Supporting Information

Synthesis new series of 4-pyrazolylquinolinones with apoptotic antiproliferative effects as dual EGFR/BRAF^{V600E} inhibitors

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Spectral data for compound 5a

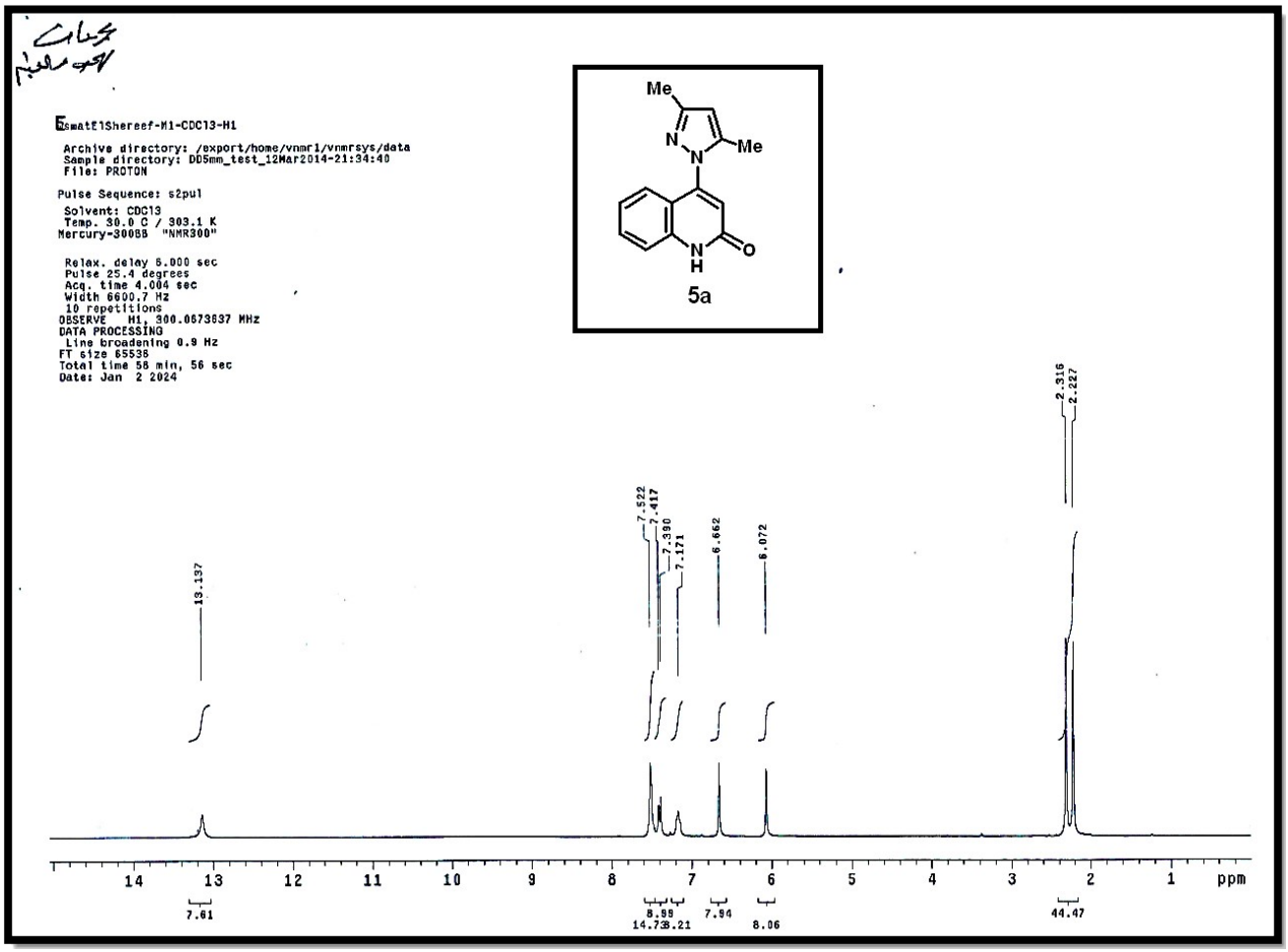


Figure 1. [1]H-NMR spectrum for compound **5a**

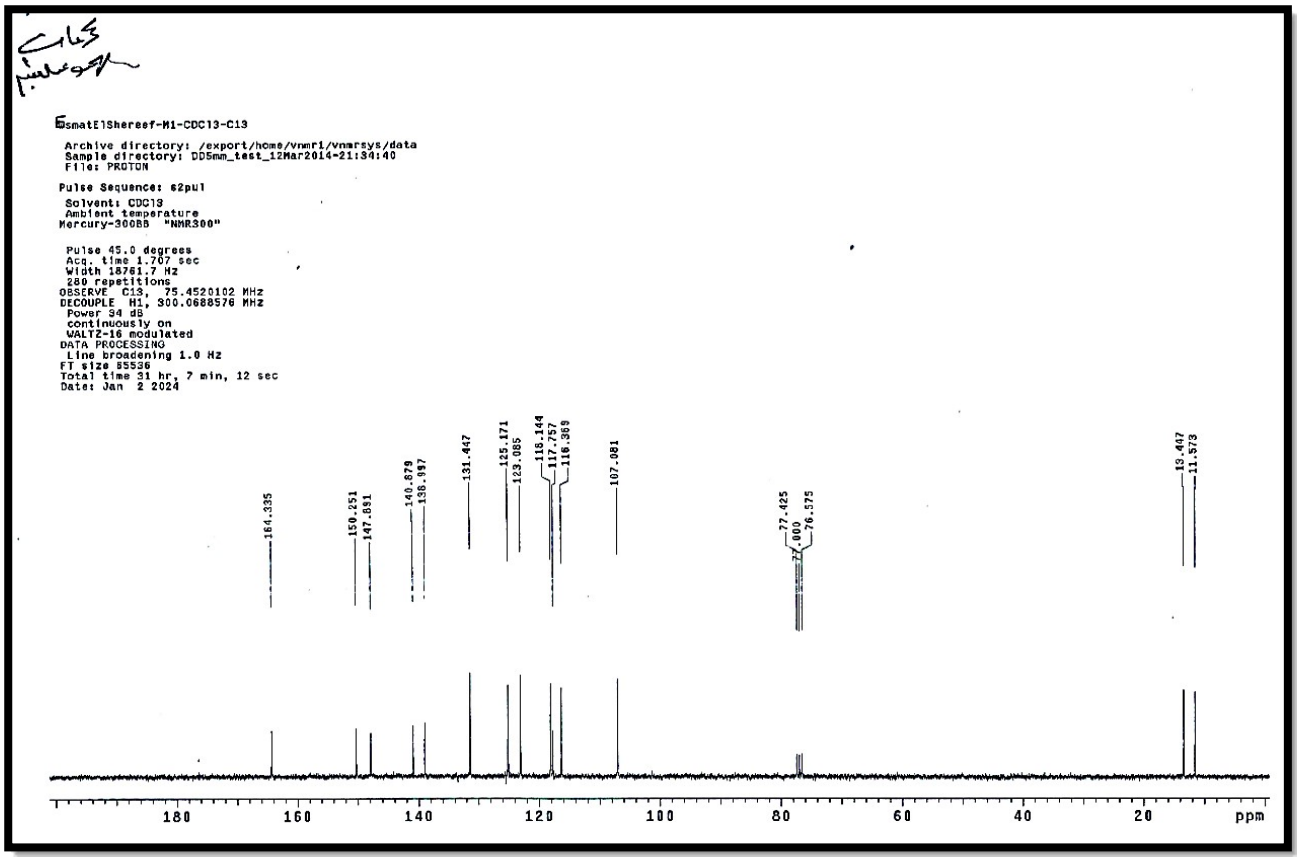


Figure 2. ^{13}C-NMR spectrums for compound **5a**

Spectral data for compound 5b

