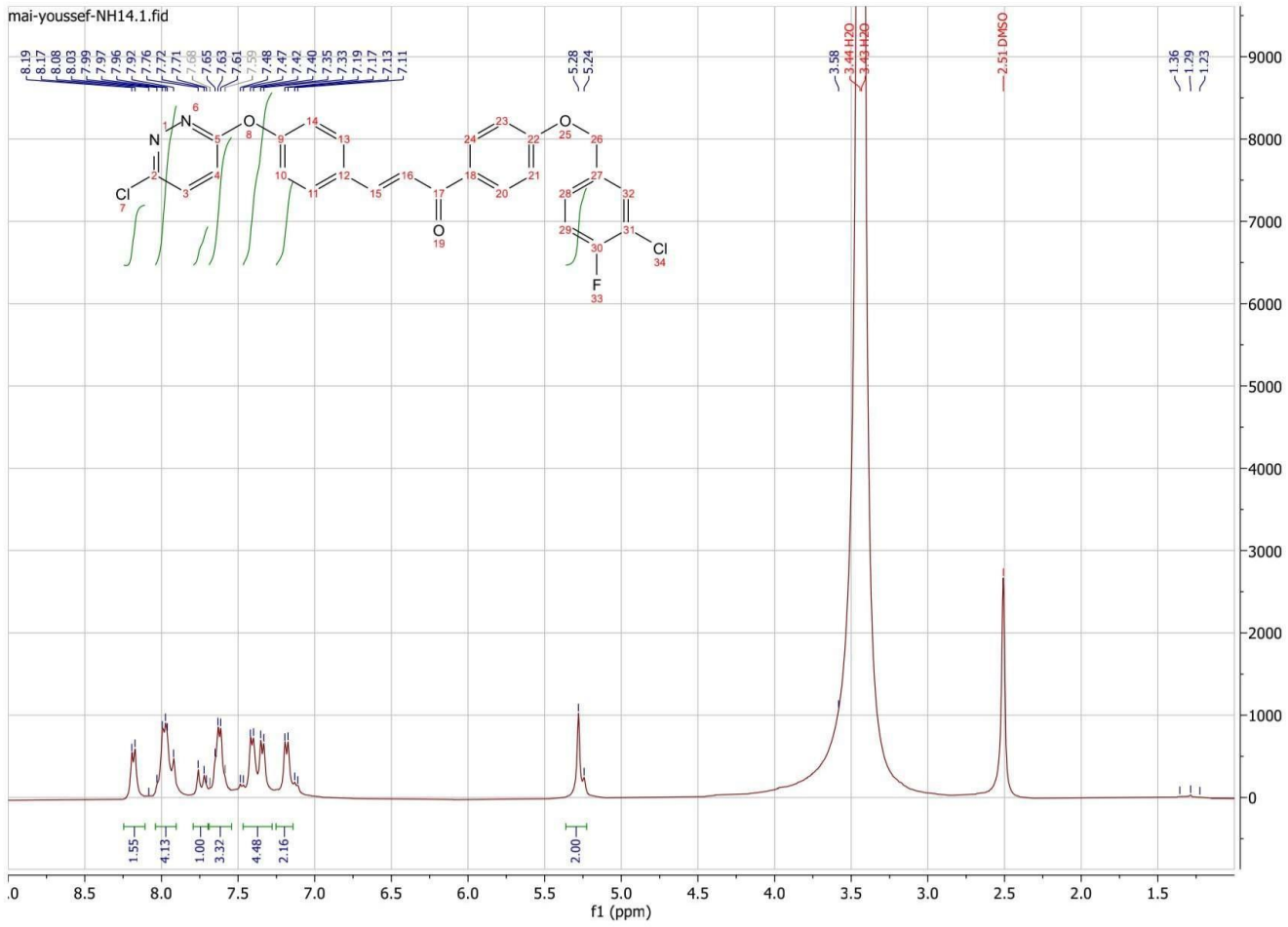


Figure S38. ^{1}H NMR (400 MHz, DMSO-d_6) spectrum of compound **4e**.

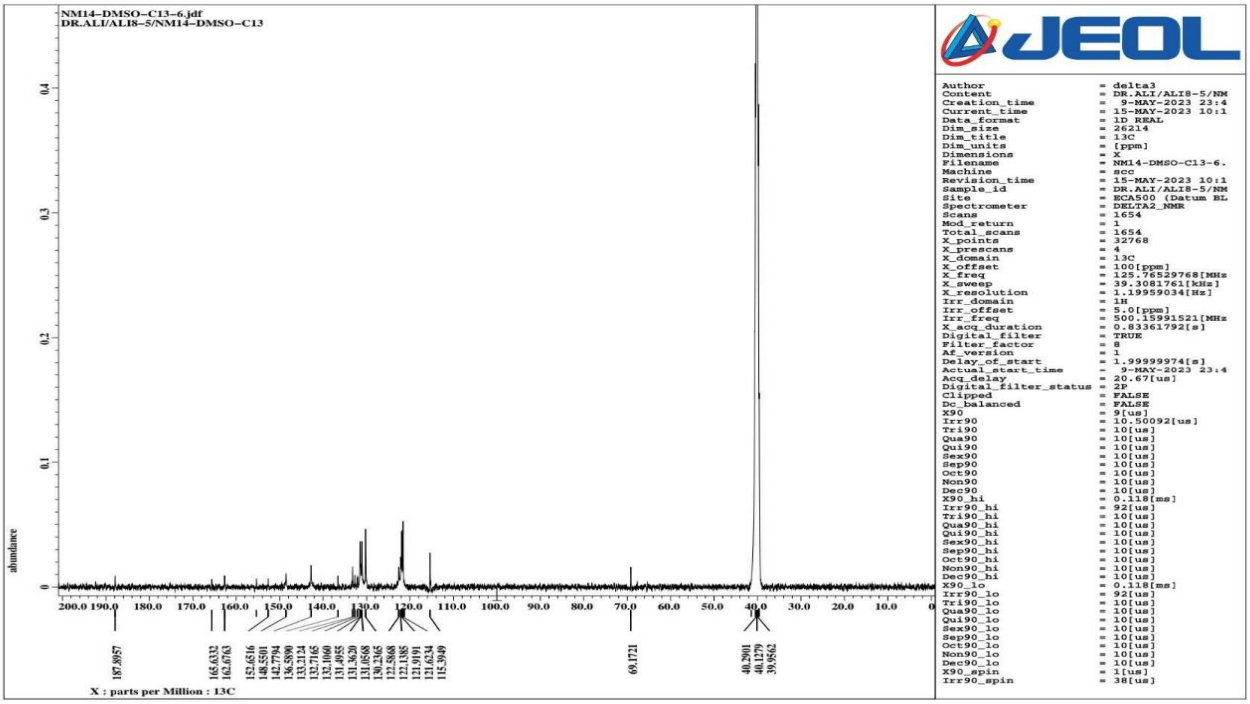


Figure S39. ^{13}C NMR (100 MHz, DMSO-d_6) spectrum of compound **4e**.

