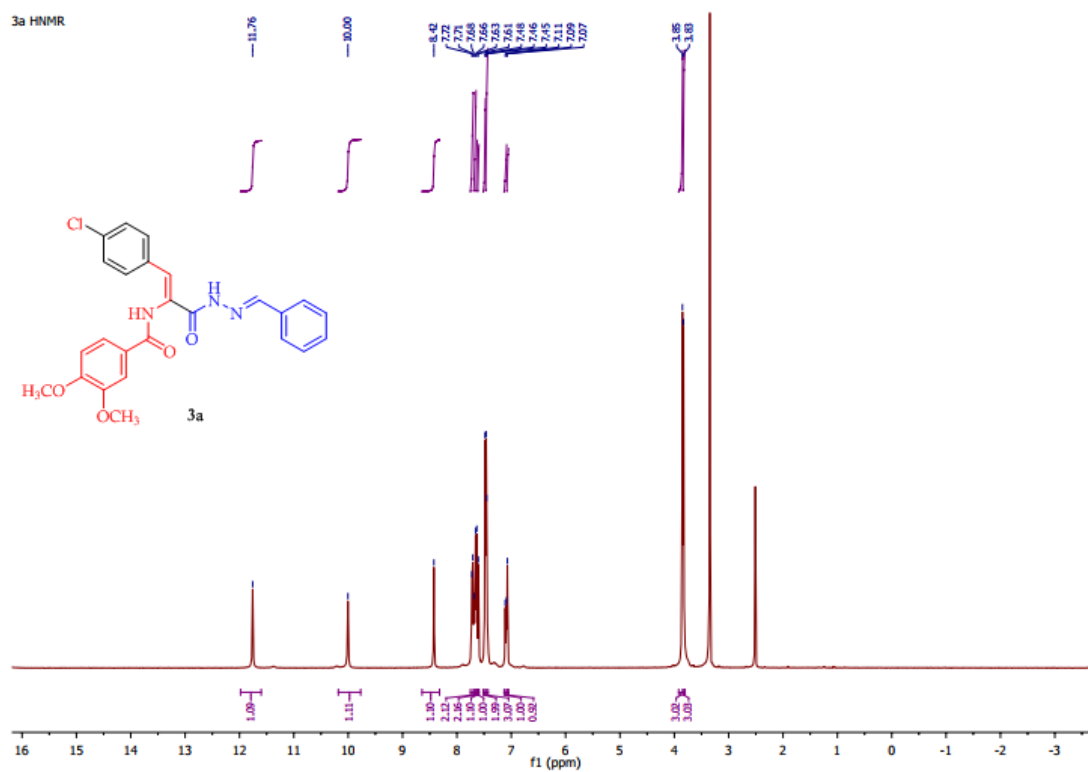
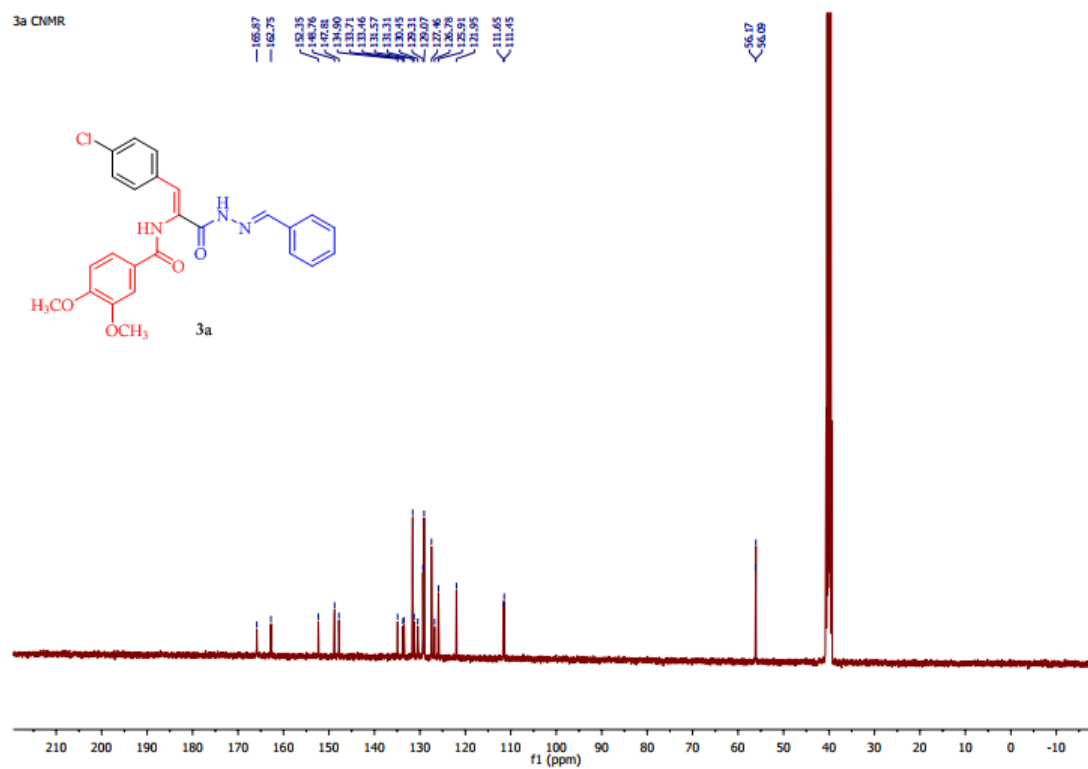


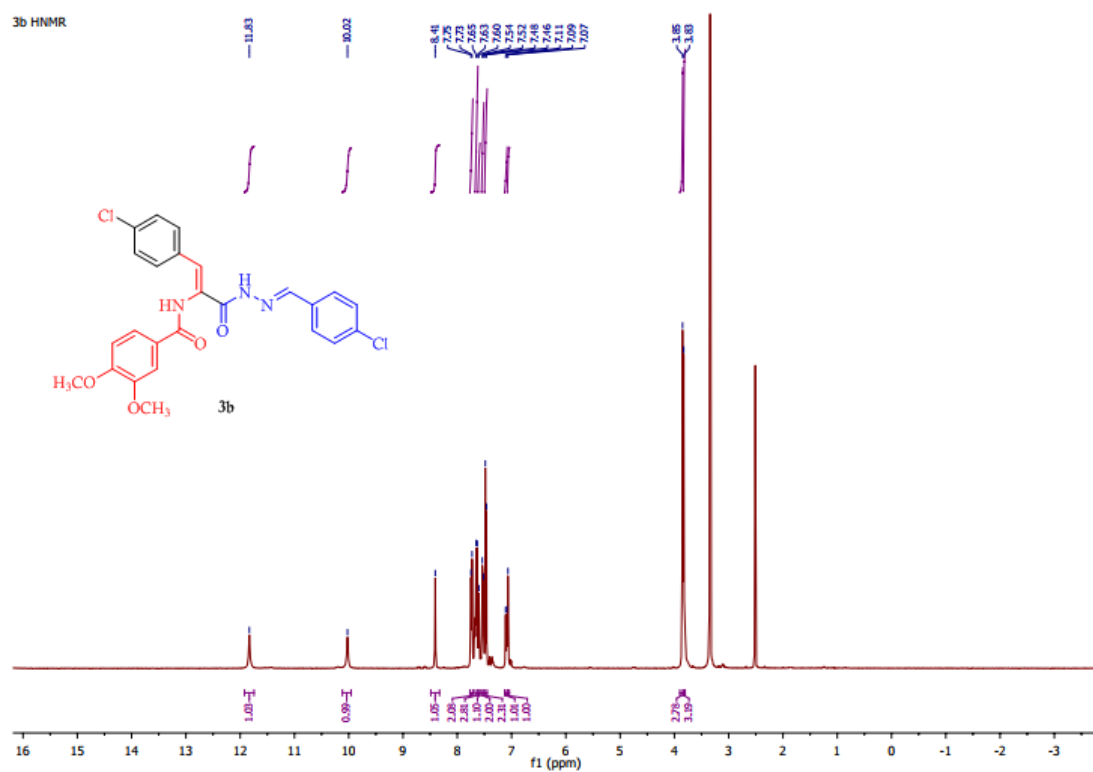
**Design, Synthesis, and *In Vitro* Antiproliferative Screening of New Hydrazone Derivatives Containing *cis*-(4-chlorostyryl) amide Moiety**



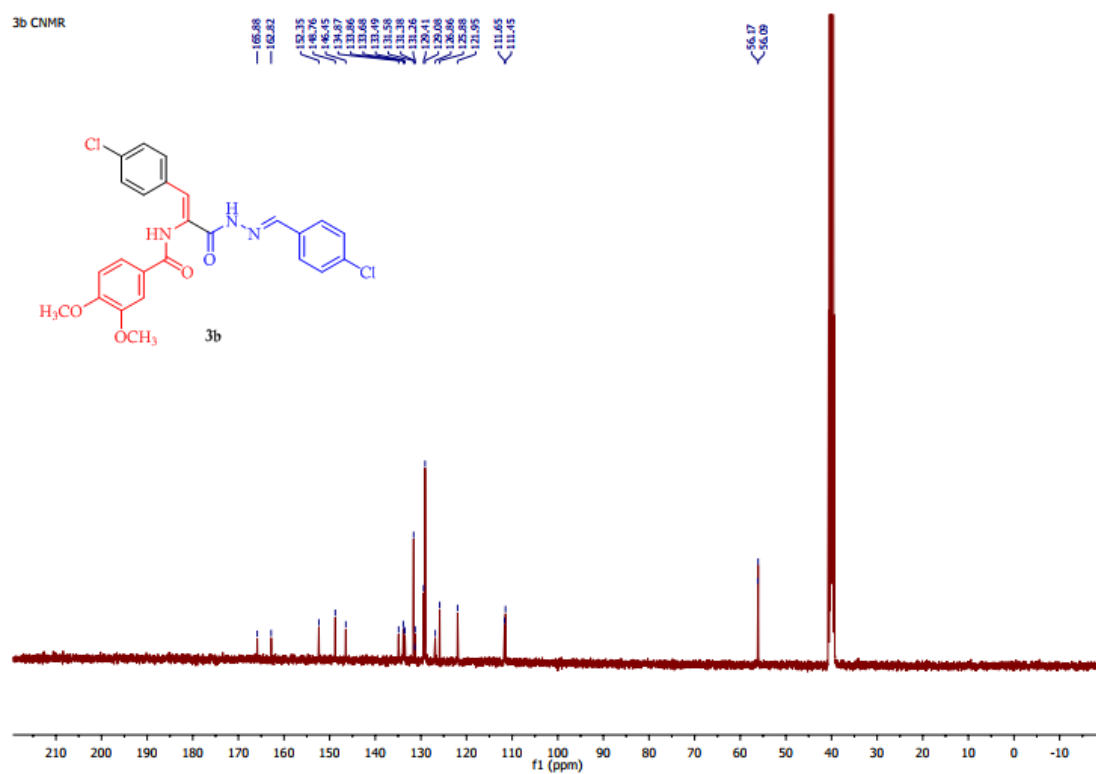
**Figure S1:** <sup>1</sup>H-NMR spectrum of compound 3a