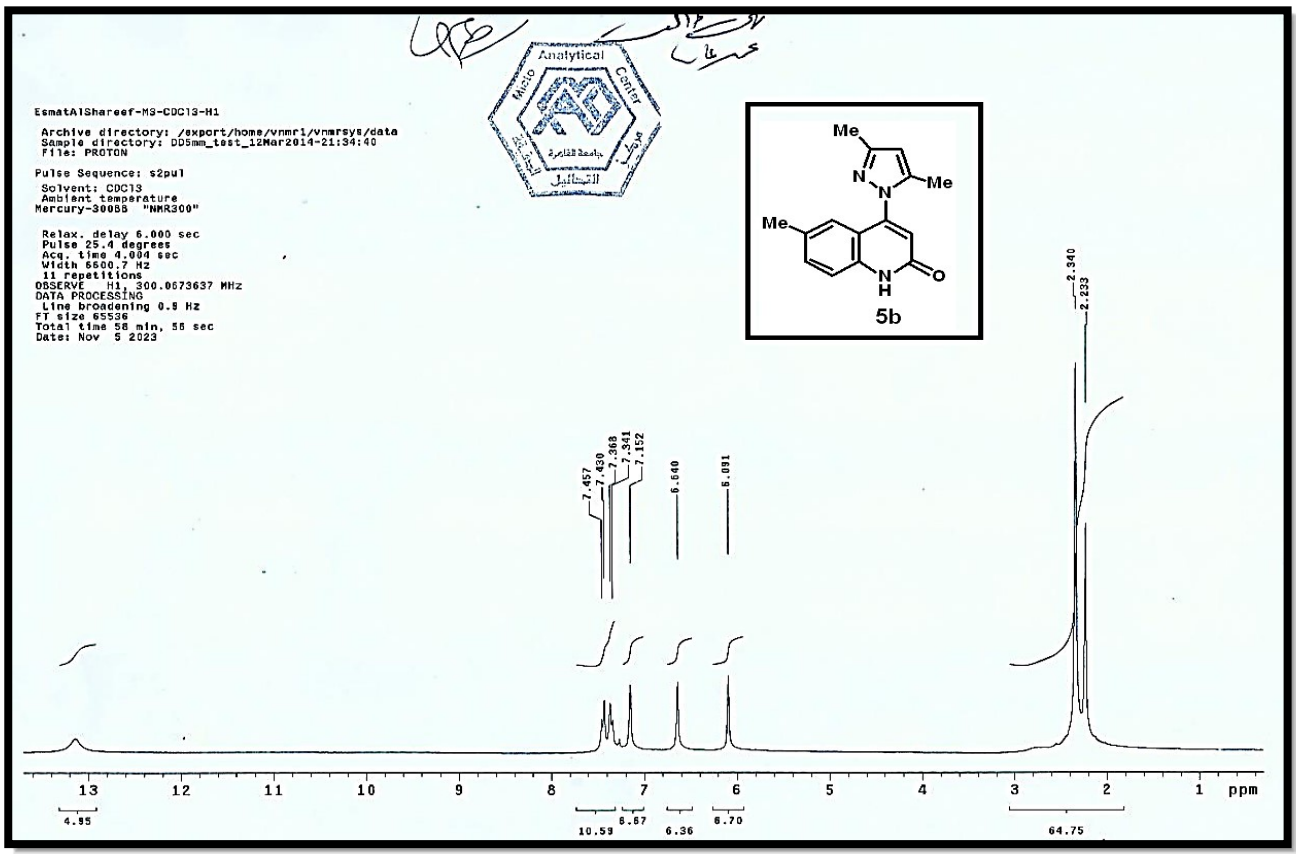


Figure 3. ^{1}H-NMR spectrum for compound **5b**

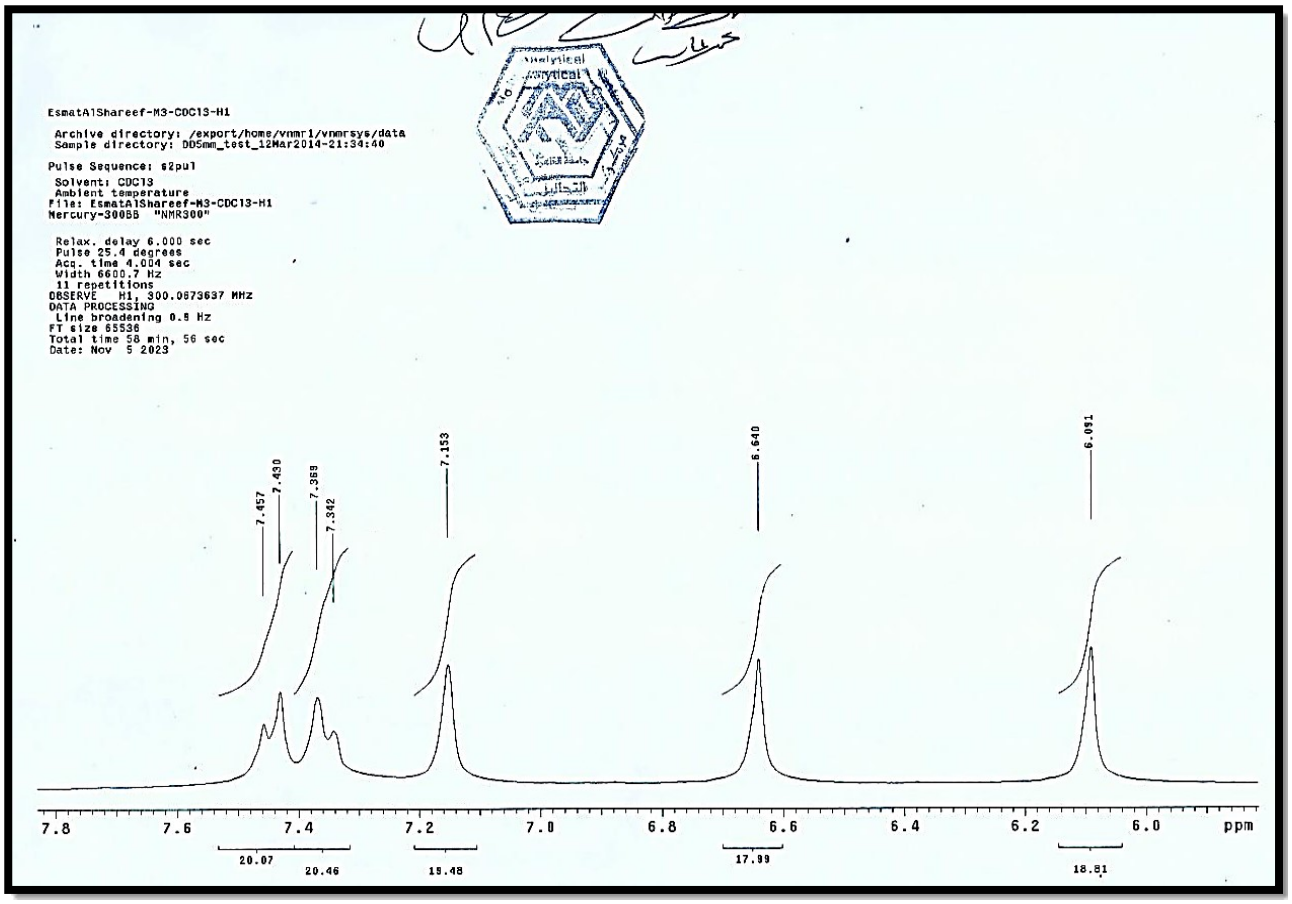


Figure 4. Part of ^{1}H-NMR spectrums for compound **5b**

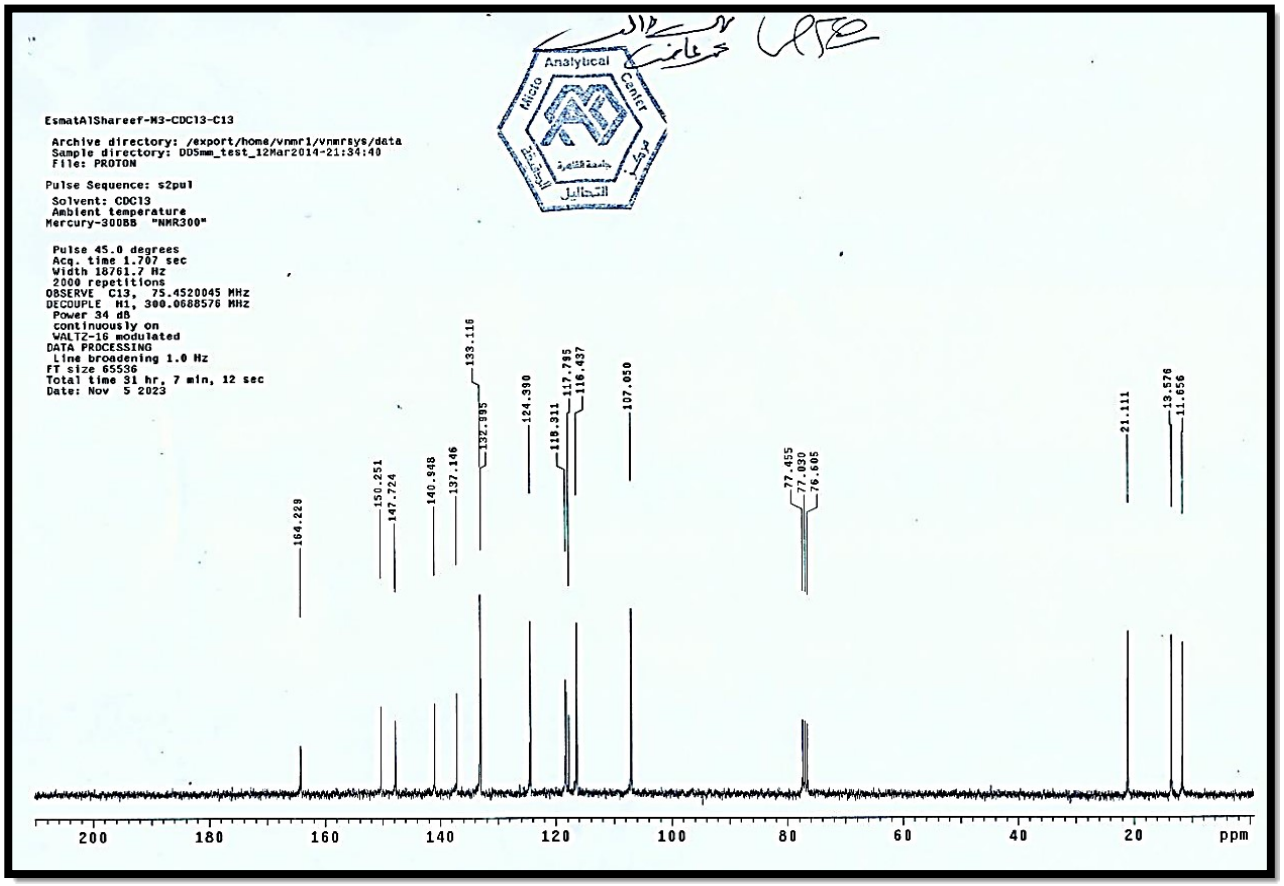


Figure 5. ^{13}C-NMR spectrum for compound **5b**

Spectral data for compound 5c

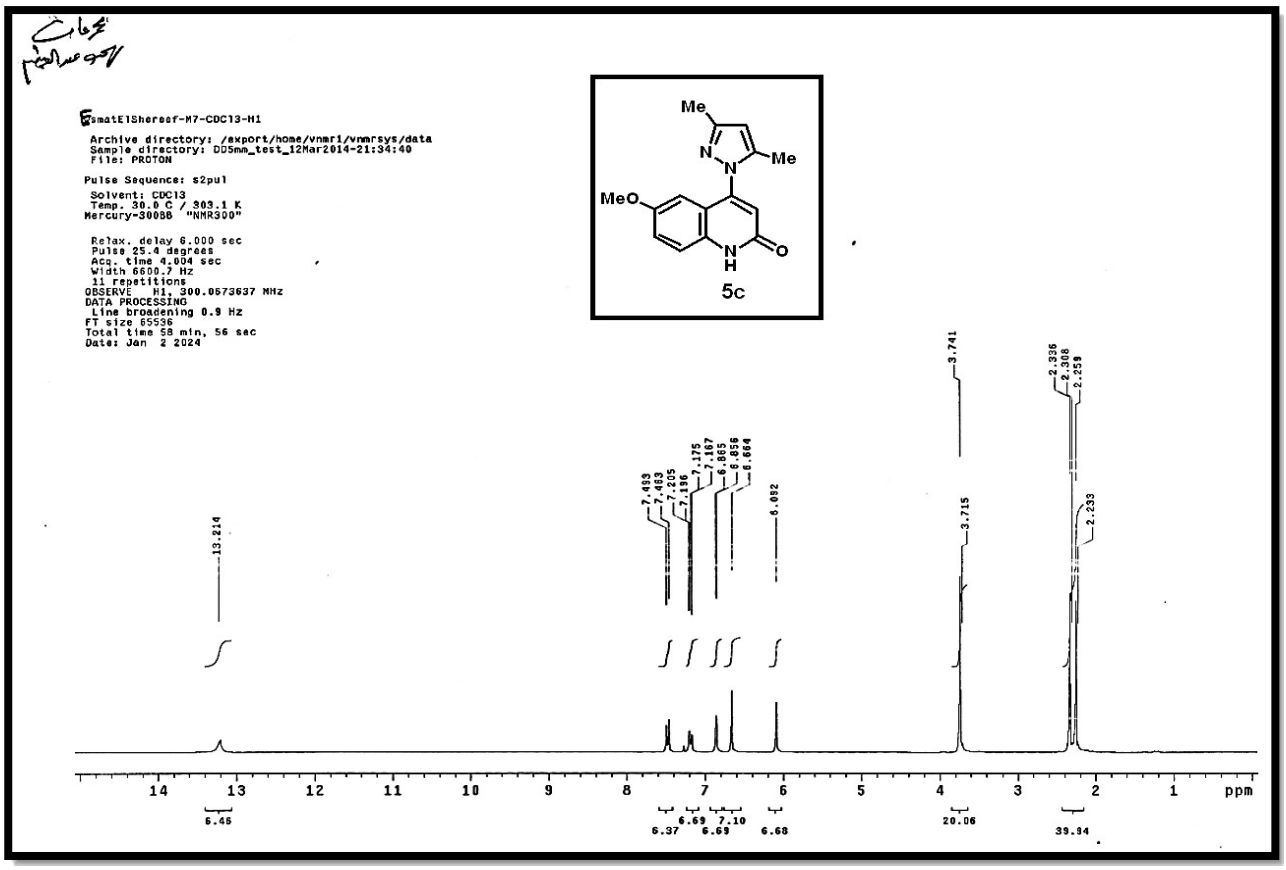


Figure 6. [1]H-NMR spectrum for compound **5c**

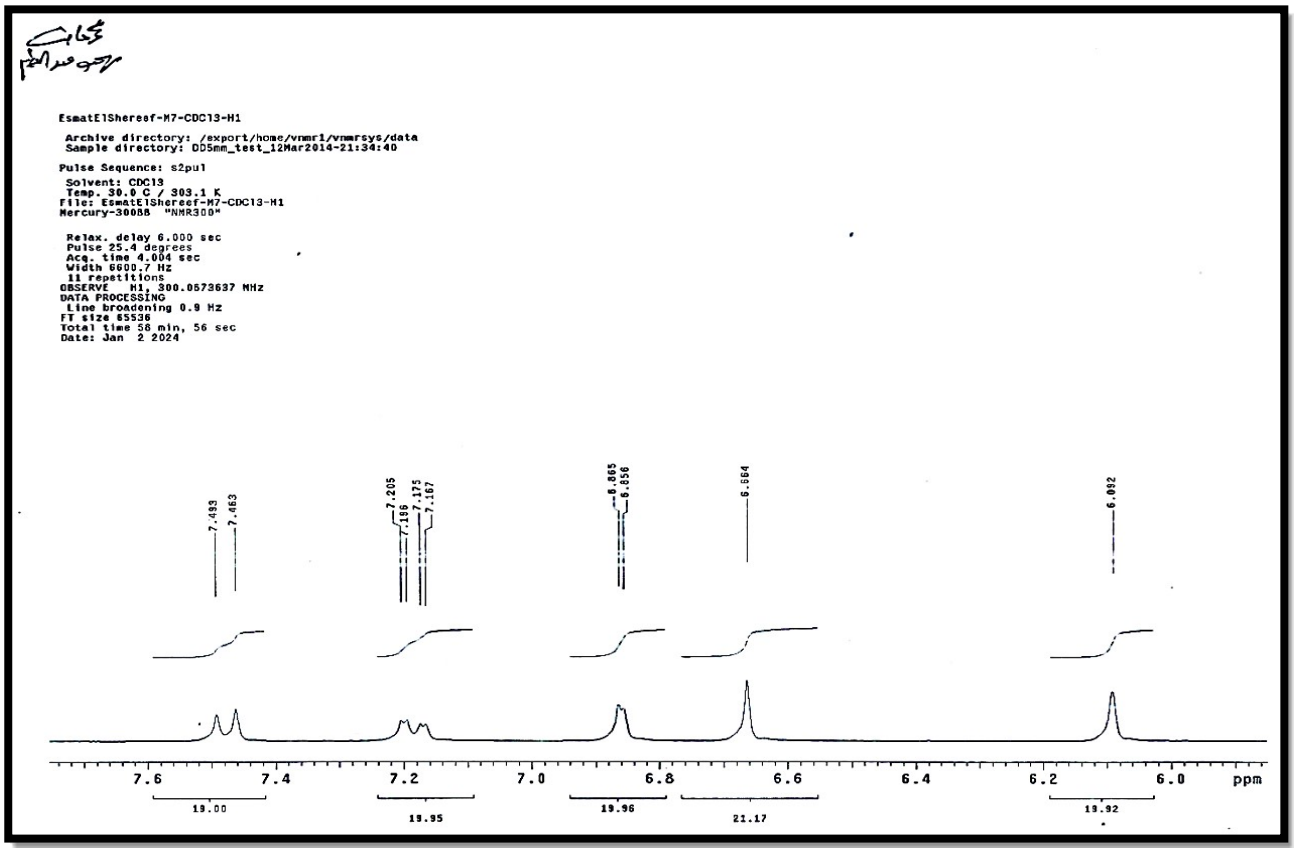