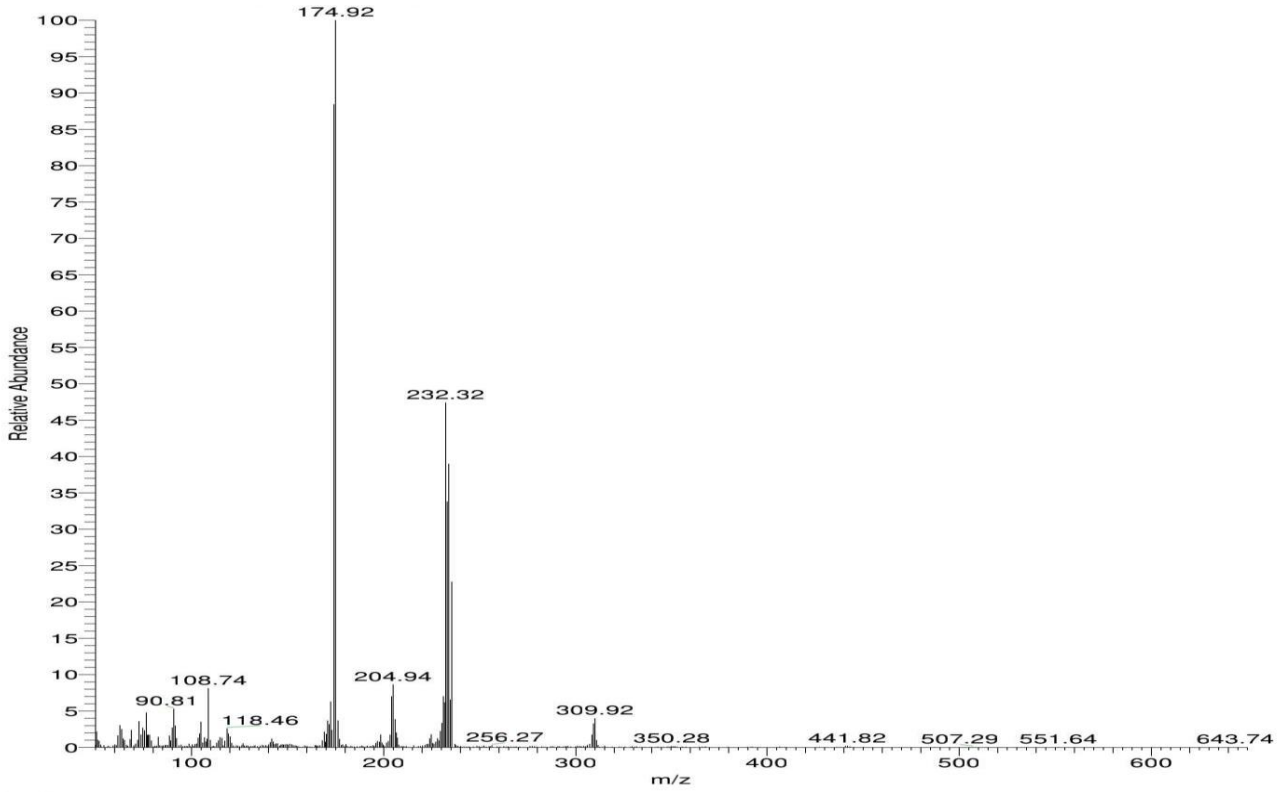
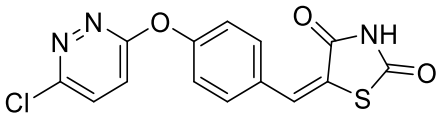


Figure S40. Mass spectrum of compound **4e**.

(E)-5-(4-((6-Chloropyridazin-3-yl)oxy)benzylidene)thiazolidine-2,4-dione (5)



5

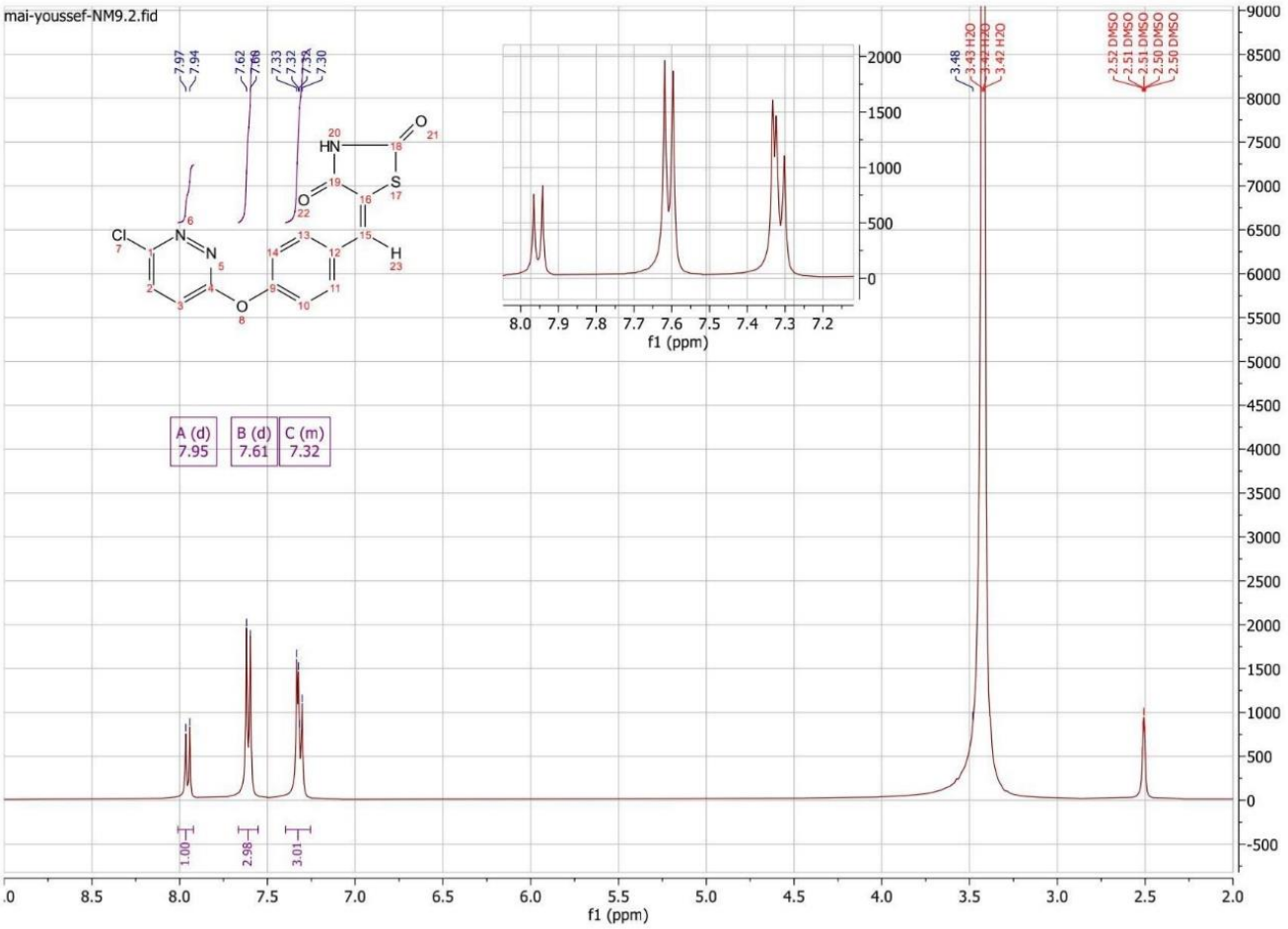


Figure S41. ^{1}H NMR (400 MHz, DMSO-d_6) spectrum of compound **5**.