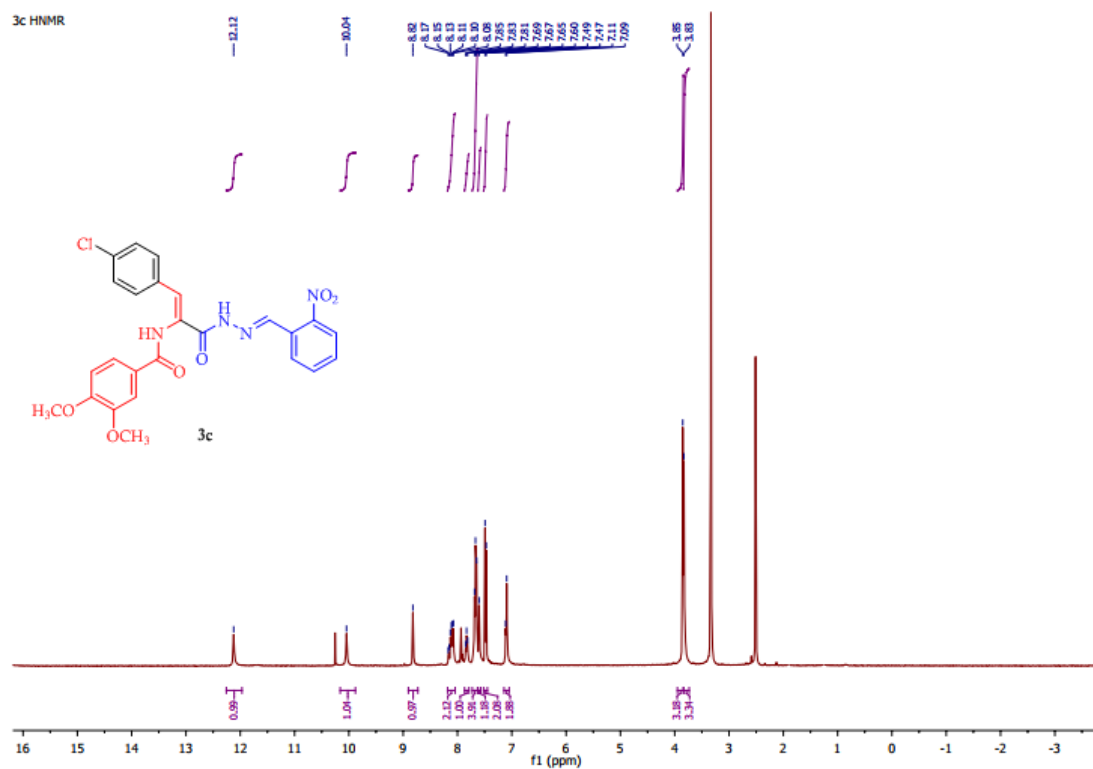
**Figure S2:**  $^{13}\text{C}$ -NMR spectrum of compound 3a



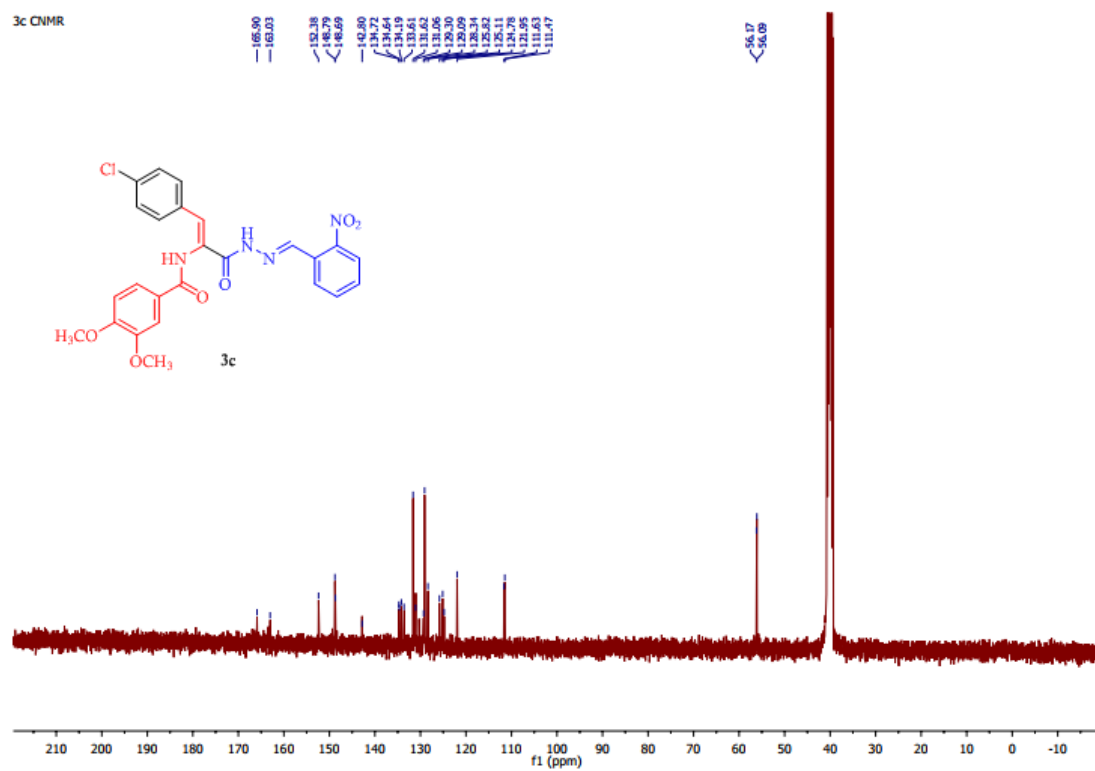
**Figure S3:**  $^1\text{H}$ -NMR spectrum of compound **3b**



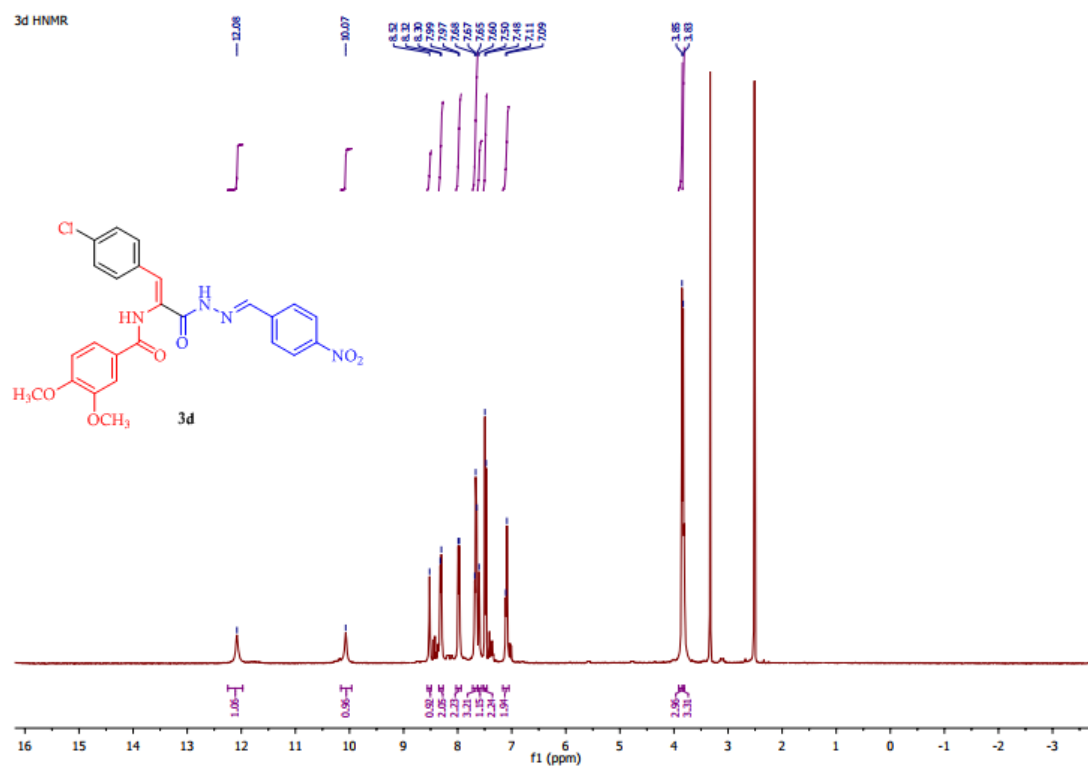
**Figure S4:** <sup>13</sup>C-NMR spectrum of compound 3b