Figure 7. Part of [1]H-NMR spectrum for compound **5c**

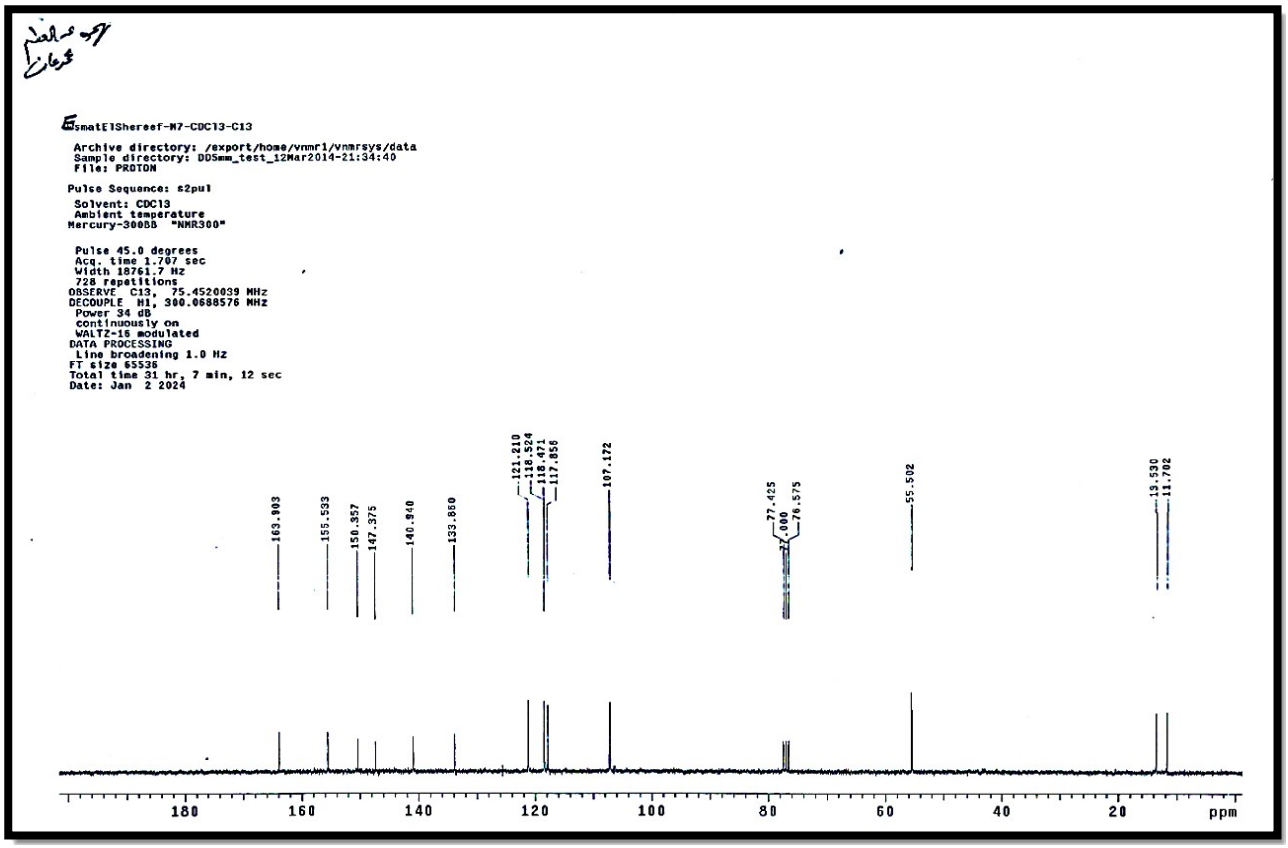


Figure 8. ^{13}C-NMR spectrum for compound **5c**

Spectral data for compound 5d

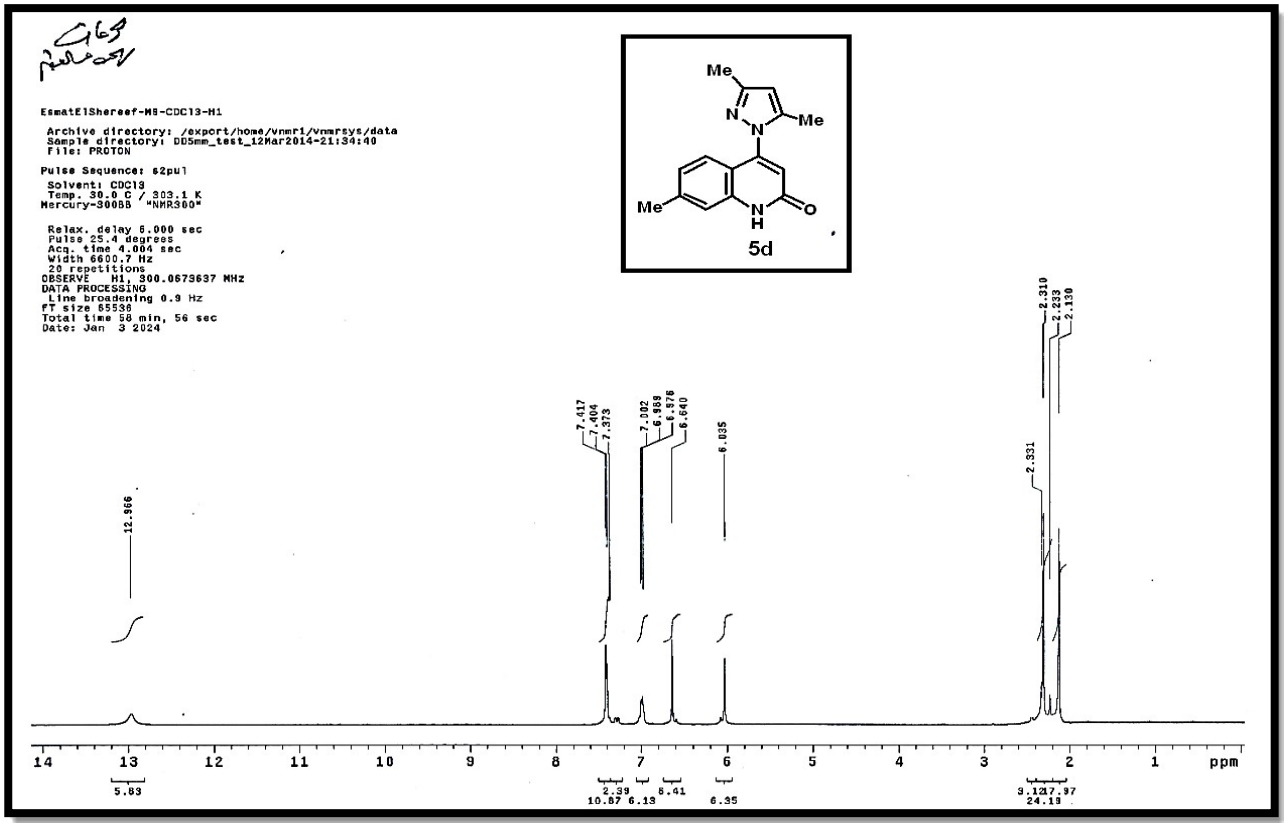


Figure 9. ^{1}H-NMR spectrum for compound **5d**

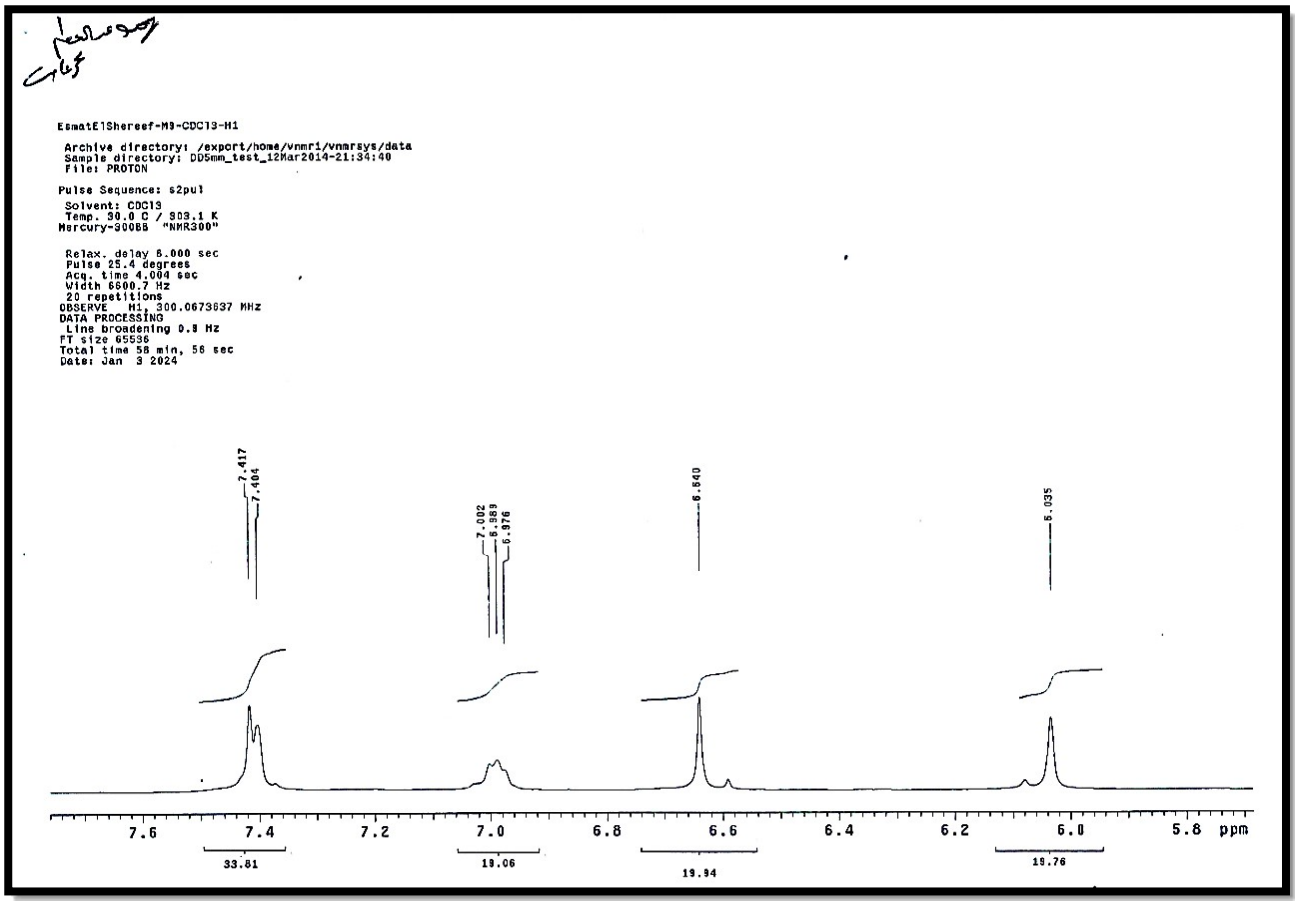


Figure 10. Part of ^{1}H-NMR spectrum for compound **5d**

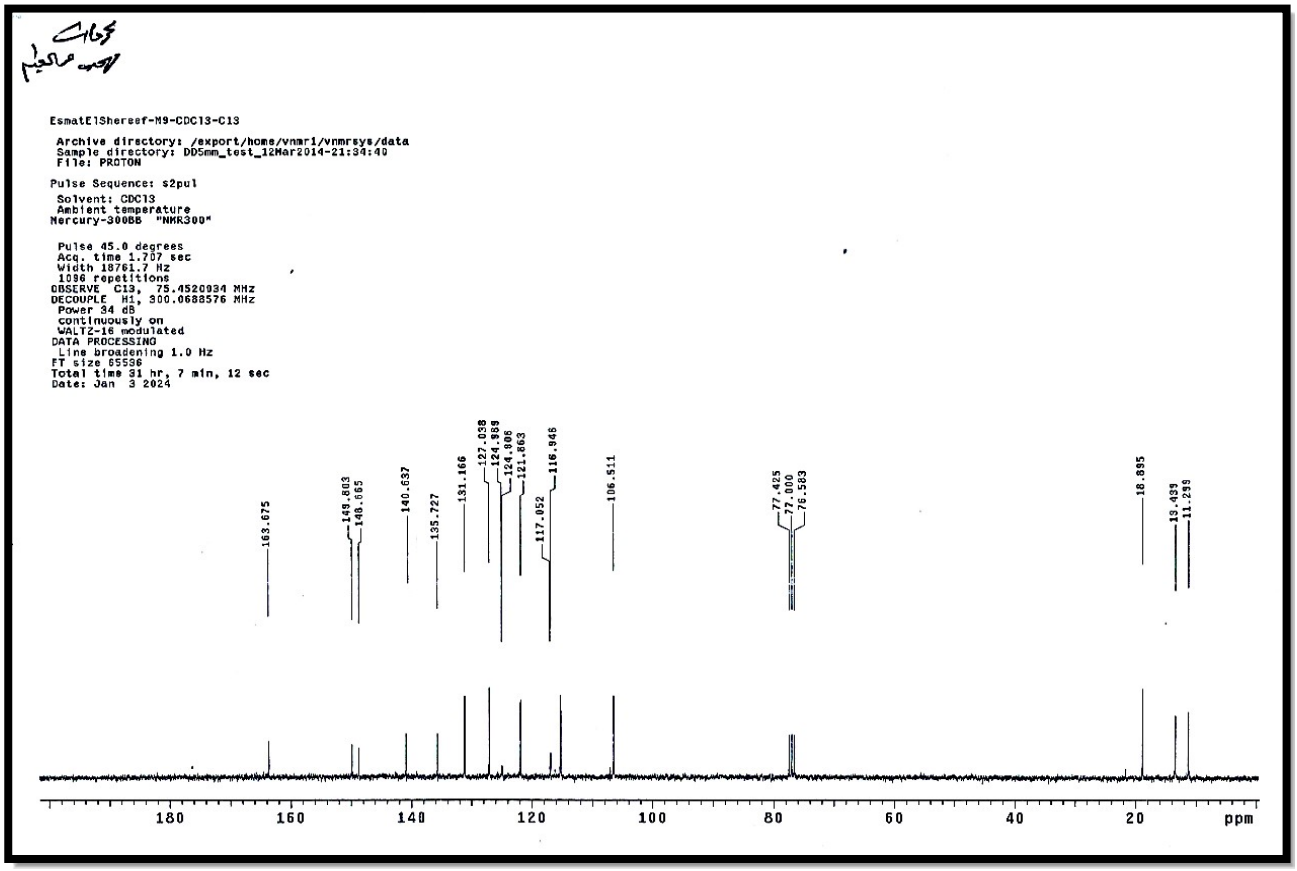