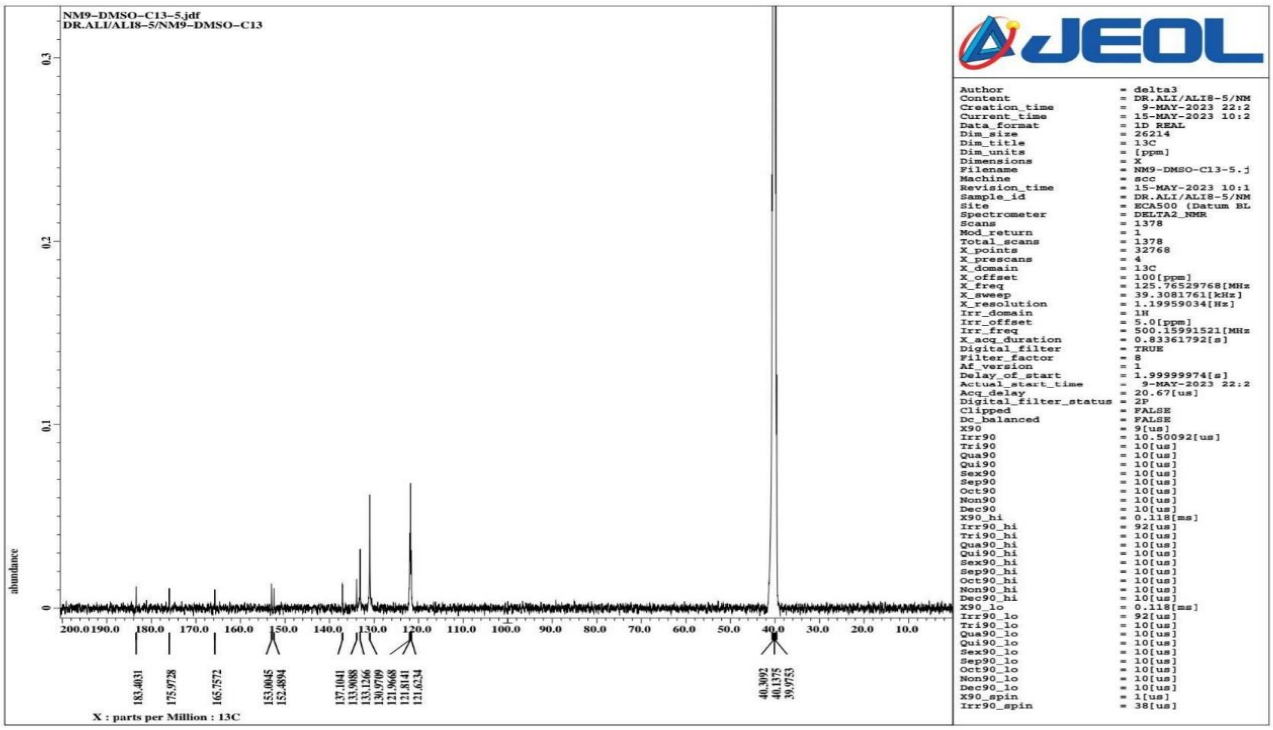


Figure S42. ^{13}C NMR (100 MHz, DMSO-d_6) spectrum of compound **5**.

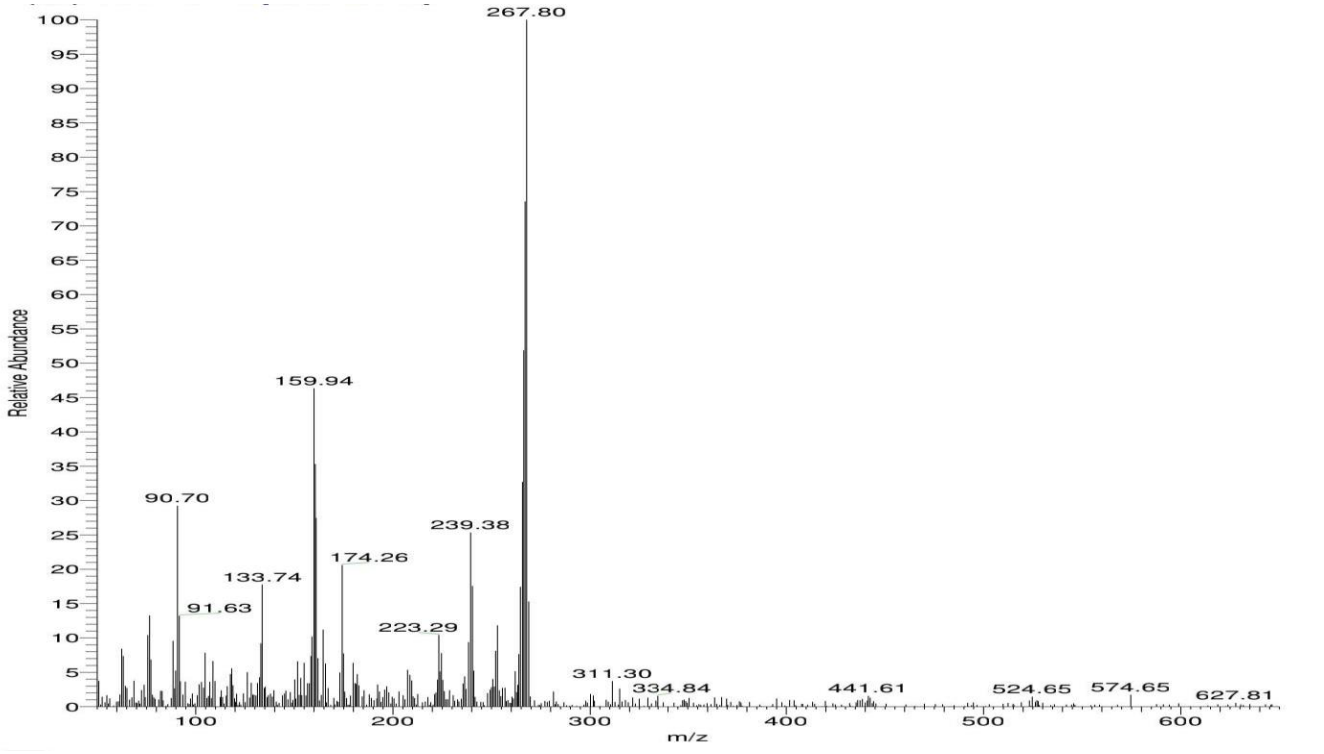


Figure S43. Mass spectrum of compound **5**.