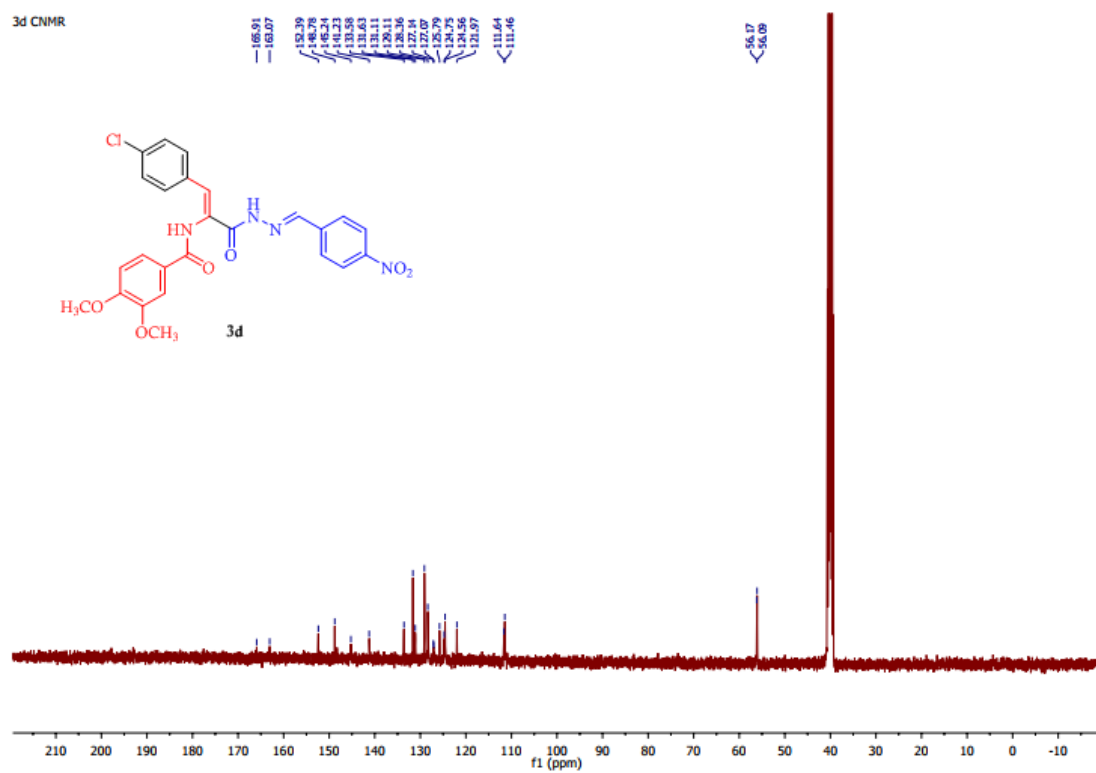
**Figure S5:**  $^1\text{H}$ -NMR spectrum of compound 3c