Figure 11. ^{13}C-NMR spectrum for compound **5d**

Spectral data for compound 5e

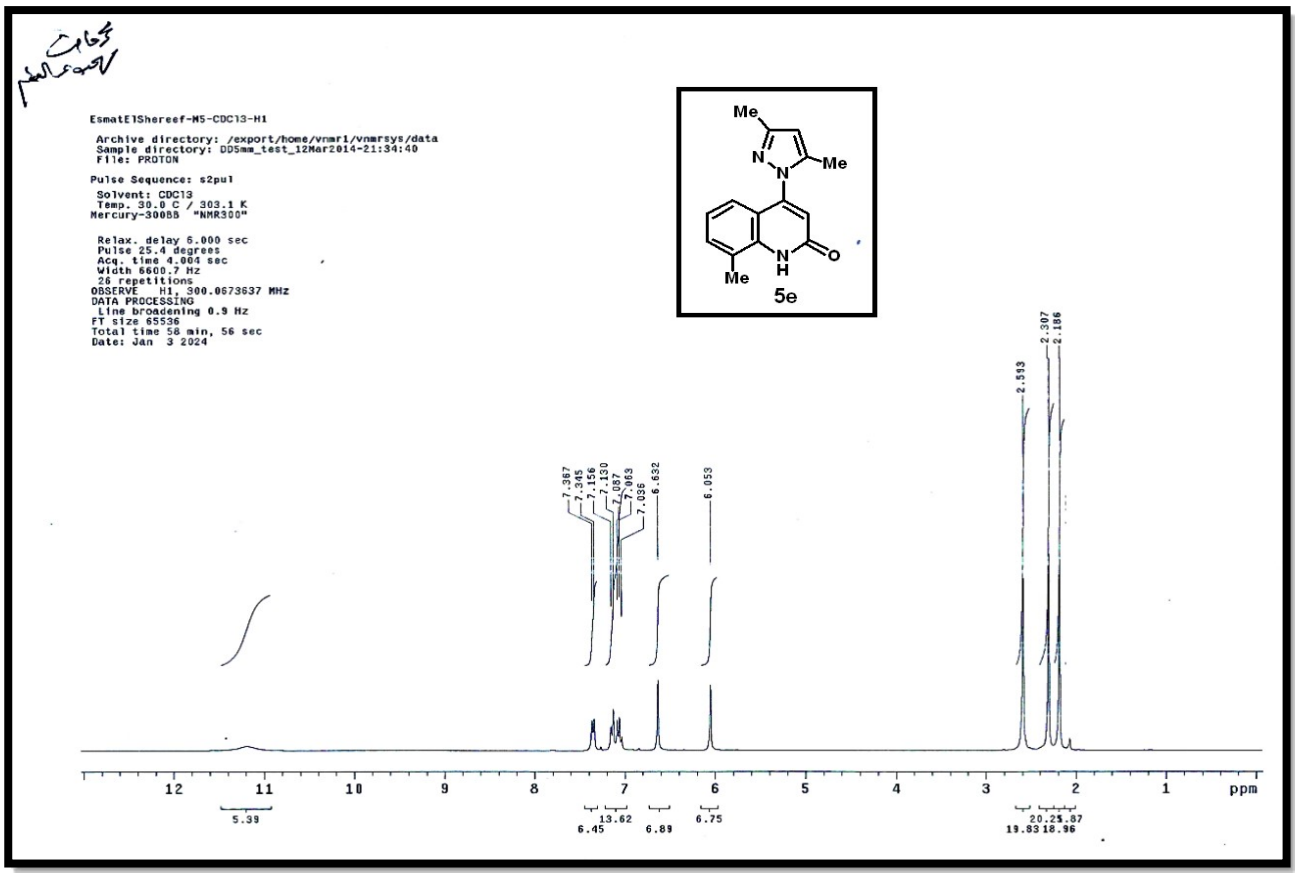


Figure 12. [1]H-NMR spectrum for compound **5e**

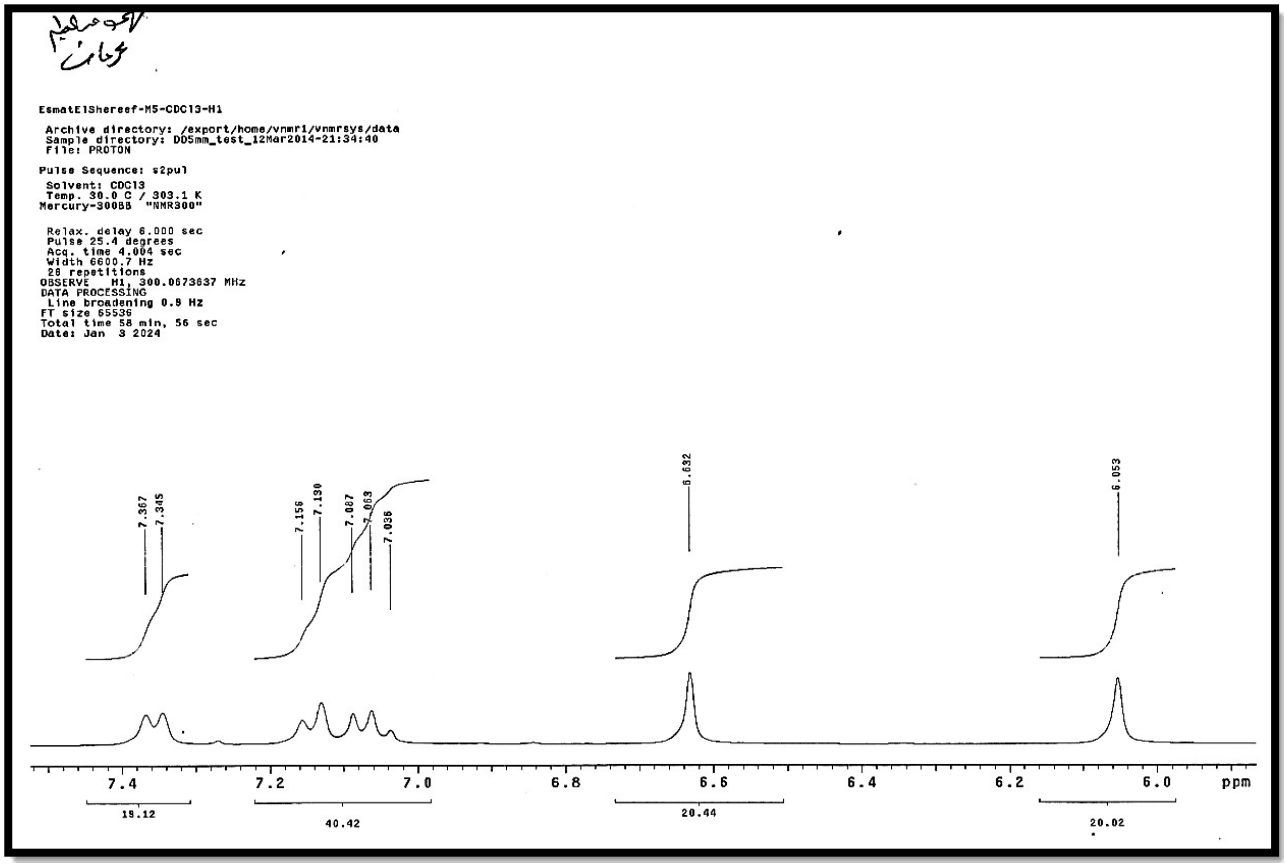


Figure 13. Part of [1]H-NMR spectrum for compound **5e**

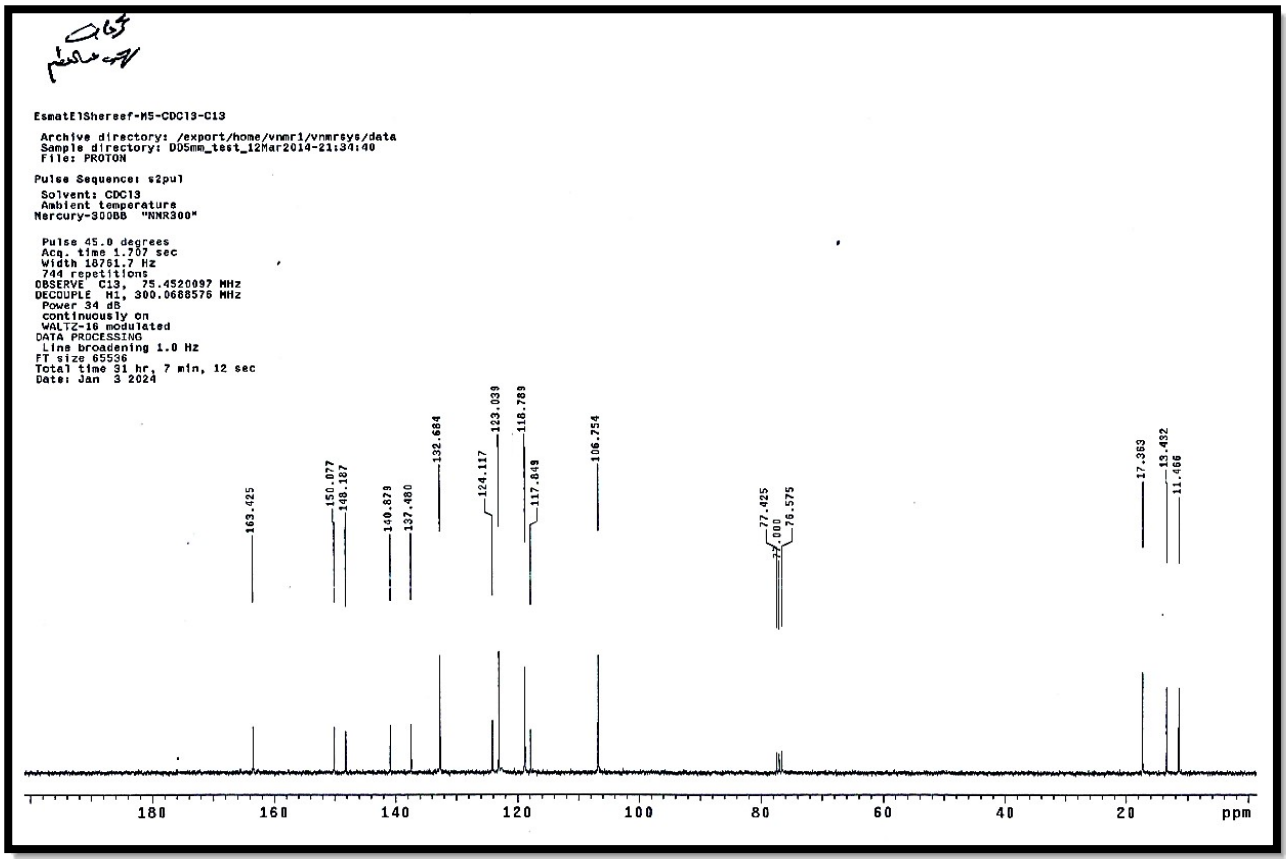


Figure 14. ^{13}C-NMR spectrum for compound **5e**

Spectral data for compound 5f

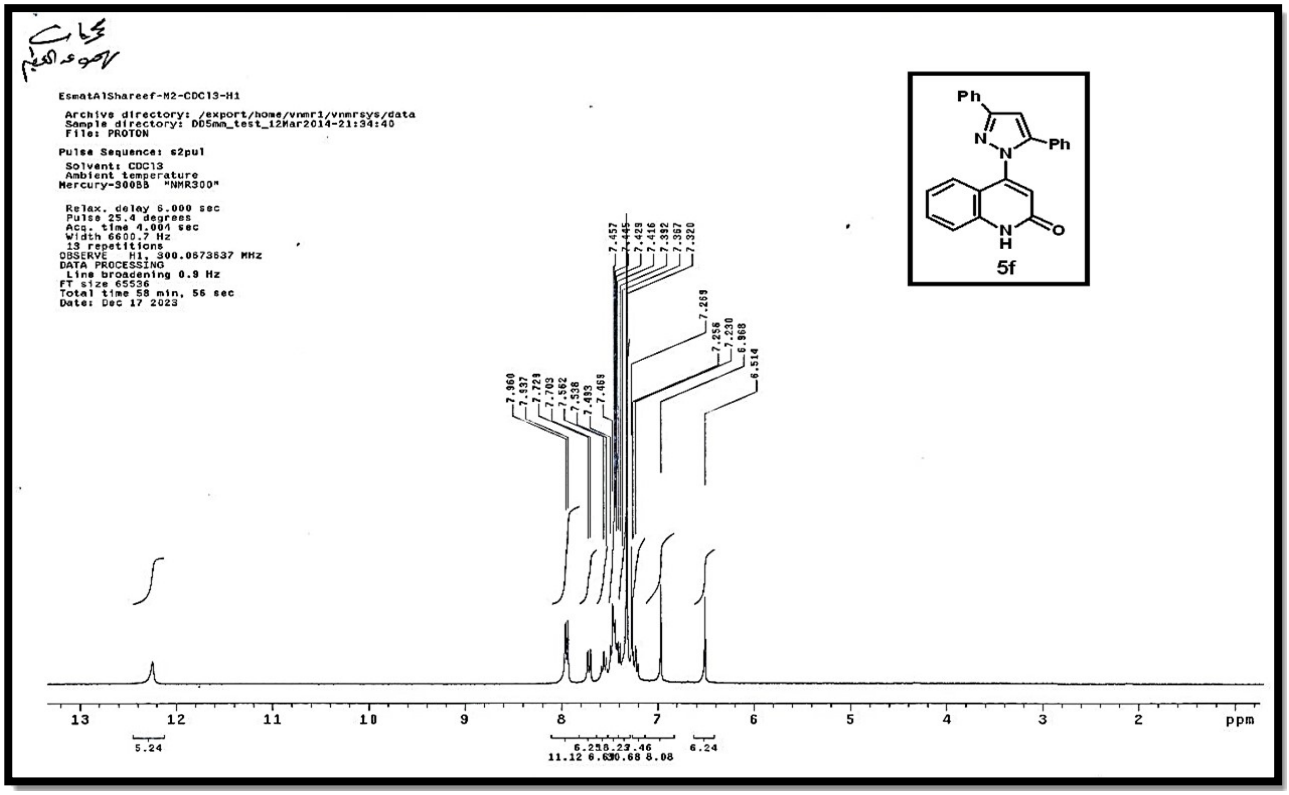