Biological Data

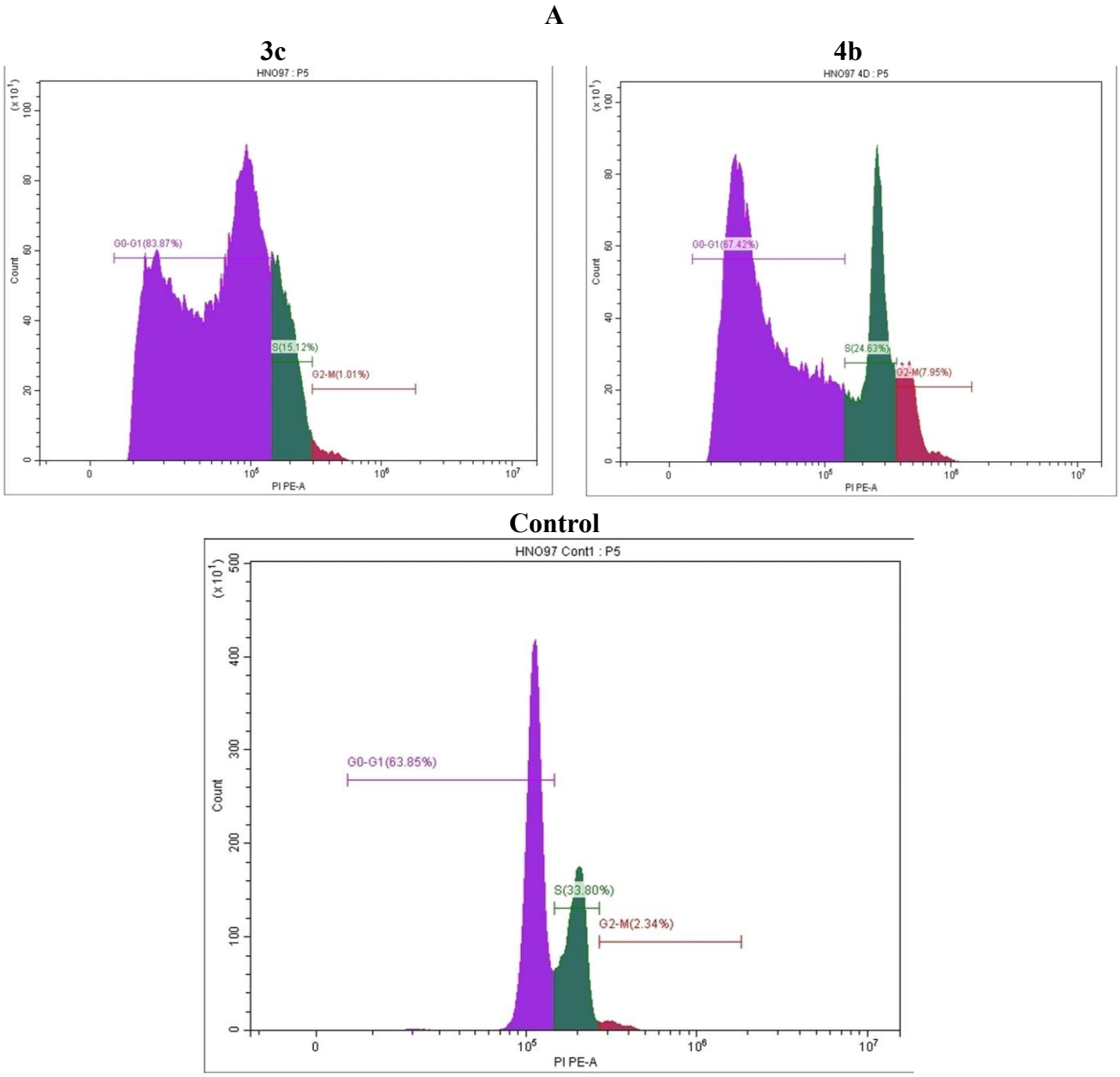
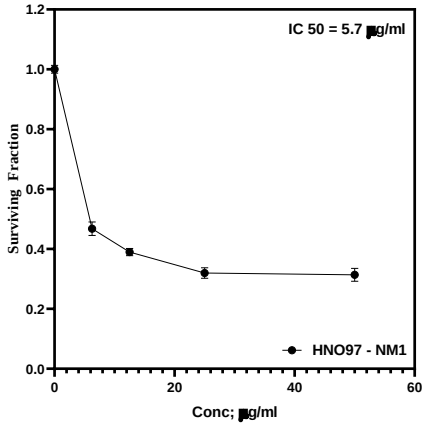
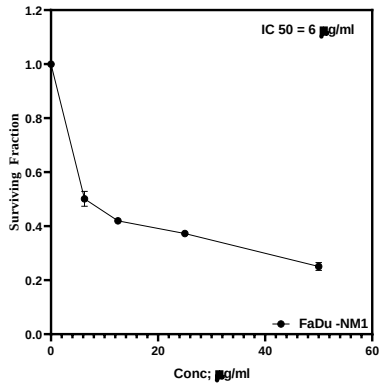
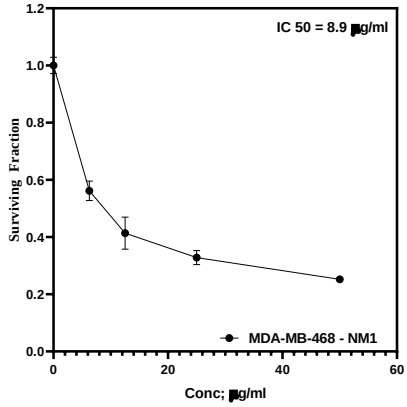
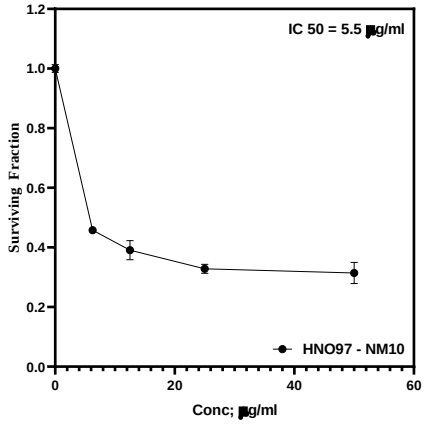
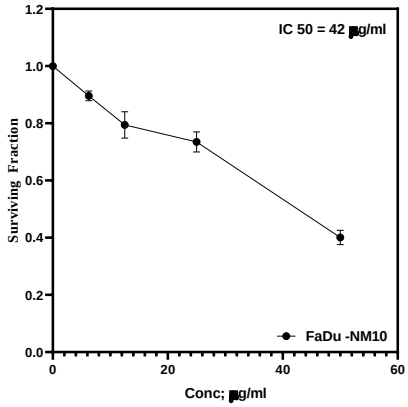
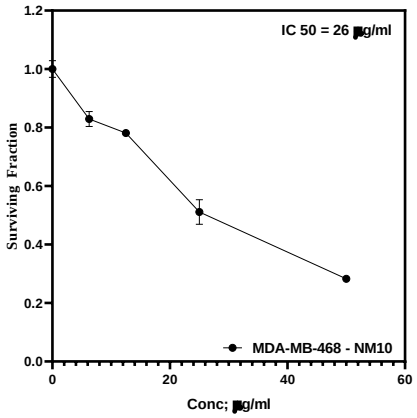
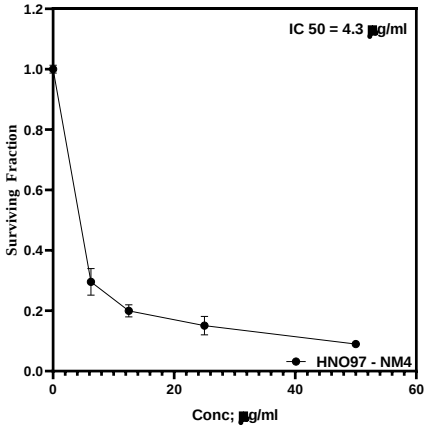
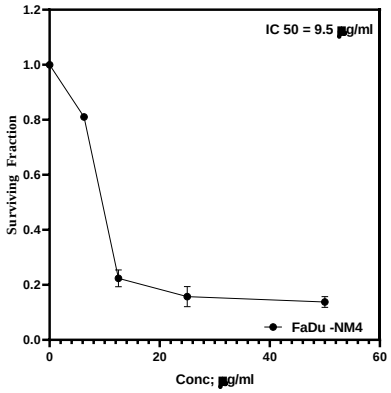
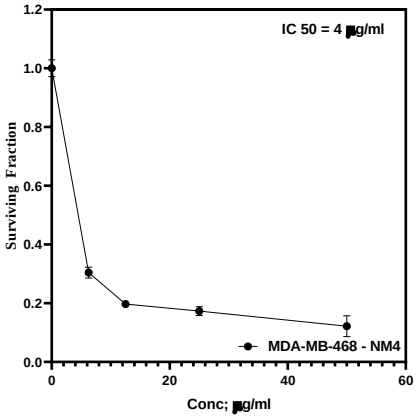
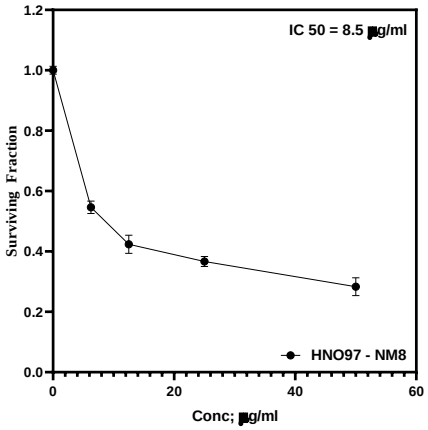
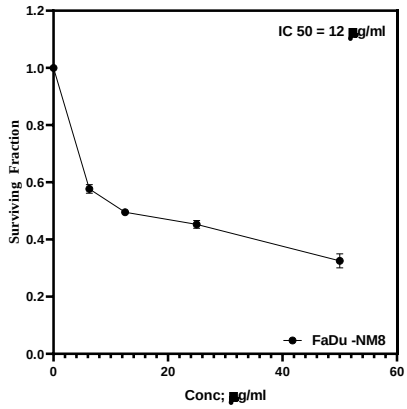
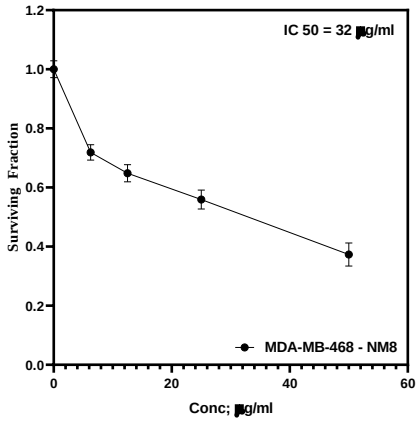
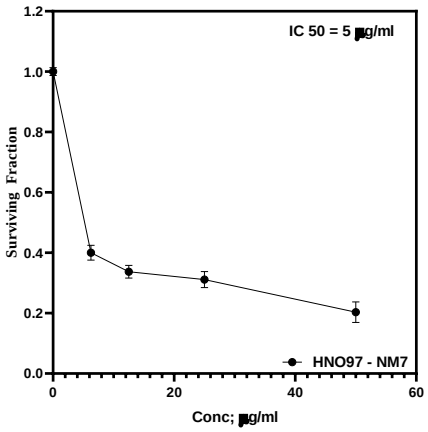