**Figure S6:**  $^{13}\text{C}$ -NMR spectrum of compound **3c**

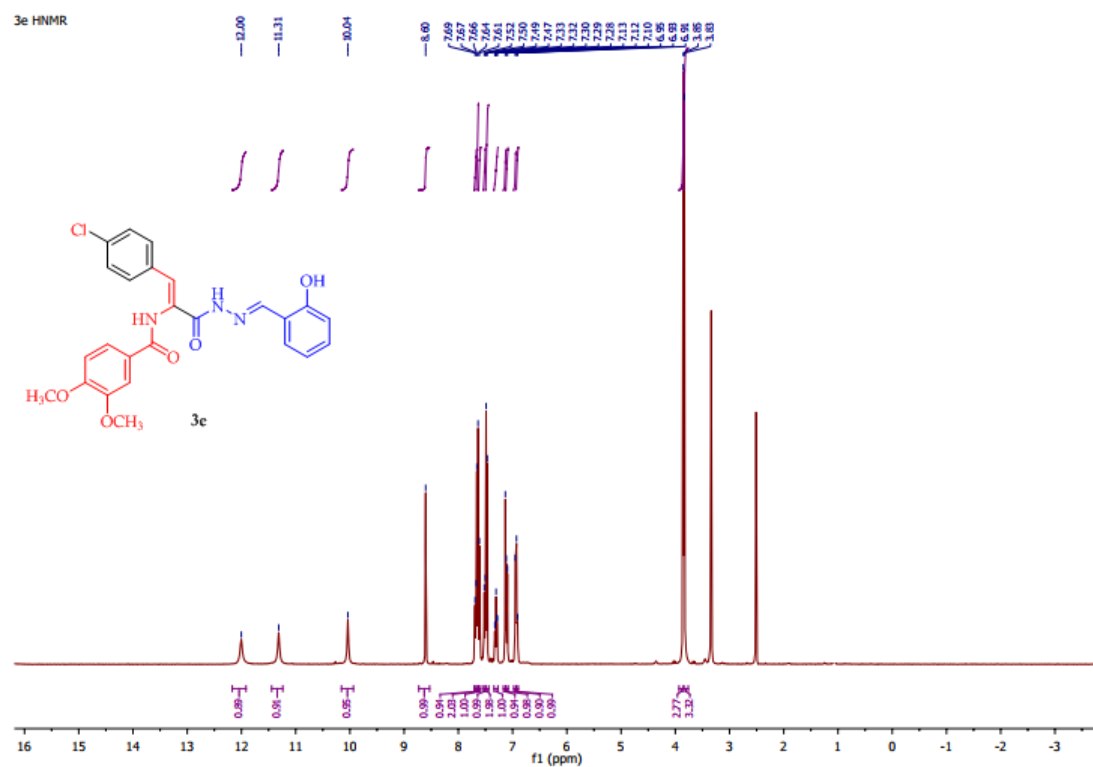


**Figure S7:**  $^1\text{H}$ -NMR spectrum of compound 3d

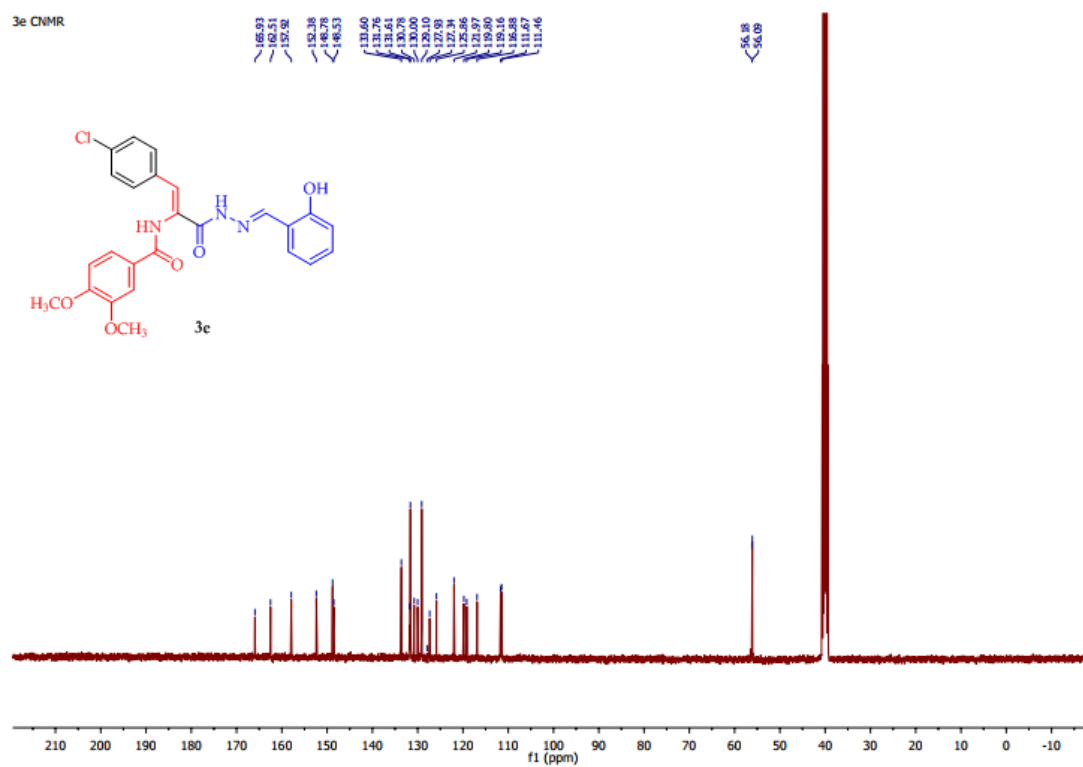


**Figure S8:**  $^{13}\text{C}$ -NMR spectrum of compound **3d**

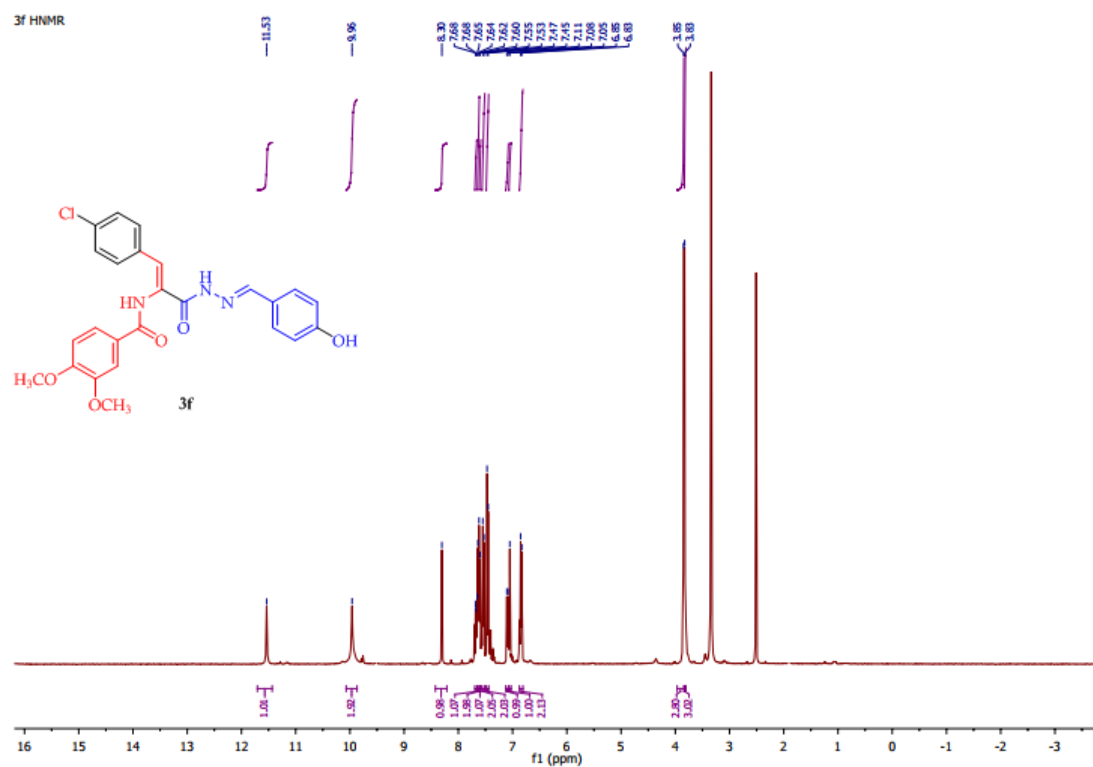




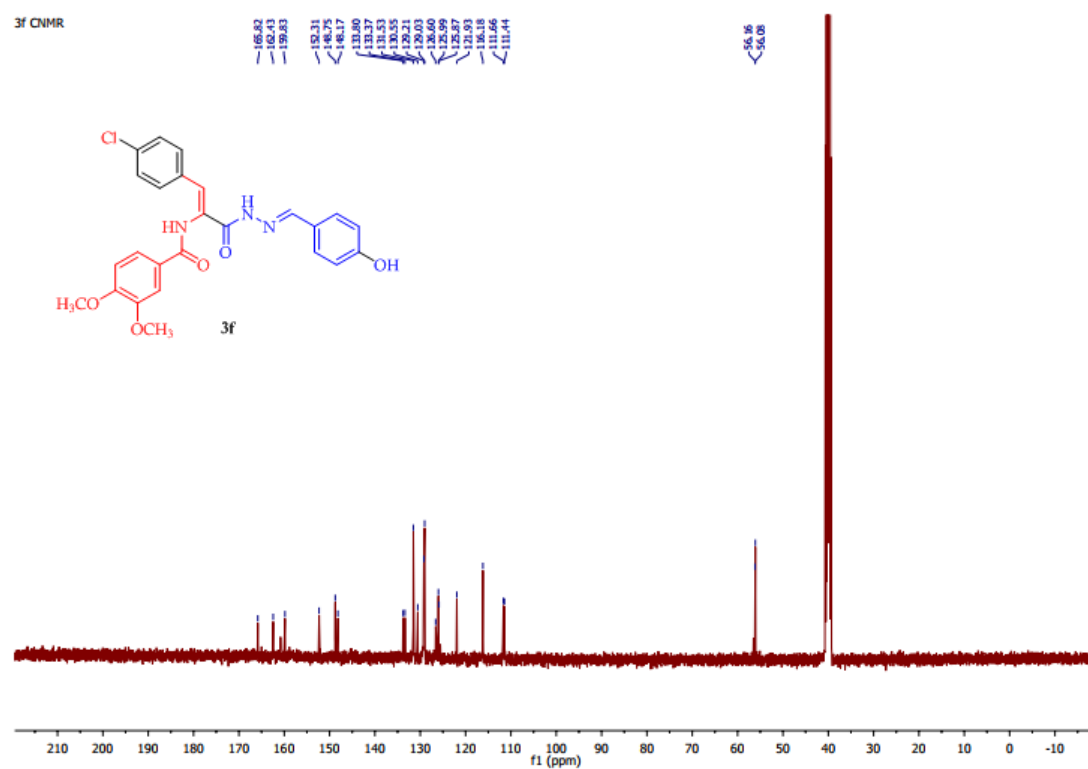