Figure 15. ^{1}H-NMR spectrum for compound **5f**

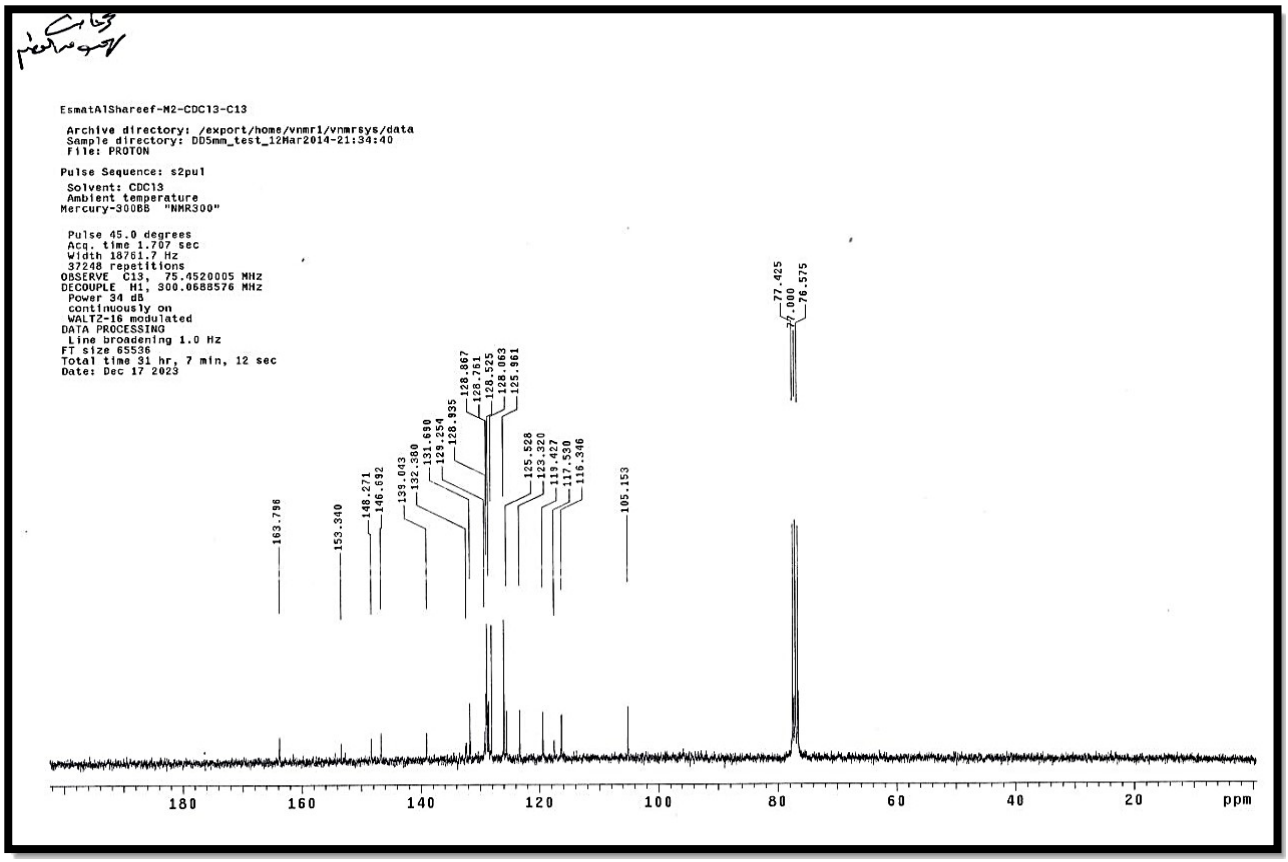


Figure 16. ^{13}C-NMR spectrum for compound **5f**

Spectral data for compound 5g

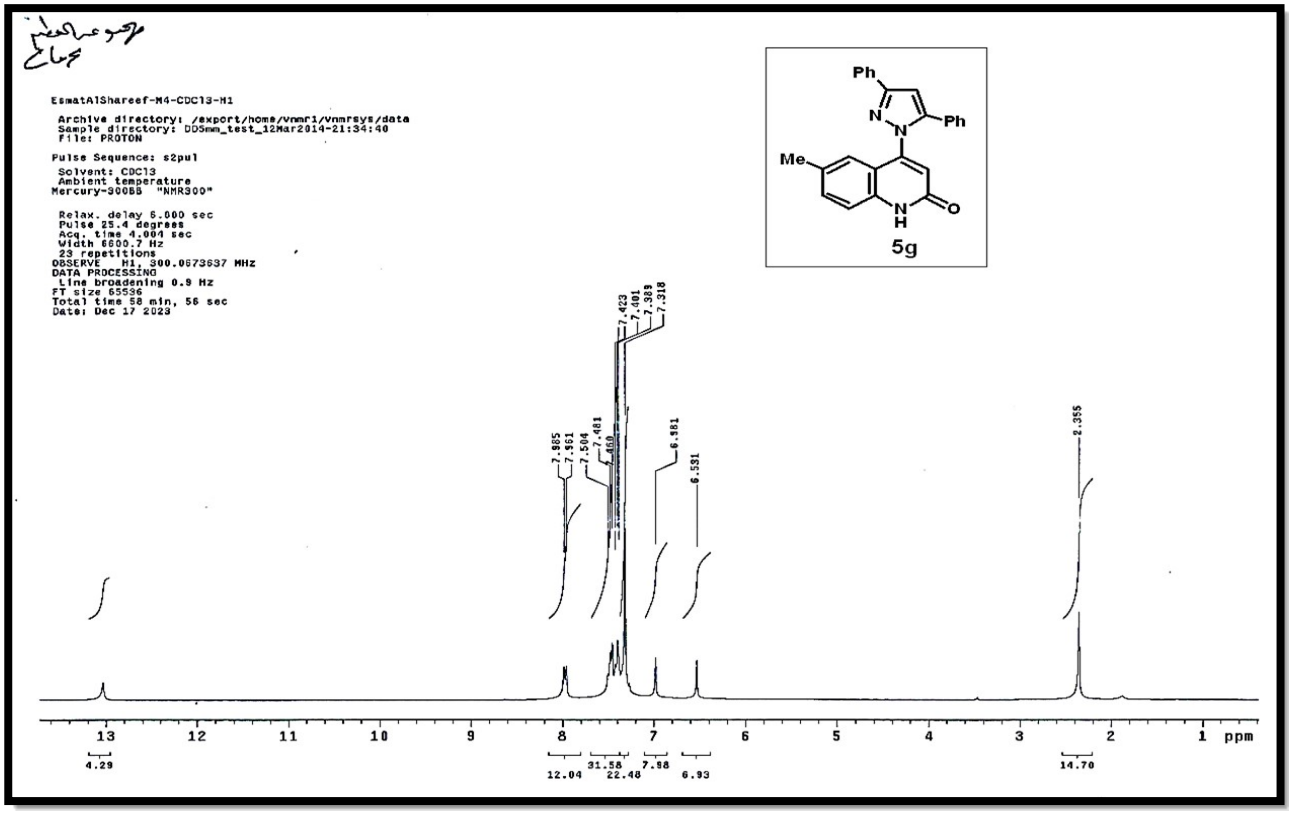


Figure 17. ^{1}H-NMR spectrum for compound **5g**

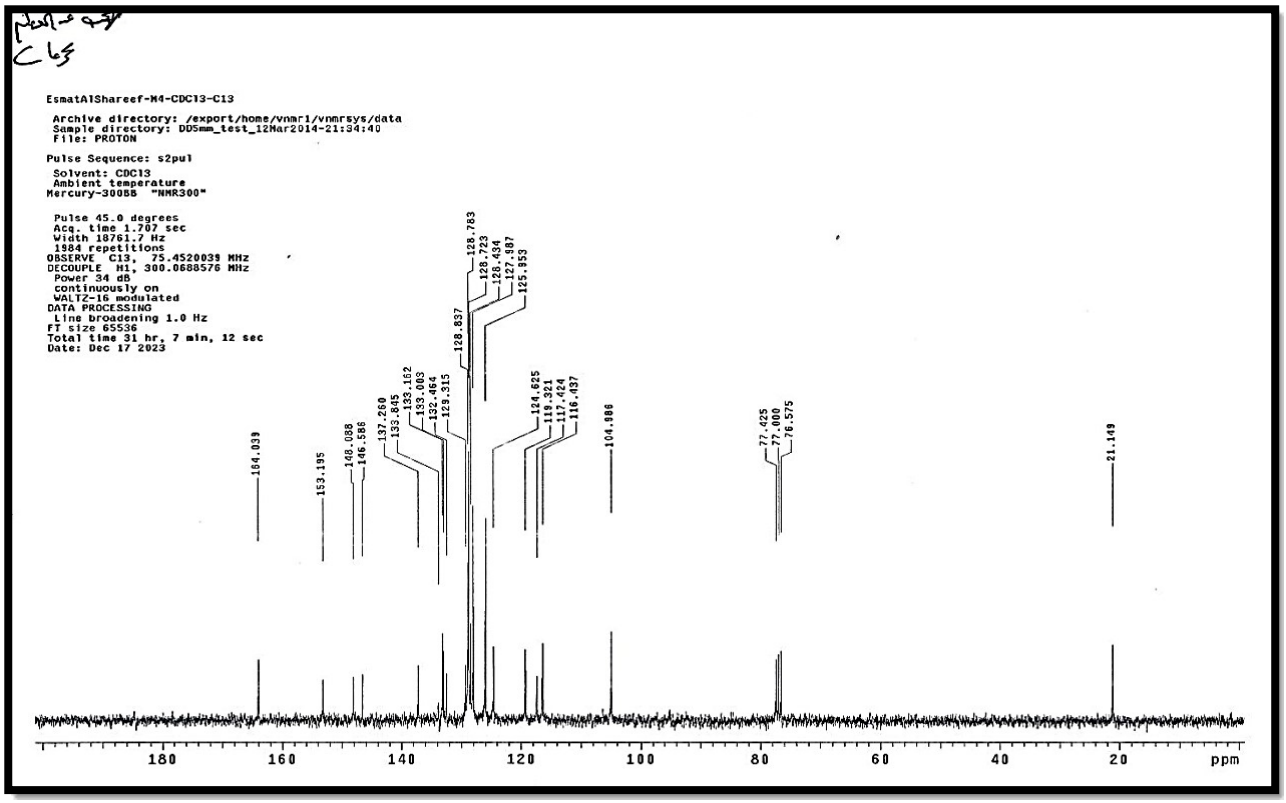


Figure 18. ^{13}C-NMR spectrum for compound **5g**

Spectral data for compound 5h

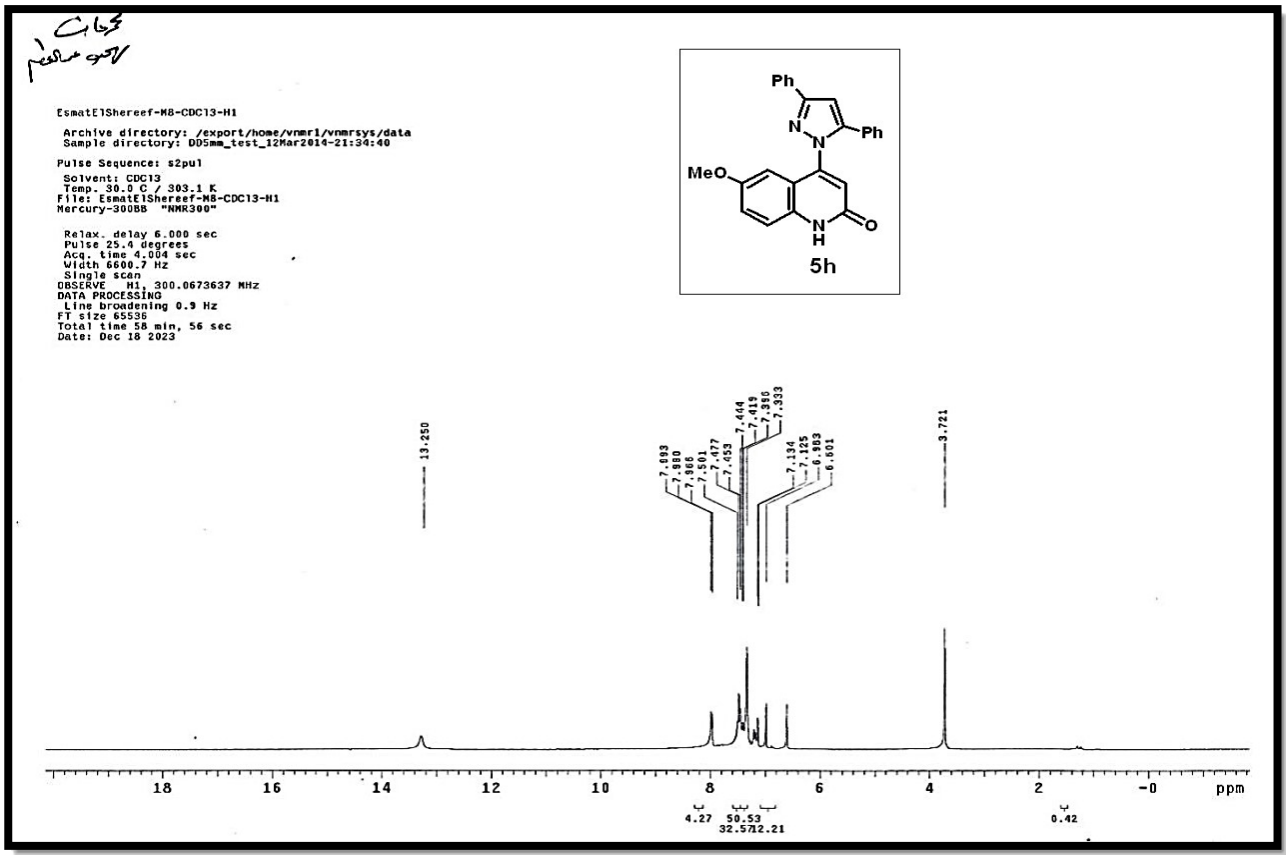