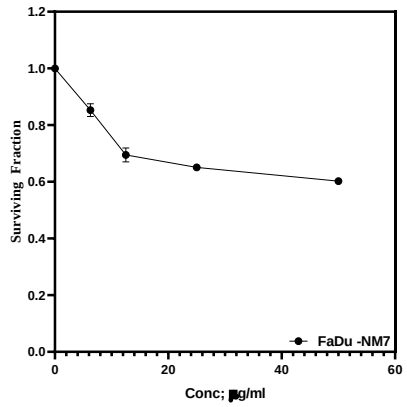
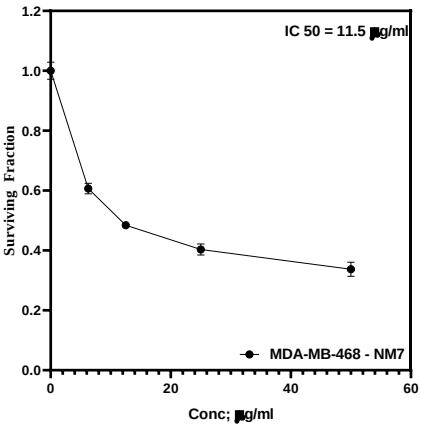
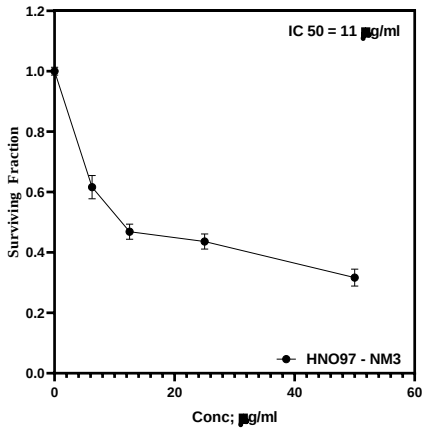
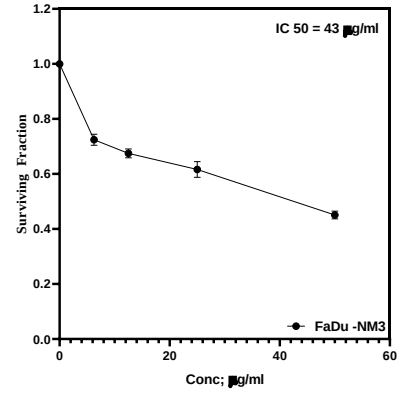
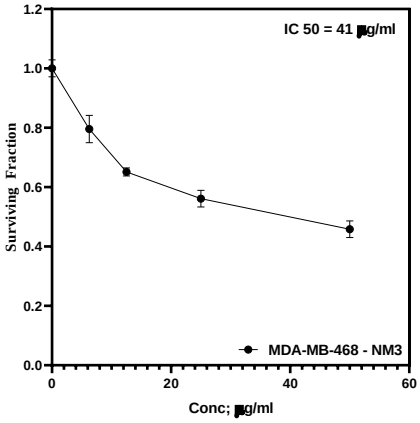
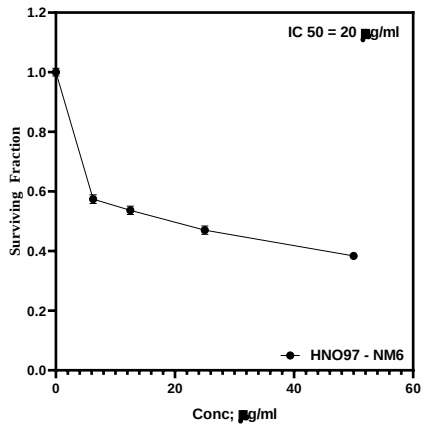
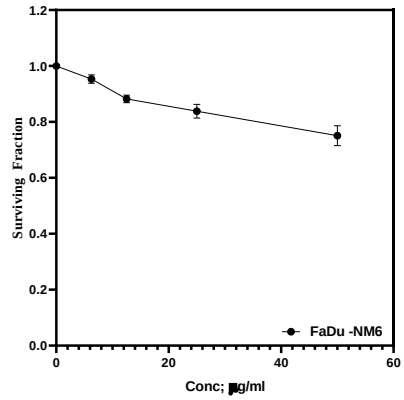
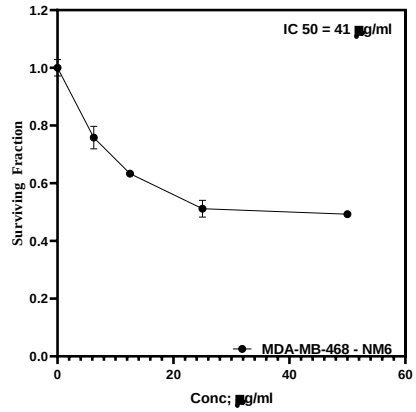
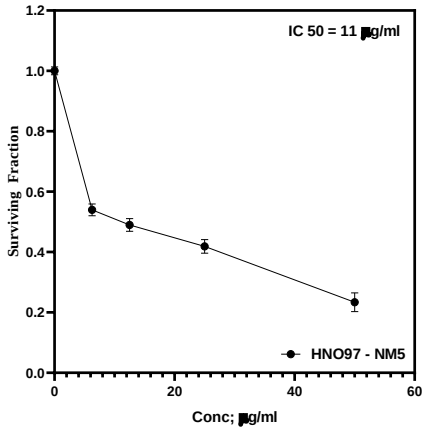
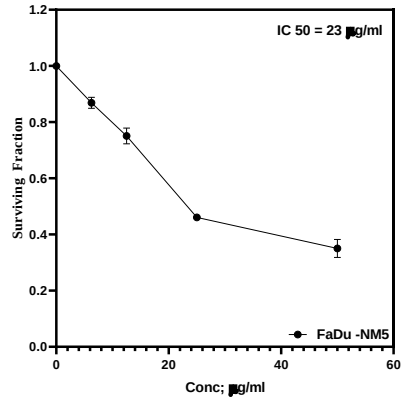
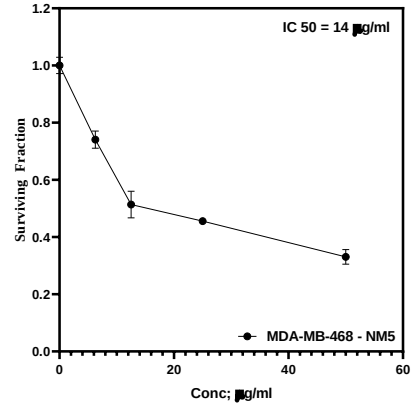
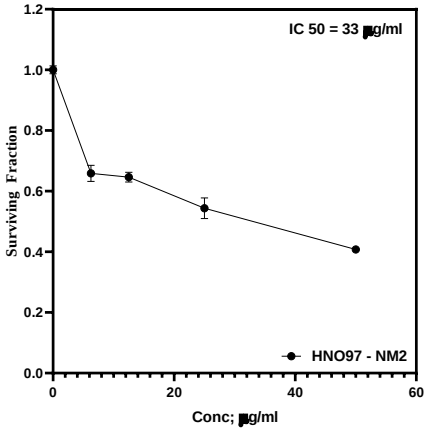
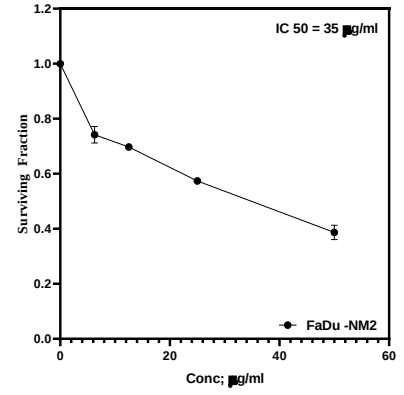
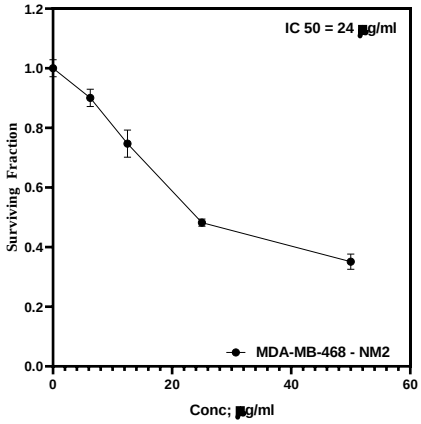