**Figure S9:**  $^1\text{H}$ -NMR spectrum of compound **3e**



**Figure S10:**  $^{13}\text{C}$ -NMR spectrum of compound **3e**

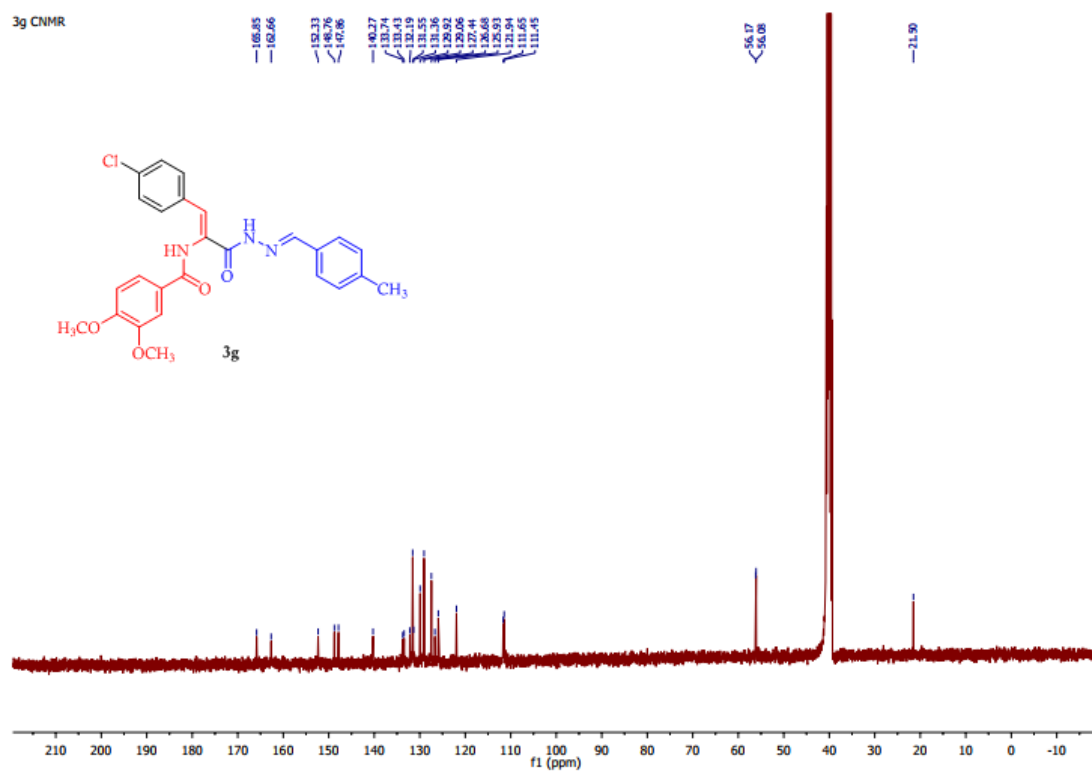


**Figure S11:**  $^1\text{H}$ -NMR spectrum of compound 3f



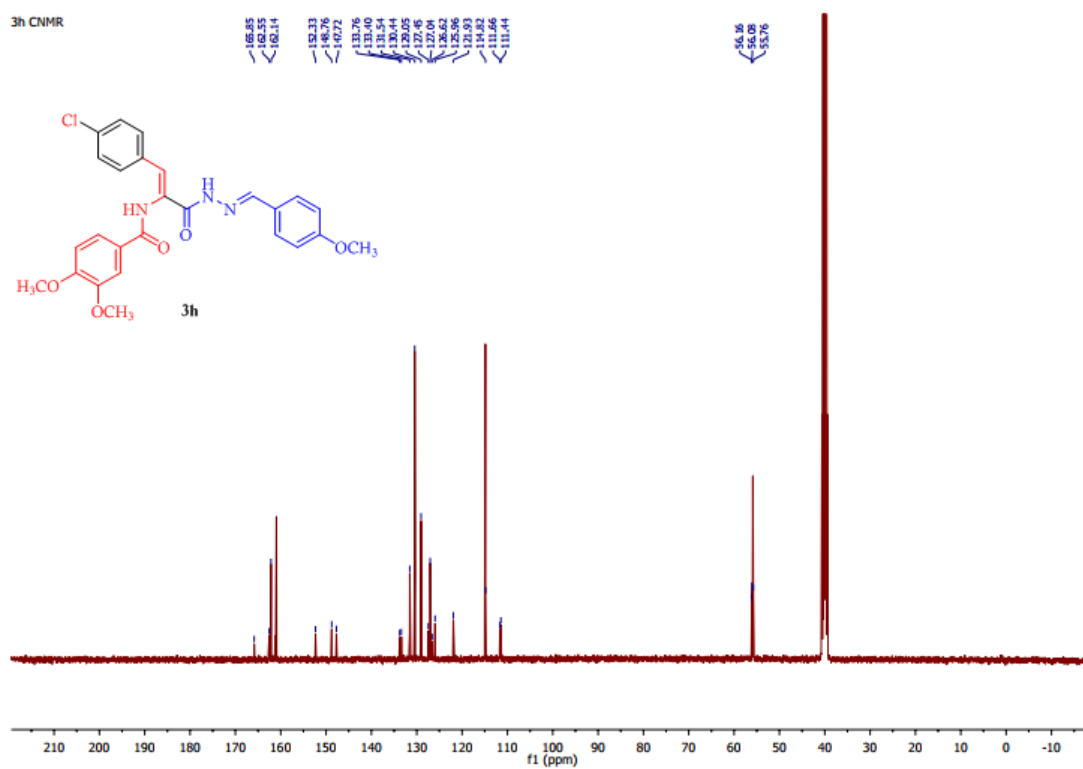
**Figure S12:** <sup>13</sup>C-NMR spectrum of compound **3f**





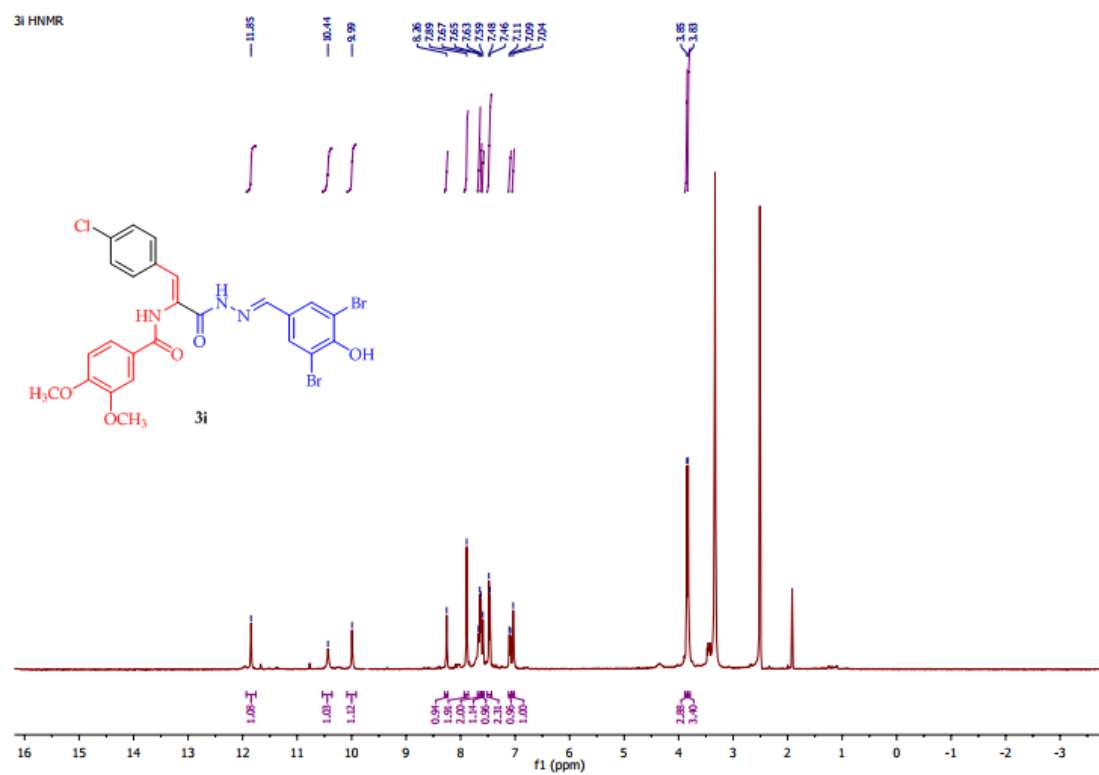
**Figure S14:**  $^{13}\text{C}$ -NMR spectrum of compound **3g**