Figure 19. ^{1}H-NMR spectrum for compound **5h**

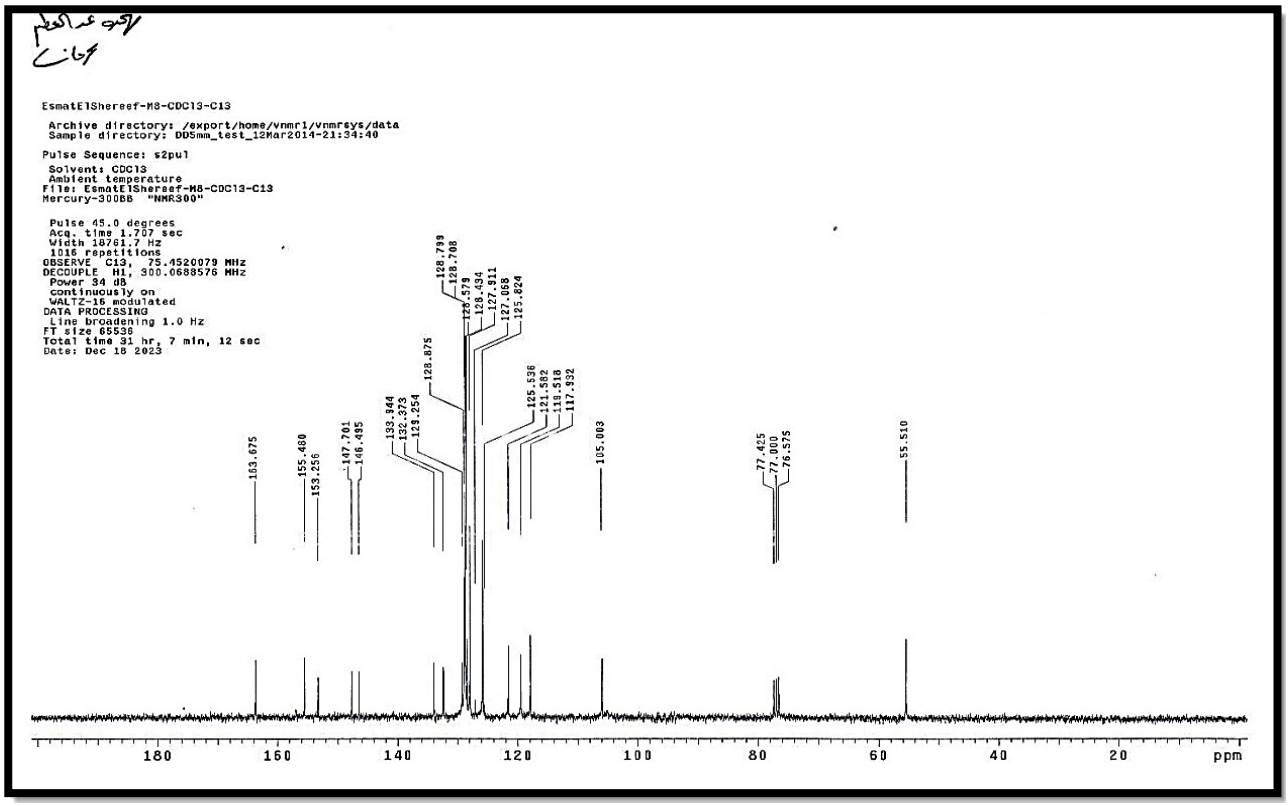


Figure 20. ^{13}C-NMR spectrum for compound **5h**

Spectral data for compound 5i

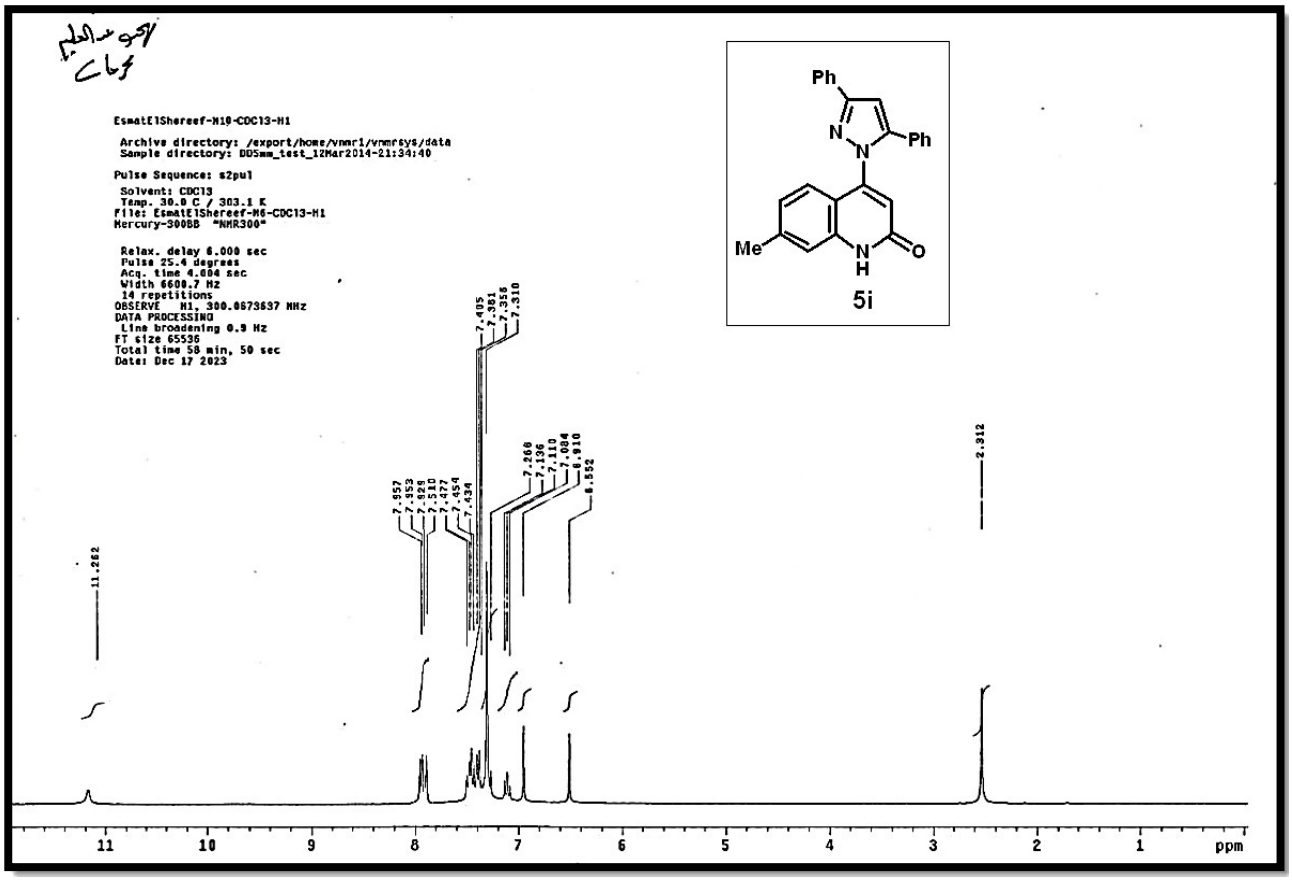


Figure 21. ^{1}H-NMR spectrum for compound **5i**

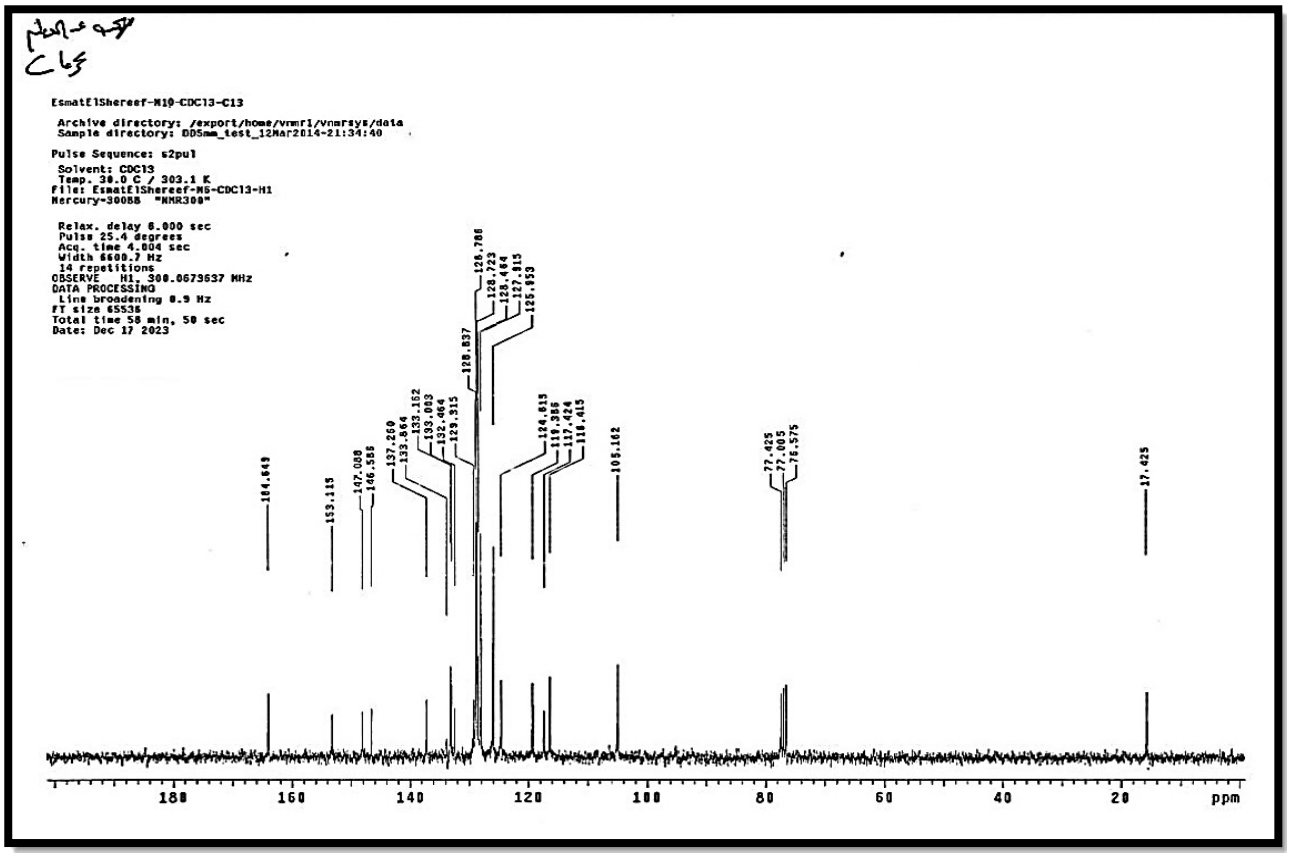


Figure 22. ^{13}C-NMR spectrum for compound **5i**

Spectral data for compound 5j

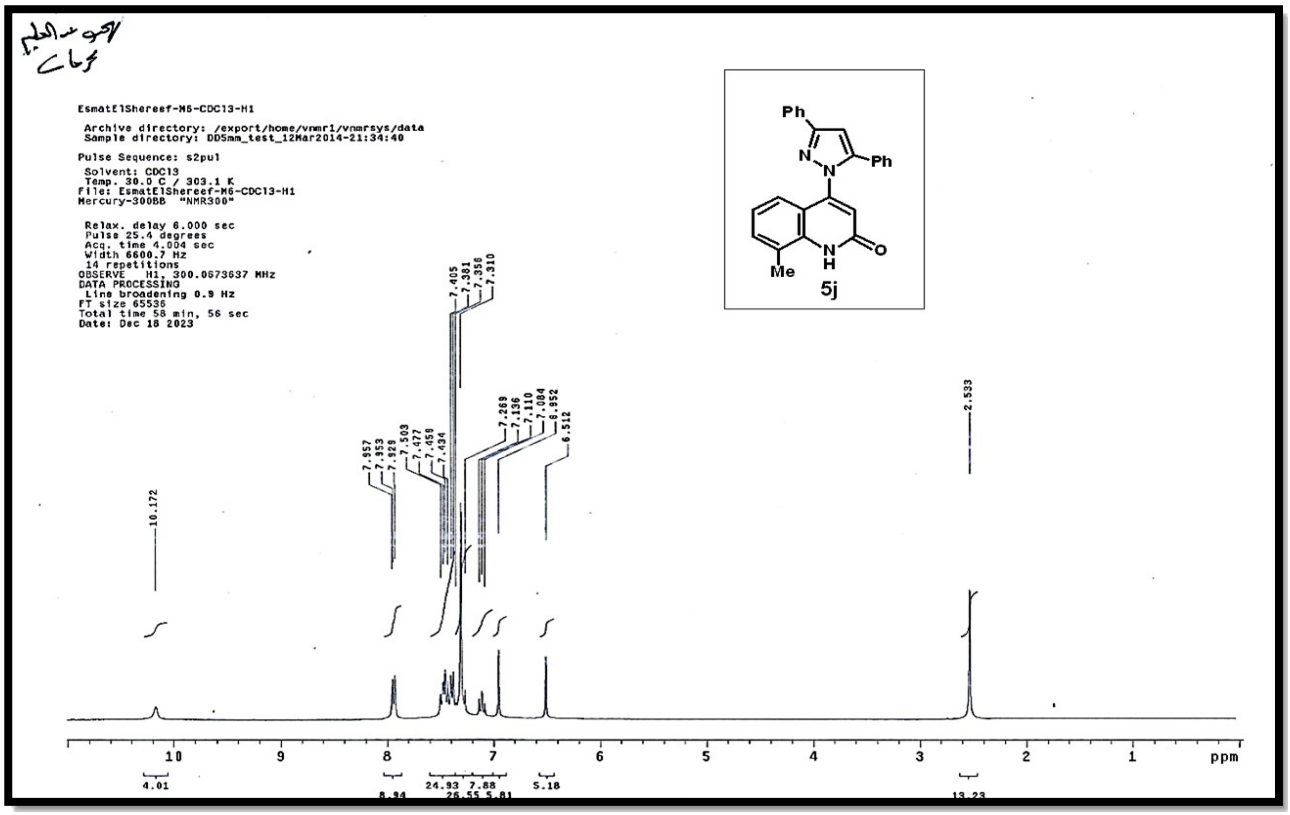


Figure 23. ^{1}H-NMR spectrum for compound **5j**