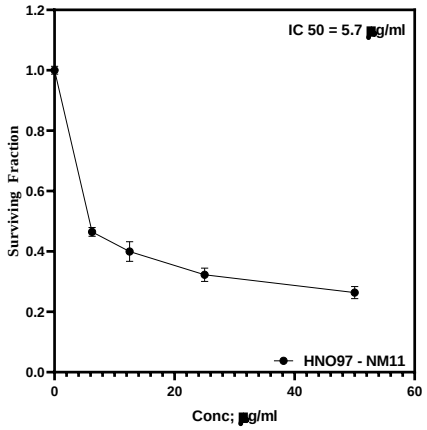
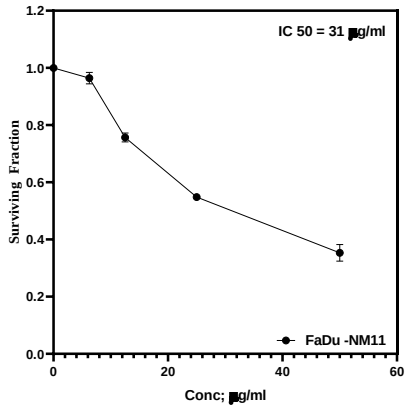
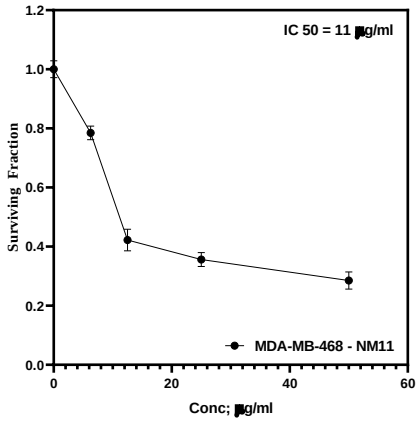
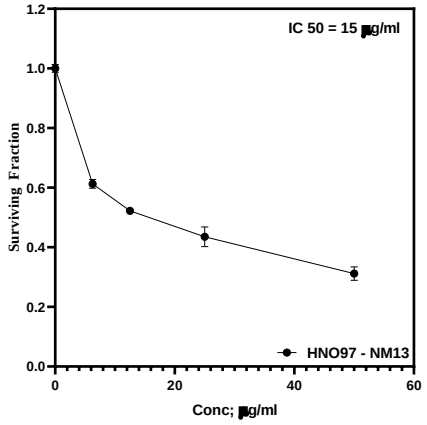
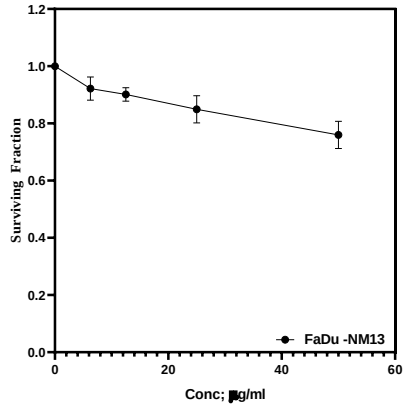
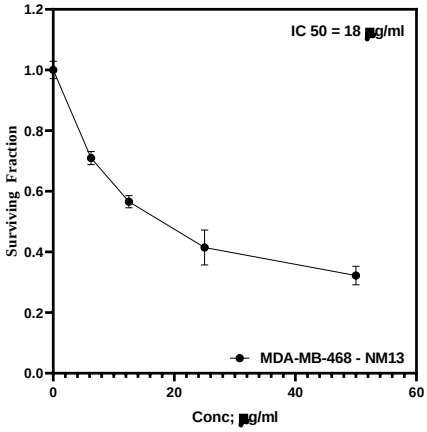
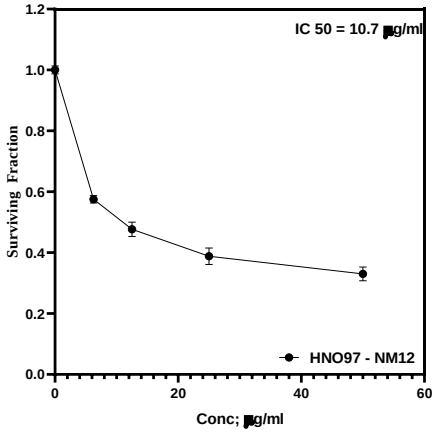
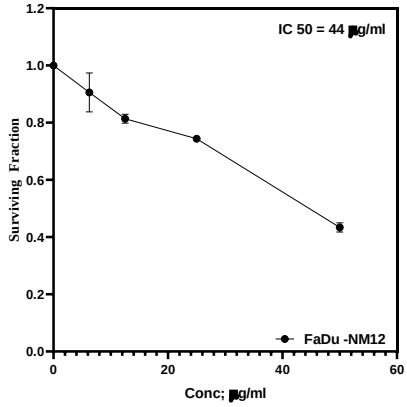
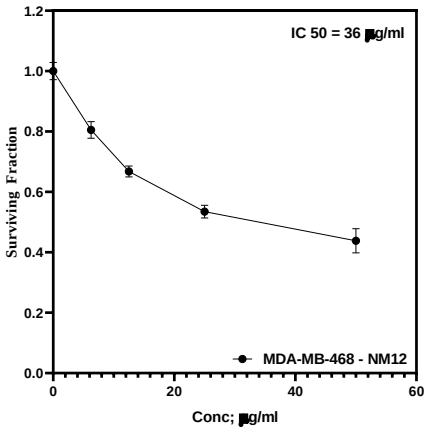
Figure S44. Repeated cell cycle analysis histograms of control, **3c**, and **4b** candidates in HNO97 cells.