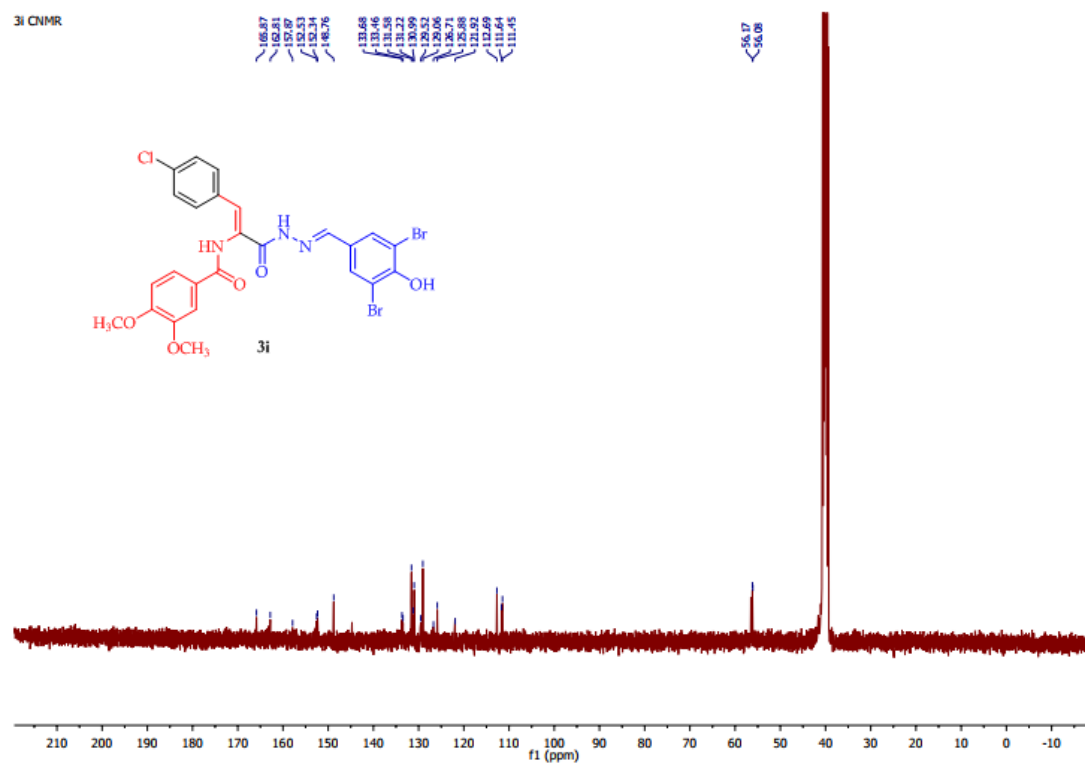


**Figure S16:** <sup>13</sup>C-NMR spectrum of compound **3h**



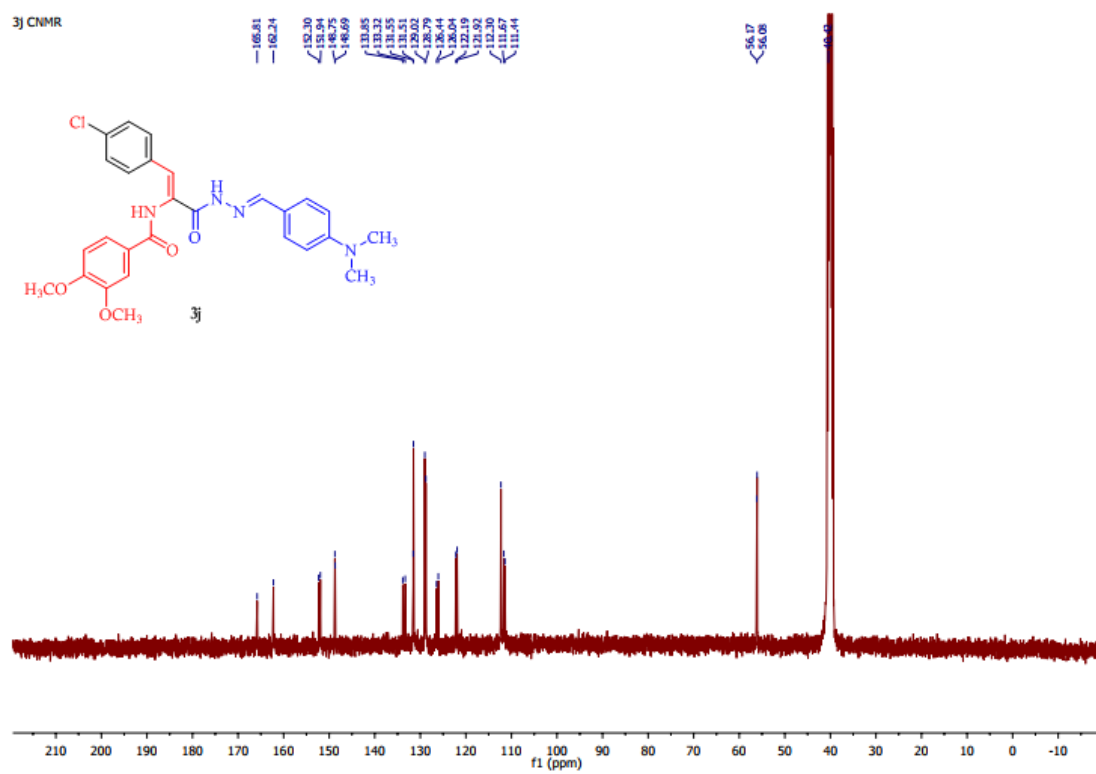


**Figure S17:** <sup>1</sup>H-NMR spectrum of compound **3i**

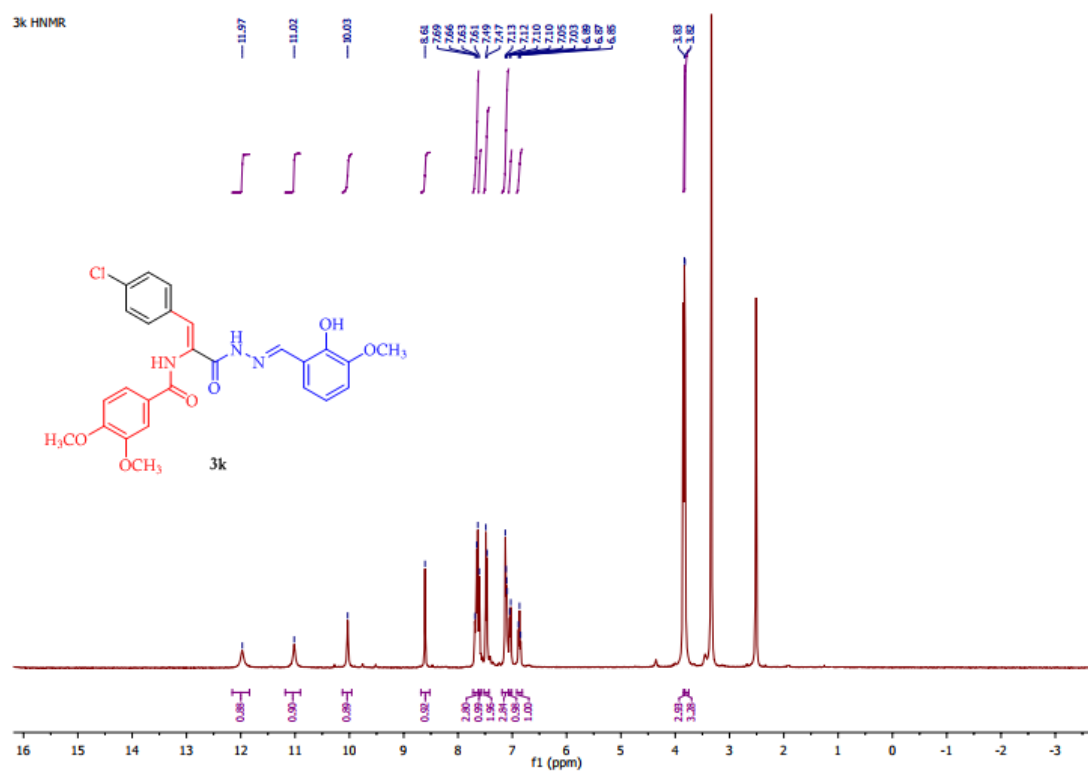


**Figure S18:** <sup>13</sup>C-NMR spectrum of compound **3i**



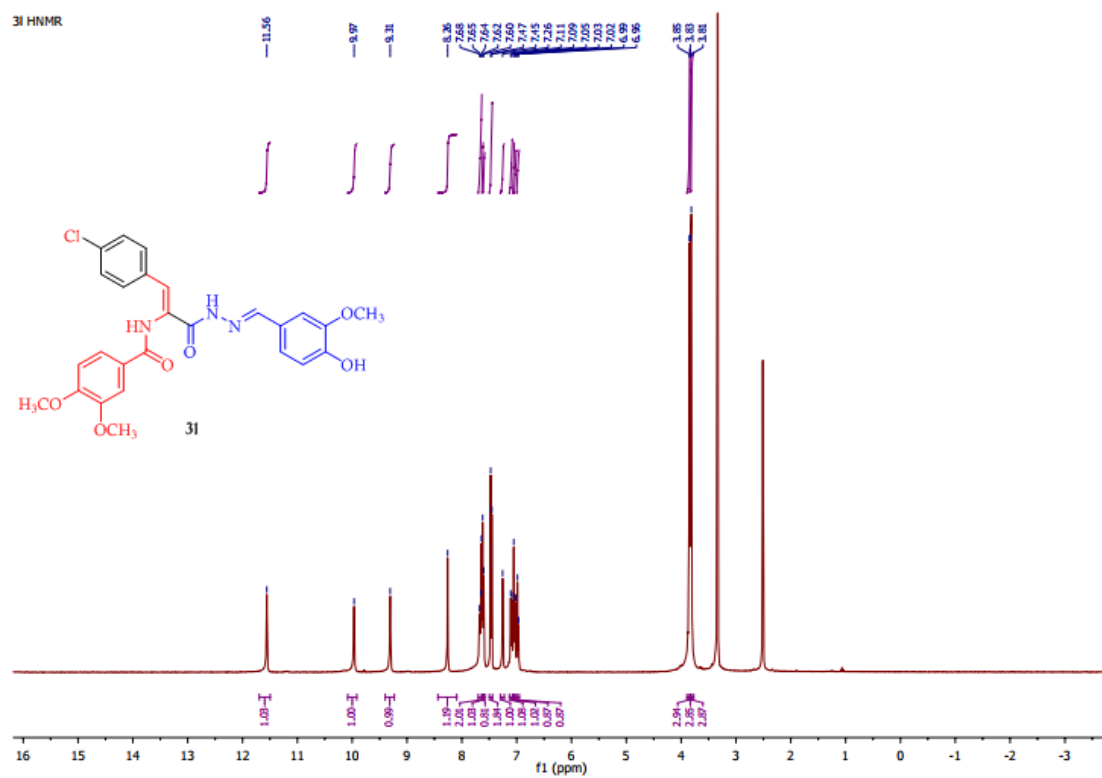


**Figure S20:**  $^{13}\text{C}$ -NMR spectrum of compound **3j**

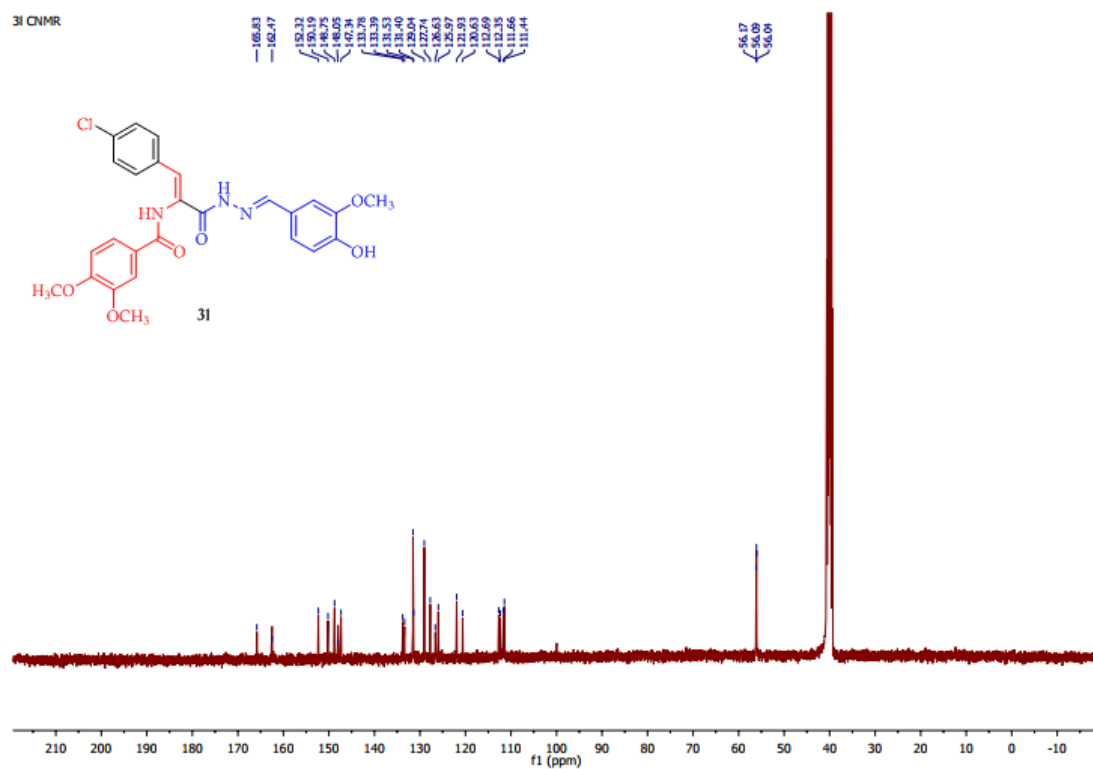