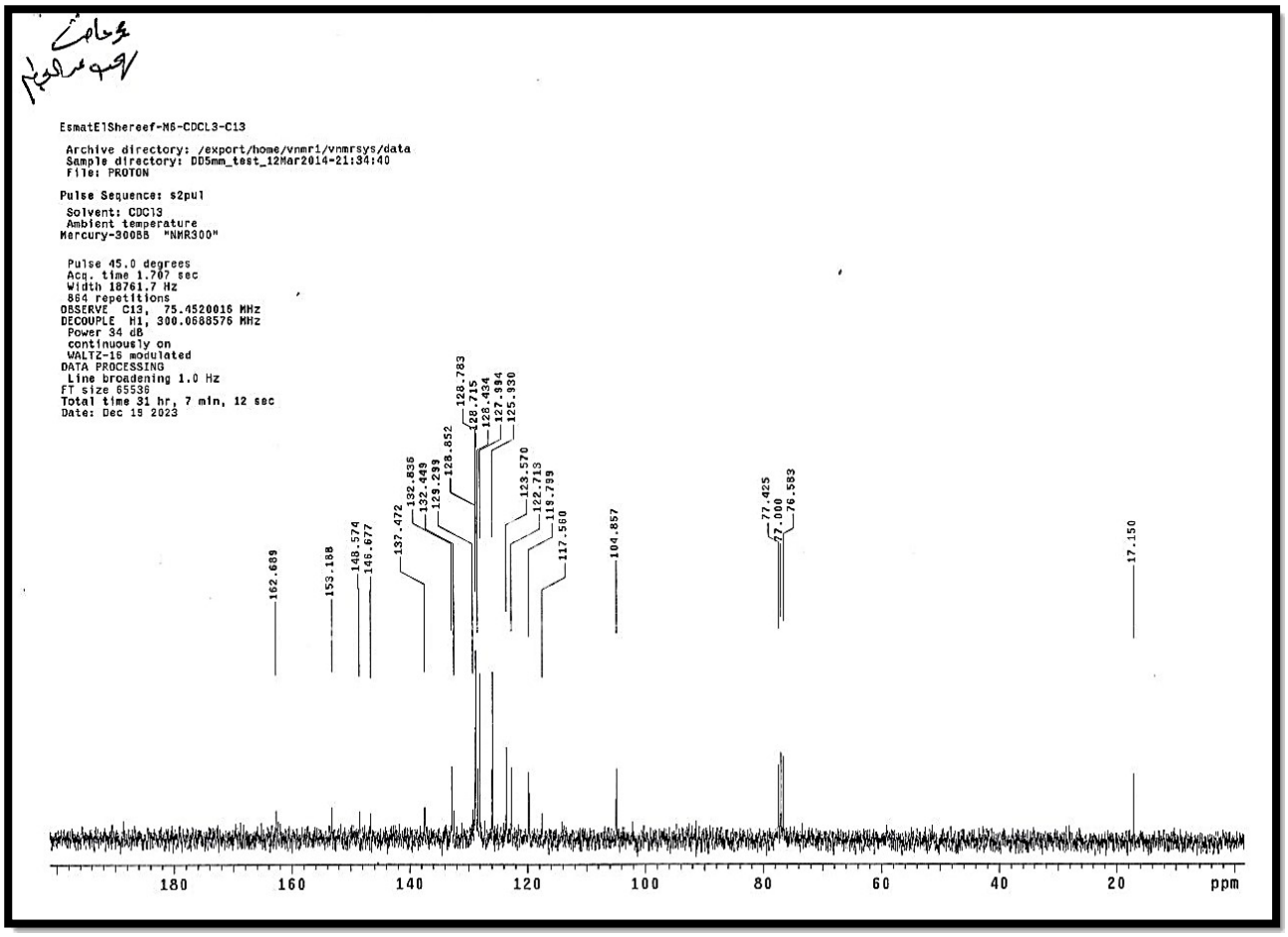
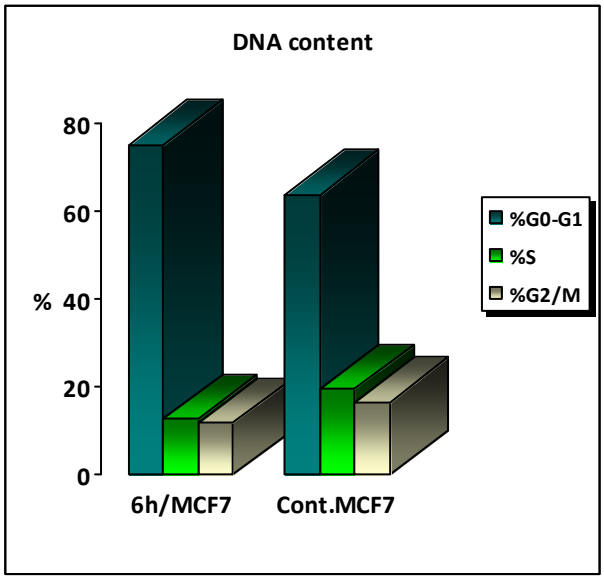


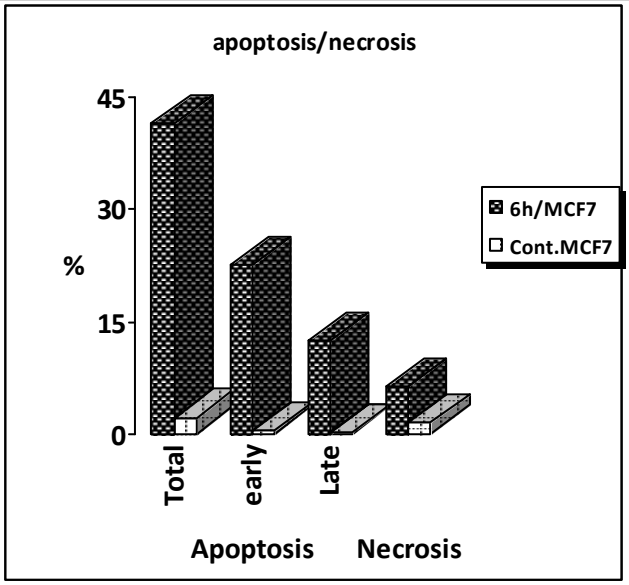
Figure 24. ^{13}C-NMR spectrum for compound **5j**

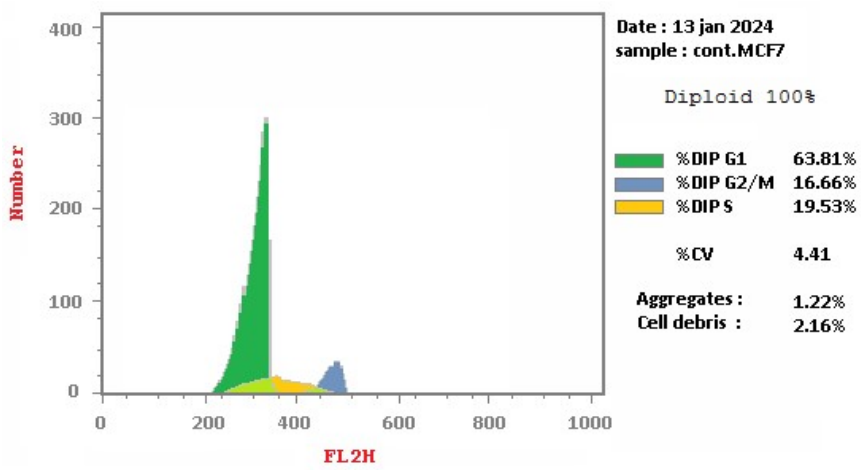
Lab Report

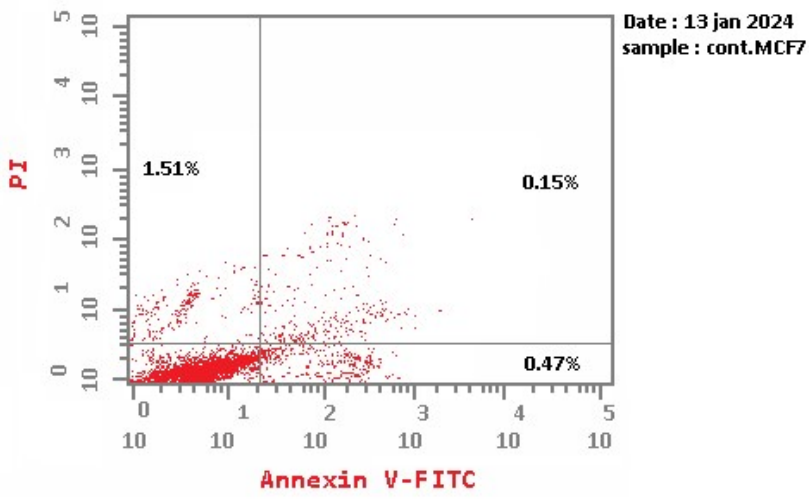
ser	Sample		DNA content			
	code	IC50 uM	%G0-G1	%S	%G2/M	Comment
1	6h/MCF7	---	75.33	12.81	11.86	cell growth arrest@ G1
2	Cont.MCF7	---	63.81	19.53	16.66	---