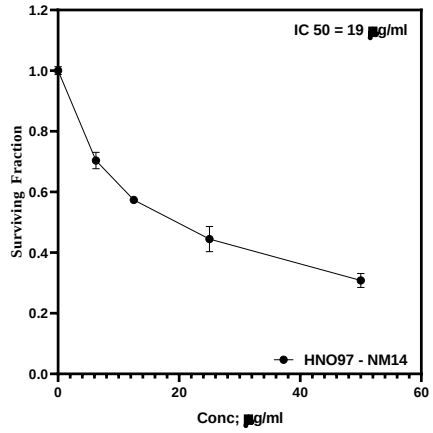
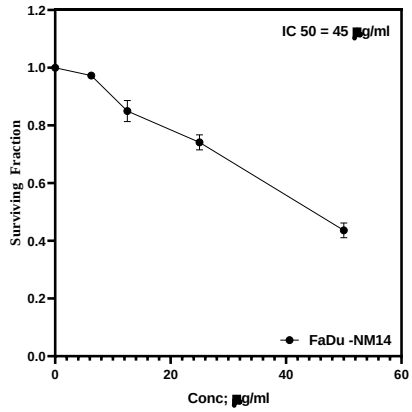
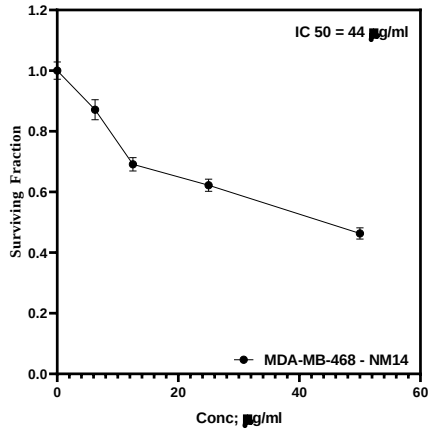
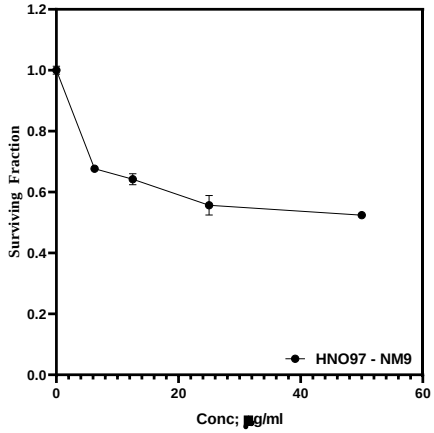
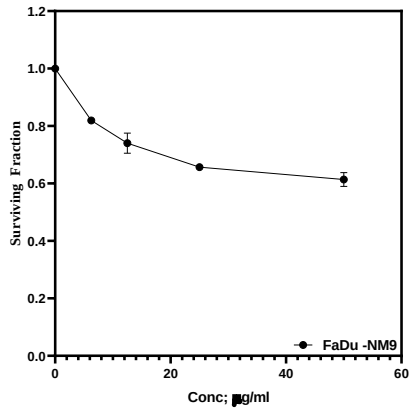
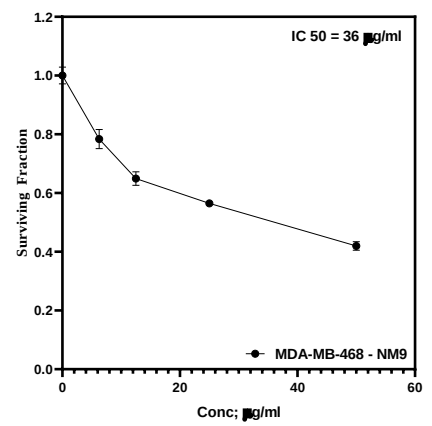
Table S2. IC_{50} curves of the new 4-chloropyridazinoxyphenyl hybrids (**3a-h**, **4a-e**, and **5**) against HNO97, FaDu, and MDA-MB-468 tested cells.