**Figure S21:**  $^1\text{H}$ -NMR spectrum of compound **3k**



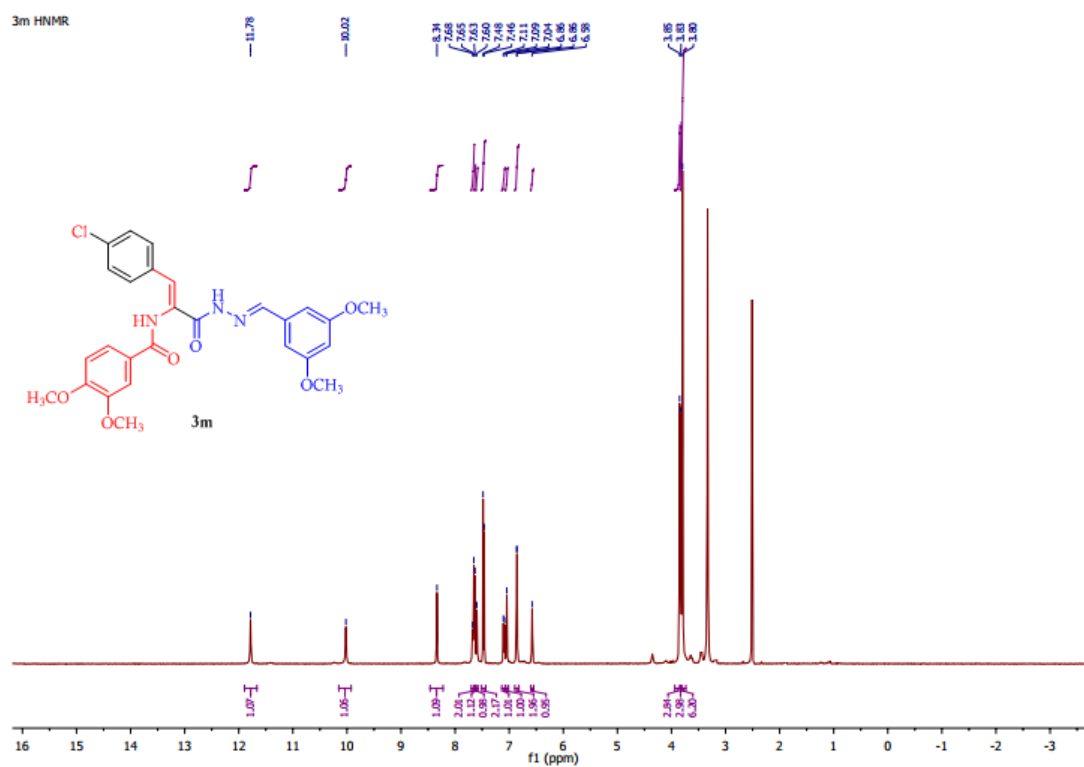


**Figure S23:**  $^1\text{H}$ -NMR spectrum of compound **31**

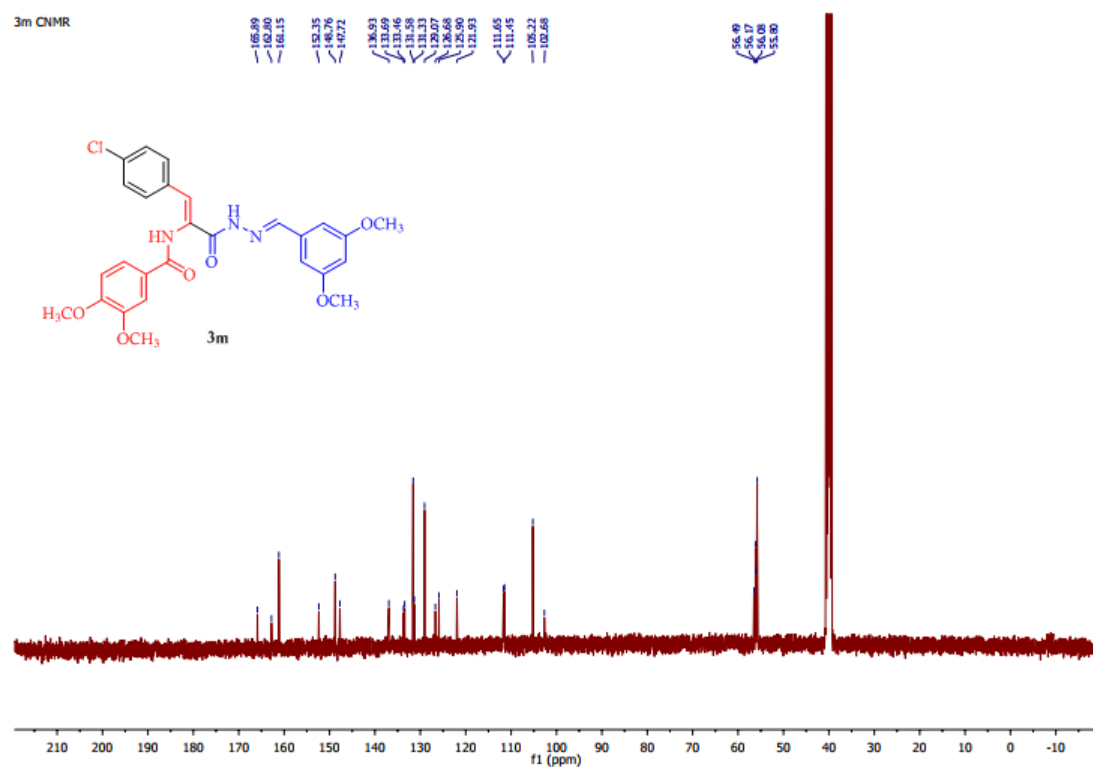


**Figure S24:**  $^{13}\text{C}$ -NMR spectrum of compound **31**

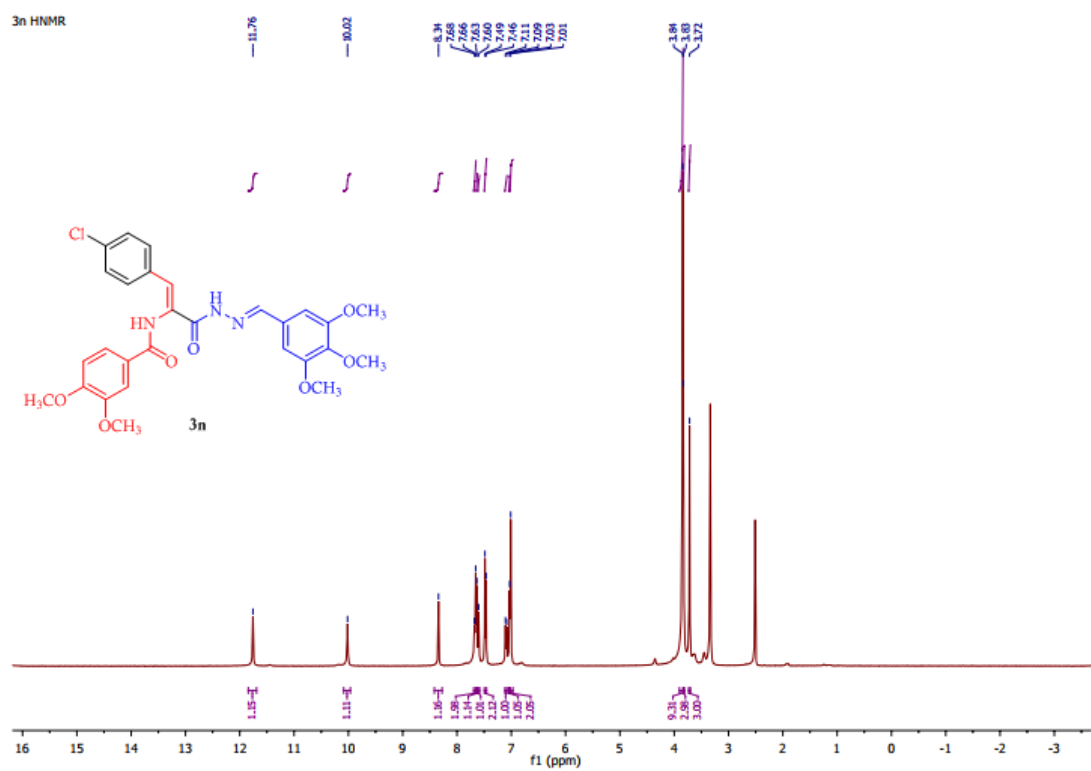




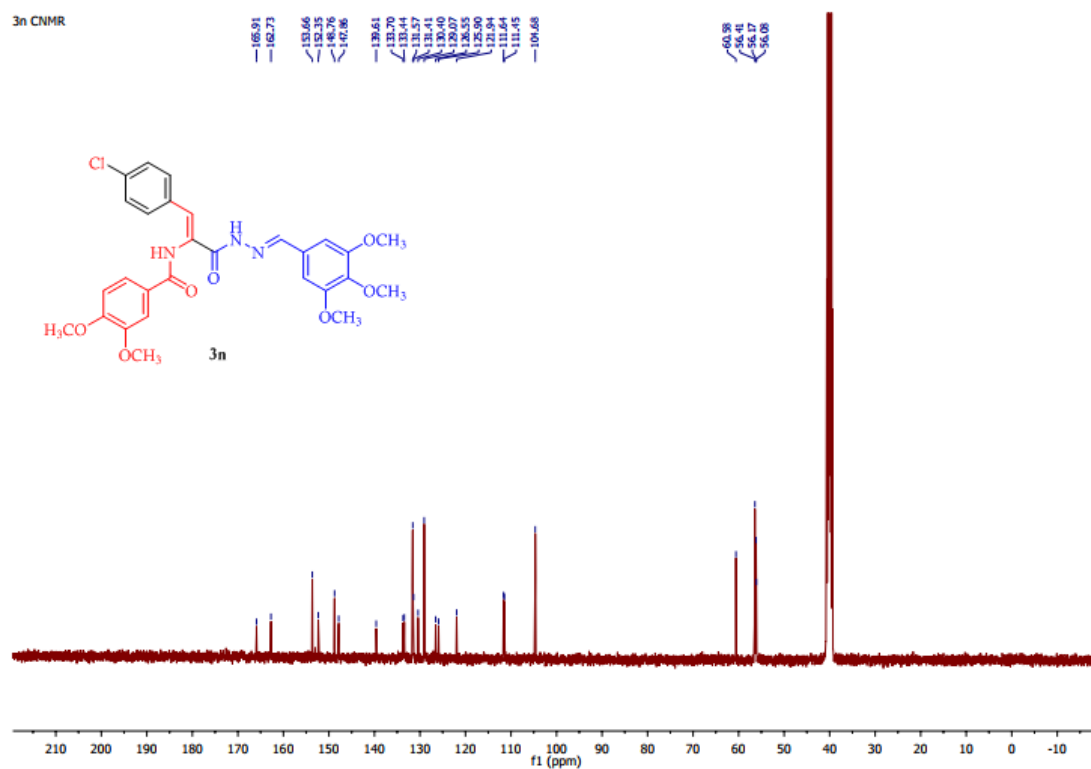
**Figure S25:**  $^1\text{H}$ -NMR spectrum of compound **3m**