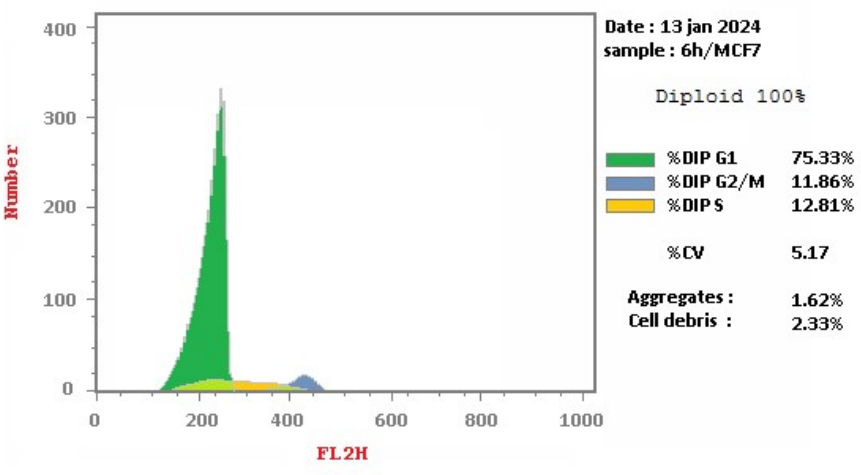


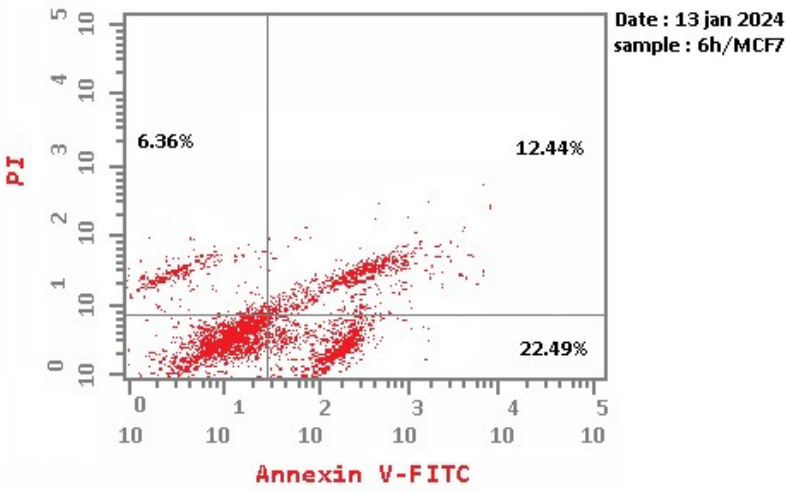
s	code	conc	Apoptosis			Necrosis
			Total	Early	Late	
1	6h/MCF7	---	41.29	22.49	12.44	6.36
2	Cont.MCF7	---	2.13	0.47	0.15	1.51




Number
FL2H
Date : 13 jan 2024
sample : cont.MCF7
Diploid 100%
%DIP G1 63.81%
%DIP G2/M 16.66%
%DIP S 19.53%
%CV 4.41
Aggregates : 1.22%
Cell debris : 2.16%


PI
Annexin V-FITC
1.51%
0.15%
0.47%
Date : 13 jan 2024
sample : cont.MCF7


Number
400
300
200
100
0
0
200
400
600
800
1000
FL2H
Date : 13 jan 2024
sample : 6h/MCF7
Diploid 100%
%DIP G1 75.33%
%DIP G2/M 11.86%
%DIP S 12.81%
%CV 5.17
Aggregates : 1.62%
Cell debris : 2.33%


PI
6.36%
12.44%
22.49%
Annexin V-FITC
Date : 13 jan 2024
sample : 6h/MCF7

4. EXPERIMENTAL

4.1. Chemistry

General details

All the reactions progress were monitored with TLC (thin-layer chromatography) on Merck alumina-backed TLC plates and visualized under UV light. All spectral data were measured in DMSO-d_6 on a Bruker AV-300 spectrometer (300 MHz for ^{1}H and 75 MHz for ^{13}C) in the Micro Analytical Center, Cairo University, Egypt. Chemical shifts are expressed in δ (ppm) versus internal Tetramethylsilane (TMS) = 0 ppm for ^{1}H and ^{13}C. Also, the chemical shifts (δ) are reported in parts per million (pm) relative to Tetramethylsilane (TMS) as internal standard, and the coupling constants (*J*) are reported in Hertz (Hz). Splitting patterns are denoted as follows: singlet (s), doublet (d), multiplet (m), triplet (t), quartet (q) and doublet of doublets (dd). Melting points (mp) were determined with a Stuart melting point instrument and are expressed in °C. Elemental analyses were carried out on Perkin device at the Microanalytical Institute of Organic Chemistry, Karlsruhe University, Germany.

4.2. Biological evaluation

4.2.1. Cytotoxic activity using MTT Assay and evaluation of IC_{50}

4.2.1.1. MTT assay

MTT assay was carried out to study the effect of compounds on mammary epithelial cells (MCF-10A). The medium in which cells were propagated contained Dulbecco's modified Eagle's medium (DMEM)/ Ham's F-12 medium (1:1) supplemented with epidermal growth factor (20 ng/mL), hydrocortisone (500 ng/mL), insulin (10 µg/mL), 2 mM glutamine and 10% foetal calf serum. After every 2-3 days, the cells were passaged using trypsin ethylenediamine tetra acetic acid (EDTA). The cells were seeded at a density of 10^4 cells mL^{-1} in flat-bottomed culture plates containing 96 wells each. After 24 h, medium was removed from the plates and the compounds in (in 0.1% DMSO) were added (in 200 µL medium to yield a final concentration of 0.1% v/v) to the wells of plates. A single compound was designated with four wells followed by incubation of plates for 96h at 37°C. After incubation, medium was removed completely from the plates followed by addition of MTT (0.4 mg/mL in medium) to each well and subsequent incubation of plates for 3h. MTT (along with the medium) was removed and DMSO (150µL) was added to each well of the culture plates, followed by vortexing and subsequent measurement of absorbance (at 540 nm) using microplate reader. The data are shown as percentage inhibition of proliferation in comparison with controls containing 0.1% DMSO.

4.2.1.2. Assay for antiproliferative effect

To explore the antiproliferative potential of compounds MTT assay was performed according to previously reported procedure using different cell lines to explore the antiproliferative potential of compounds propidium iodide fluorescence assay was performed using different cell lines. To calculate the total nuclear DNA, a fluorescent dye (propidium iodide, PI) is used which can attach to the DNA, thus offering a quick and precise technique. PI cannot pass through the cell membrane and its signal intensity can be considered as directly proportional to quantity of cellular DNA. Cells whose

cell membranes are damaged or have changed permeability are counted as dead ones. The assay was performed by seeding the cells of different cell lines at a density of 3000-7500 cells/well (in 200µl medium) in culture plates followed by incubation for 24h at 37 °C in humidified 5% CO_2/95% air atmospheric conditions. The medium was removed; the compounds were added to the plates at 10 µM concentrations (in 0.1% DMSO) in triplicates, followed by incubation for 48 h. DMSO (0.1%) was used as control. After incubation, medium was removed followed by the addition of PI (25 µl, 50µg/mL in water/medium) to each well of the plates. At -80 °C, the plates were allowed to freeze for 24 h, followed by thawing at 25°C. A fluorometer (Polar-Star BMG Tech) was used to record the readings at excitation and emission wavelengths of 530 and 620 nm for each well. The percentage cytotoxicity of compounds was calculated using the following formula:

$$\% \text{ Cytotoxicity} = \frac{A_C - A_{TC}}{A_C} \times 100$$

Where A_{TC}= Absorbance of treated cells and A_C= Absorbance of control. Erlotinib was used as positive control in the assay.

4.2.1.3. EGFR inhibitory assay

EGFR-TK assay was performed to evaluate the inhibitory potency of the most potent compounds against EGFR. Baculoviral expression vectors including pBlueBacHis2B and pFASTBacHTc were used separately to clone 1.6 kb cDNA coding for EGFR cytoplasmic domain (EGFR-CD, amino acids 645–1186). 5′ upstream to the EGFR sequence comprised a sequence that encoded $(His)_6$. Sf-9 cells were infected for 72h for protein expression. The pellets of Sf-9 cells were solubilized in a buffer containing sodium vanadate (100 µM), aprotinin (10 µg/mL), triton (1%), HEPES buffer (50mM), ammonium molybdate (10 µM), benzamidine HCl (16 µg/mL), NaCl (10 mM), leupeptin (10 µg/mL) and pepstatin (10 µg/mL) at 0°C for 20 min at pH 7.4, followed by centrifugation for 20 min. To eliminate the non-specifically bound material, a Ni-NTA super flow packed column was used to pass through and wash the crude extract supernatant first with 10 mM and then with 100 mM

imidazole. Histidine-linked proteins were first eluted with 250 and then with 500 mM imidazole subsequent to dialysis against NaCl (50 mM), HEPES (20 mM), glycerol (10%) and 1 µg/mL each of aprotinin, leupeptin and pepstatin for 120 min. The purification was performed either at 4 °C or on ice. To record autophosphorylation level, EGFR kinase assay was carried out on the basis of DELFIA/Time-Resolved Fluorometry. The compounds were first dissolved in DMSO absolute, subsequent to dilution to appropriate concentration using HEPES (25 mM) at pH 7.4. Each compound (10 µL) was incubated with recombinant enzyme (10 µL, 5 ng for EGFR, 1:80 dilution in 100 mM HEPES) for 10 min at 25°C, subsequent to the addition of 5X buffer (10 µL, containing 2 mM $MnCl_2$, 100 µM Na_3VO_4, 20 mM HEPES and 1 mM DTT) and ATP-$MgCl_2$ (20 µL, containing 0.1 mM ATP and 50 mM $MgCl_2$) and incubation for 1h. The negative and positive controls were included in each plate by the incubation of enzyme either with or without ATP-$MgCl_2$. The liquid was removed after incubation and the plates were washed thrice using wash buffer. Europium-tagged antiphosphotyrosine antibody (75 µL, 400 ng) was added to each well followed by incubation of 1h and then washing of the plates using buffer. The enhancement solution was added to each well and the signal was recorded at excitation and emission wavelengths of 340 at 615 nm. The autophosphorylation percentage inhibition by compounds was calculated using the following equation:

$$100\% - [(negative\ control)/(positive\ control) - (negative\ control)]$$

Using the curves of percentage inhibition of eight concentrations of each compound, IC_{50} was calculated. Majority of signals detected by antiphosphotyrosine antibody were from EGFR because the enzyme preparation contained low impurities.

4.2.1.4. BRAF kinase assay

V^{600E} mutant BRAF kinase assay was performed to investigate the activity of tested compounds against BRAF. Mouse full-length GST-tagged $BRAF^{V600E}$ (7.5 ng, Invitrogen, PV3849) was pre-incubated with drug (1 µL) and assay dilution buffer (4 µL) for 60 min at 25°C. In assay dilution

buffer, a solution (5 µL) containing $MgCl_2$ (30 mM), ATP (200 µM), recombinant human full length (200 ng) and *N*-terminal His-tagged MEK1 (Invitrogen) was added to start the assay, subsequent to incubation for 25 min at 25°C. The assay was stopped using 5X protein denaturing buffer (LDS) solution (5 µL). To further denature the protein, heat (70° C) was applied for 5 min. 4-12% precast NuPage gel plates (Invitrogen) were used to carry out electrophoresis (at 200 V). 10 µL of each reaction was loaded into the precast plates and electrophoresis was allowed to proceed. After completion of electrophoresis, the front part of the precast gel plate (holding hot ATP) was cut and afterwards cast-off. The dried gel was developed using a phosphor screen. A reaction without active enzyme was used as negative control while that containing no inhibitor served as positive control. To study the effect of compounds on cell-based pERK1/2 activity in cancer cells, commercially available ELISA kits (Invitrogen) were used according to manufacturer's instructions.

4.2.1.6. Cell apoptosis assay

Apoptosis was determined by flow cytometry based on the Annexin-V-fluoresce in isothiocyanate (FITC) and propidium iodide (PI) staining kit (BD Pharmingen, San Diego, USA) [25, 26]. Apoptosis was determined by flow cytometry based on the Annexin-V-fluoresce in isothiocyanate (FITC) and propidium iodide (PI) staining kit (BD Pharmingen, San Diego, USA). Apoptotic cells were defined as Annexin-V-positive. Cells were grown to approximately ~70% confluence and exposed to different concentrations of compounds (0, 2, 4, 6 and8 µmol/L) for 24 h. Treated cells were trypsinzed, washed twice with PBS and transferred into micro centrifuge tubes for centrifugation at1000 rpm for 5 min at room temperature, then resuspended in binding buffer, 5 µL of FITC and PI were added to Eppendorf tube, cells were vortexed, incubated for 15 min at room temperature in dark. Subsequently, cells were analyzed by flow cytometry (Becton Dickinson, Franklin Lakes, and USA).

4.3. Statistical analysis

Computerized Prism 5 program was used to statistically analyzed data using one-way ANOVA test followed by Tukey's as post ANOVA for multiple comparison at $P \leq .05$. Data were presented as mean ± SEM.