Comp.	IC_{50} (µg/mL)		
	HNO97	**FaDu**	**MDA-MB-468**
3a			
3b			
3c			

3d	 IC 50 = 8.5 µg/ml Surviving Fraction HNO97 - NM8 Conc; µg/ml	 IC 50 = 12 µg/ml Surviving Fraction FaDu -NM8 Conc; µg/ml	 IC 50 = 32 µg/ml Surviving Fraction MDA-MB-468 - NM8 Conc; µg/ml
3e	 IC 50 = 5 µg/ml Surviving Fraction HNO97 - NM7 Conc; µg/ml	 Surviving Fraction FaDu -NM7 Conc; µg/ml	 IC 50 = 11.5 µg/ml Surviving Fraction MDA-MB-468 - NM7 Conc; µg/ml
3f	 IC 50 = 11 µg/ml Surviving Fraction HNO97 - NM3 Conc; µg/ml	 IC 50 = 43 µg/ml Surviving Fraction FaDu -NM3 Conc; µg/ml	 IC 50 = 41 µg/ml Surviving Fraction MDA-MB-468 - NM3 Conc; µg/ml

3g	 IC 50 = 20 µg/ml Surviving Fraction HNO97 - NM6 Conc; µg/ml	 Surviving Fraction FaDu -NM6 Conc; µg/ml	 IC 50 = 41 µg/ml Surviving Fraction MDA-MB-468 - NM6 Conc; µg/ml
3h	 IC 50 = 11 µg/ml Surviving Fraction HNO97 - NM5 Conc; µg/ml	 IC 50 = 23 µg/ml Surviving Fraction FaDu -NM5 Conc; µg/ml	 IC 50 = 14 µg/ml Surviving Fraction MDA-MB-468 - NM5 Conc; µg/ml
4a	 IC 50 = 33 µg/ml Surviving Fraction HNO97 - NM2 Conc; µg/ml	 IC 50 = 35 µg/ml Surviving Fraction FaDu -NM2 Conc; µg/ml	 IC 50 = 24 µg/ml Surviving Fraction MDA-MB-468 - NM2 Conc; µg/ml

4b	 IC 50 = 5.7 µg/ml Surviving Fraction HNO97 - NM11 Conc; µg/ml	 IC 50 = 31 µg/ml Surviving Fraction FaDu -NM11 Conc; µg/ml	 IC 50 = 11 µg/ml Surviving Fraction MDA-MB-468 - NM11 Conc; µg/ml
4c	 IC 50 = 15 µg/ml Surviving Fraction HNO97 - NM13 Conc; µg/ml	 Surviving Fraction FaDu -NM13 Conc; µg/ml	 IC 50 = 18 µg/ml Surviving Fraction MDA-MB-468 - NM13 Conc; µg/ml
4d	 IC 50 = 10.7 µg/ml Surviving Fraction HNO97 - NM12 Conc; µg/ml	 IC 50 = 44 µg/ml Surviving Fraction FaDu -NM12 Conc; µg/ml	 IC 50 = 36 µg/ml Surviving Fraction MDA-MB-468 - NM12 Conc; µg/ml

4e	 IC 50 = 19 µg/ml Surviving Fraction HNO97 - NM14 Conc; µg/ml	 IC 50 = 45 µg/ml Surviving Fraction FaDu -NM14 Conc; µg/ml	 IC 50 = 44 µg/ml Surviving Fraction MDA-MB-468 - NM14 Conc; µg/ml
5	 Surviving Fraction HNO97 - NM9 Conc; µg/ml	 Surviving Fraction FaDu -NM9 Conc; µg/ml	 IC 50 = 36 µg/ml Surviving Fraction MDA-MB-468 - NM9 Conc; µg/ml

Materials and Methods

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

The antitumor activities of compounds (**3a-h, 4a-e,** and **5**) 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 **3a-h, 4a-e,** and **5** 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 HNO97, FaDu, and MDA-MB-468 cancer cell lines and normal HSF cell line

The antitumor activities of compounds (**3a-h, 4a-e,** and **5**) against HNO97, FaDu, and MDA-MB-468 cells, besides the normal HSF cell line, 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 **3a-h, 4a-e,** and **5** 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 p53, BAX, caspase 3, caspase 6, BCL-2, and CK 18. Standards or samples are then added to the appropriate microplate wells with a biotin-conjugated antibody specific to p53, BAX, caspase 3, caspase 6, BCL-2, and CK 18. 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 p53, BAX, caspase 3, caspase 6, BCL-2, and CK 18, 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 p53, BAX, caspase 3, caspase 6, BCL-2, and CK 18 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 p53, BAX, caspase 3, caspase 6, BCL-2, and CK 18 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. Prepare 7 wells for standard, 1 well for blank. Add 100 µL each of dilutions of standard (read Reagent Preparation), blank, and samples into the appropriate wells. Cover with the Plate sealer. Incubate for 1 h at 37 ºC.

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 ºC.

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 50μL 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. Western blot assays

Western blots were carried out as described previously [2]. In brief, cells were grown under the specific condition with the tested drug in 6 well plate, after the desired incubation period, the growth medium was removed from the cells, then rinse cells in PBS, and remove the PBS. 100μl 1X SR reagent into a culture dish and scrape the attached cells, then transfer them to a microtube. Measure protein concentration using a BCA kit, prepare the sample by adding loading buffer and heating at 95°C for 5 min. Cell lysates (30 μg) were separated by SDS-polyacrylamide gel electrophoresis, transferred onto a polyvinylidene difluoride membrane 12.5% or 15%, and then exposed to the appropriate antibodies.

Primary antibodies for Anti-PARP1 antibody [EPR18461] (ab191217), cleaved caspase-3 from Cell Signaling Technology Inc. (Beverly, MA, USA), Bax monoclonal antibody from Santa Cruz Biotechnology (Santa Cruz, CA, USA), anti-alpha tubulin antibody from Sigma (Saint-Quentin-Fallavier, France), Anti-phospho-Histone H2A.X (Ser139) Antibody, clone JBW301 were used at 1:1000, Horseradish peroxidase-conjugated anti-rabbit, or anti-mouse secondary antibodies were purchased from GE Healthcare (Amersham, UK) and used at 1:10,000. Proteins were visualized with the ECL system (Amersham, UK). Membranes were developed using Odyssey Fc Imager (LI-COR, U.S.). The western blot assays were representative of at least three independent experiments.

SI5. Cell cycle analysis

Human head and neck squamous cell carcinoma (HNO97) cells 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 each compound (**3c** or **4b**), 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] P. Skehan, R. Storeng, D. Scudiero, A. Monks, J. McMahon, D. Vistica, J.T. Warren, H. Bokesch, S. Kenney, M.R. Boyd, New colorimetric cytotoxicity assay for anticancer-drug screening, JNCI: Journal of the National Cancer Institute 82(13) (1990) 1107-1112.
[2] B.T. Kurien, R.H. Scofield, Western blotting, Methods 38(4) (2006) 283-293.