**Figure S26:**  $^{13}\text{C}$ -NMR spectrum of compound **3m**

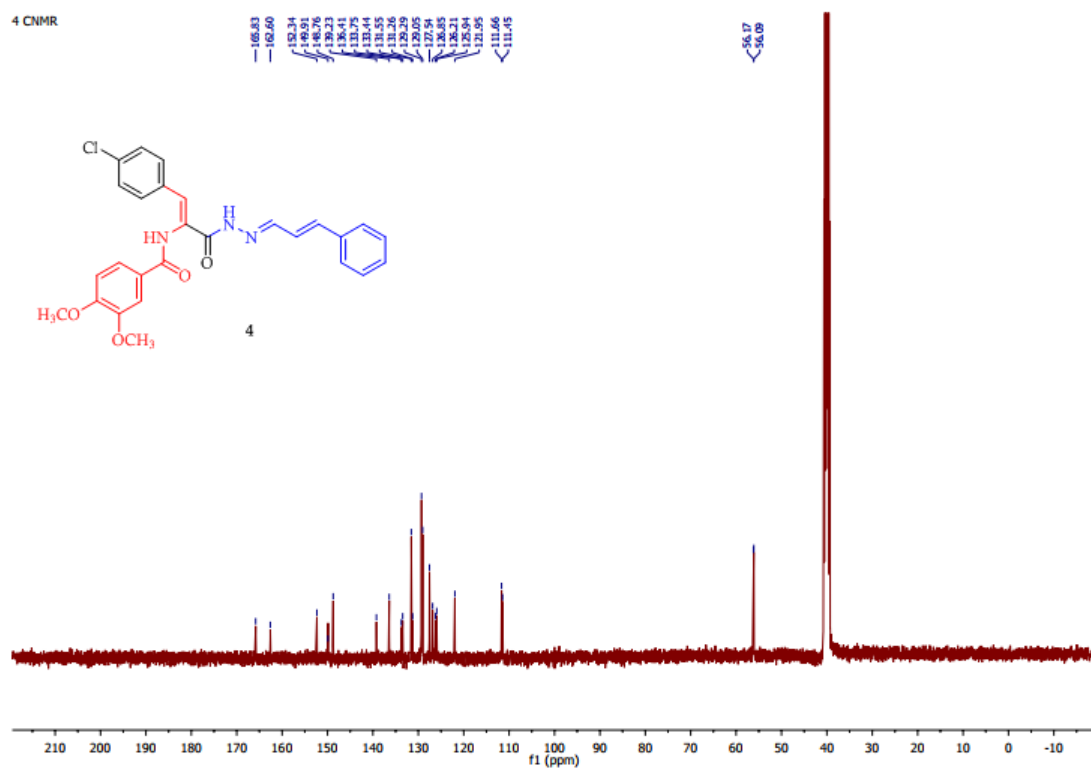


**Figure S27:**  $^1\text{H}$ -NMR spectrum of compound **3n**

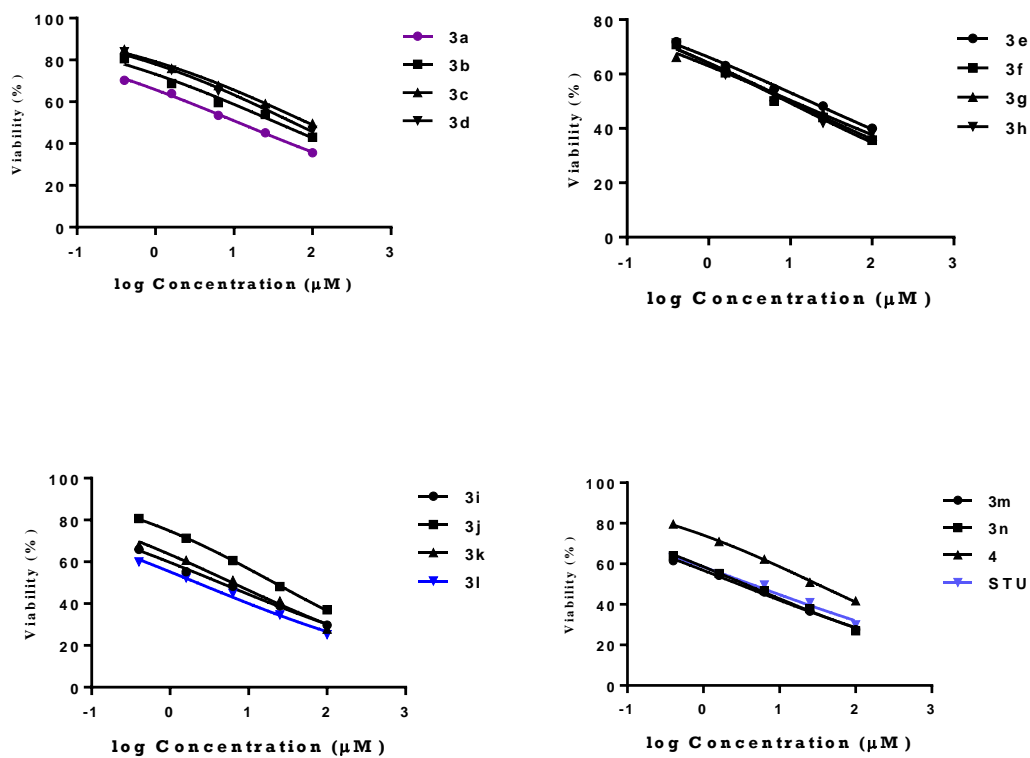


**Figure S28:**  $^{13}\text{C}$ -NMR spectrum of compound **3n**

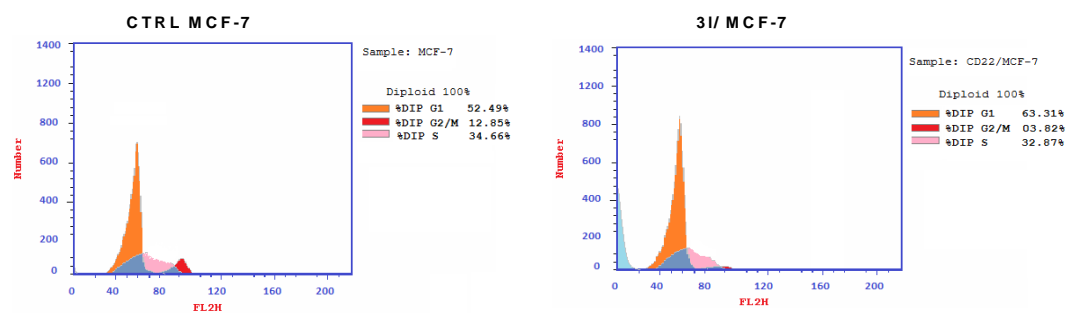




**Figure S30:** <sup>13</sup>C-NMR spectrum of compound 4



**Figure S31:** Dose-response curves for the effect of the prepared hydrazone derivatives **3a-n** and **4** at five different concentrations (μM) on the MCF-7 cell line for 24 h.



**Figure S32:** FACS analysis of compound **3I** on the cell cycle distribution of MCF-7 cancer cells after 48 h.





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**Data Sheet**  
**VEGFR2(KDR) Kinase Assay Kit**  
Catalog # 40325

**DESCRIPTION:** Vascular endothelial growth factor receptor 2 (VEGFR2), also called Kinase Insert Domain receptor (KDR), is a tyrosine kinase (TK) receptor for VEGFs that plays a central role in tumor angiogenesis; therefore, the inhibition of VEGFR2 is a promising therapeutic strategy for inhibiting angiogenesis and tumor growth. The VEGFR2 Kinase Assay Kit is designed to measure VEGFR2 kinase activity for screening and profiling applications using Kinase-Glo<sup>®</sup> MAX as a detection reagent. The VEGFR2 Kinase Assay Kit comes in a convenient 96-well format, with enough purified recombinant VEGFR2 enzyme, VEGFR2 substrate, ATP and Kinase Buffer 1 for 100 enzyme reactions.

**COMPONENTS:**

Catalog #	Reagent	Amount	Storage	
40301	VEGFR2 (KDR)	3 µg	-80°C	Avoid multiple freeze/thaw cycles!
79334	5x Kinase Buffer 1	1.5 ml	-20°C	
	ATP (500 µM)	100 µl	-20°C	
40217	50x PTK substrate Poly (GluTyr 4:1)	100 µl	-20°C	
	96-well plate, white	1	Room Temp.	

**MATERIALS OR INSTRUMENTS REQUIRED BUT NOT SUPPLIED:**  
Kinase-Glo MAX (Promega, #V6071)

Dithiothreitol (DTT, 1 M; optional)  
Microplate reader capable of reading luminescence  
Adjustable micropipettor and sterile tips  
30°C incubator

**APPLICATIONS:** Useful for studying enzyme kinetics and screening small molecular inhibitors for drug discovery and HTS applications.

**STABILITY:** Up to 6 months when stored as recommended.

**REFERENCE:**

Sharma, K., et al. *Biomol Chromatogr.* 2015 Jun; 28(6):803-34.  
Fontanella, C., et al. *Ann Transl Med.* 2014 Dec; 2(12):123

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130321



DRG® Caspase-9 (human) ELISA (EIA-4860)

Revised 27 Dec. 2011 rm (Vers. 2.1)



*This kit is intended for Research Use Only.*

*Not for use in diagnostic procedures.*

*Please use only the valid version of the package insert provided with the kit.*

**INTENDED USE**

The human Caspase-9 ELISA is an enzyme-linked immunosorbent assay for measurement of human Caspase-9. The human Caspase-9 ELISA is for research use only. Not for diagnostic or therapeutic procedures.

**PRINCIPLES OF THE TEST**

An anti-human Caspase-9 coating antibody is adsorbed onto microwells.

Human Caspase-9 present in the sample or standard binds to antibodies adsorbed to the microwells. The polyclonal detection antibody (rabbit) binds to human Caspase-9 captured by the first antibody.

Following incubation unbound detection antibody is removed during a wash step. Anti-rabbit-IgG-HRP is added and binds to the Detection Antibody.

Following incubation unbound anti-rabbit-IgG-HRP is removed during a wash step, and substrate solution reactive with HRP is added to the wells.

A coloured product is formed in proportion to the amount of human Caspase-9 present in the sample or standard. The reaction is terminated by addition of acid and absorbance is measured at 450 nm. A standard curve is prepared from 7 human Caspase-9 standard dilutions and human Caspase-9 concentration determined.

**REAGENTS PROVIDED**

Reagents for human Caspase-9 ELISA (96 tests)

- 1 aluminium pouch with a Microwell Plate coated with monoclonal antibody to human Caspase-9
- 1 vial (100 µL) anti-human Caspase-9 polyclonal Detection Antibody (rabbit)
- 1 vial (10 µL) Anti-rabbit-IgG-HRP
- 2 vials human Caspase-9 Standard lyophilized, 200 ng/mL upon reconstitution
- 1 vial (12 mL) Sample Diluent
- 1 vial (5 mL) Assay Buffer Concentrate 20x (PBS with 1% Tween 20 and 10% BSA)
- 1 bottle (50 mL) Wash Buffer Concentrate 20x (PBS with 1% Tween 20)
- 1 bottle (15 mL) Lysis Buffer 10x
- 1 vial (15 mL) Substrate Solution (tetramethyl-benzidine)
- 1 vial (15 mL) Stop Solution (1M Phosphoric acid)
- 1 vial (0.4 mL) Blue-Dye

DRG International Inc., USA

Fax: (908) 233-0758 • E-mail: [corp@drg-international.com](mailto:corp@drg-international.com) • Web: [www.drg-international.com](http://www.drg-international.com)

1

#### **4.1.1. Chemistry: General**

Melting points were determined in open capillaries tube using Electrothermal Digital melting point apparatus and were uncorrected.  $^1\text{H}$ -NMR and  $^{13}\text{C}$ -NMR spectra were obtained with a Bruker 400 MHz DRX-Avance NMR spectrometer; peak positions are given in ppm downfield from tetramethylsilane (TMS) as the internal standard. Elemental analyses were performed on Elementar, Vario El, Microanalytical unit, Cairo, Egypt, and were found within  $\pm 0.4\%$  of the theoretical values.

## 4.2. Biological studies

### 4.1.1. Cytotoxic activity evaluation

To measure the cytotoxic activity of the newly synthesized hydrazone derivatives **3a-n** and **4** in the breast carcinoma (MCF-7) cell line, cell viability assay was assessed using the MTT assay method. Cells at a density of  $1 \times 10^4$  were seeded in a 96-well plate at 37 °C for 24 h under 5% CO<sub>2</sub>. After incubation, the cells were treated with different concentrations of the test compounds **3a-n** and **4** and incubated for 24 h, then 20 µl of MTT solution at 5 mg/mL was applied and incubated for 4 h at 37 °C. Dimethyl sulphoxide (DMSO) in the volume of 100 µl was added to each well to dissolve the purple formazan that had formed. The color intensity of the formazan product, which represents the growth condition of the cells, is quantified by using an ELISA plate reader (EXL 800, USA) at 570 nm absorbance. The experimental conditions were carried out with at least three replicates, and the experiments were repeated at least three times.

### 4.2.2. VEGFR-2 inhibition assay

Hydrazinyl compound **3l** and Sorafenib were evaluated for their VEGFR-2 inhibitory activity according to the manufacturer's instructions using # VEGFR-2 (KDR) Kinase Assay Kit Catalog # 40325 (BPS Bioscience).

### 4.2.3. Cell cycle analysis of compound **3l**

Cell cycle analysis in MCF-7 cells was investigated using a fluorescent Annexin V-FITC/ PI detection kit (*BioVision EZCell™* Cell Cycle Analysis Kit Catalog #K920) by flow cytometry assay. MCF-7 cells at a density of  $2 \times 10^5$  per well were harvested and washed twice in PBS. After that, the cells were incubated at 37 °C and 5% CO<sub>2</sub>. The medium was incubated with the tested compound **3l** at its IC<sub>50</sub> (µM) for 48 h, washed twice in PBS, fixed with 70% ethanol, and rinsed again with PBS. Afterward, the medium was stained with DNA fluorochrome PI for 15 min at 37 °C. The samples were immediately analyzed using a FACS Calibur flow cytometer (Becton and Dickinson, Heidelberg, Germany).

### 4.2.4. Apoptosis assay for compound **3l**

Apoptosis in MCF-7 cells was investigated using a fluorescent Annexin V-FITC/ PI detection kit (*BioVision Annexin V-FITC Apoptosis Detection Kit*, Catalog #: K101) by flow cytometry assay. MCF-7 cells at a density of  $2 \times 10^5$  per well were treated with compound **3l** at its IC<sub>50</sub> (µM) for 48 h; the cells were harvested and stained with

Annexin V-FITC/ PI dye for 15 min in the dark at 37 °C. The samples were immediately analyzed using a *FACS Calibur* flow cytometer (Becton and Dickinson, Heidelberg, Germany).

#### **4.2.5. Caspase 9 assay for compound 3I**

To determine the effect of the synthesized hydrazone molecule **3I** on apoptosis, the active caspase 9 level was measured using ELISA analysis according to the manufacturer's instructions. Briefly, MCF-7 cells at a density of  $2 \times 10^4$  per well were treated with compound **3I** at its IC<sub>50</sub> (μM) for 48 h; then, the cells were lysed with cell extraction buffer. This lysate was diluted in standard diluent buffer over the range of the assay. The optical density of each well was determined within 30 min using a microplate plate reader set at 450 nm to determine the human active caspase 9 level.