Supplementary data

Discovery of Thieno[2,3-*d*]pyrimidine-Based Dual Aurora-B/VEGFR-2 Inhibitors with Potent Anticancer Activity: Molecular Docking, Mechanistic Studies, and *In Vivo* Validation in a Breast Cancer Model

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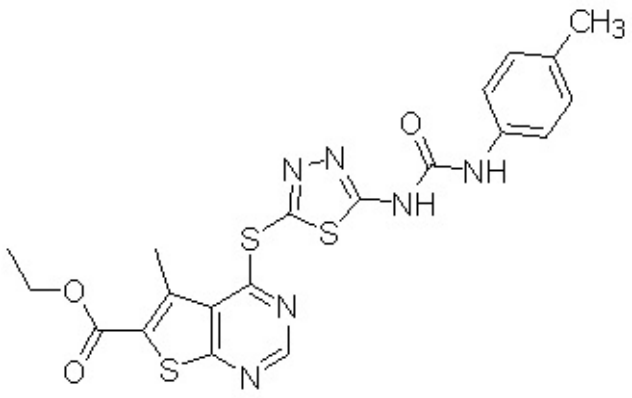
Eman Nasr_CH3
single_pulse



M1

Eman Nasr_OCH3
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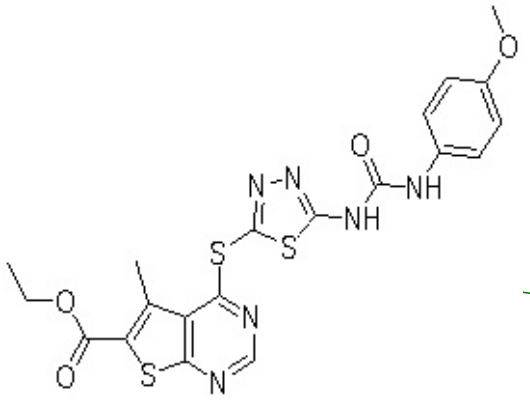


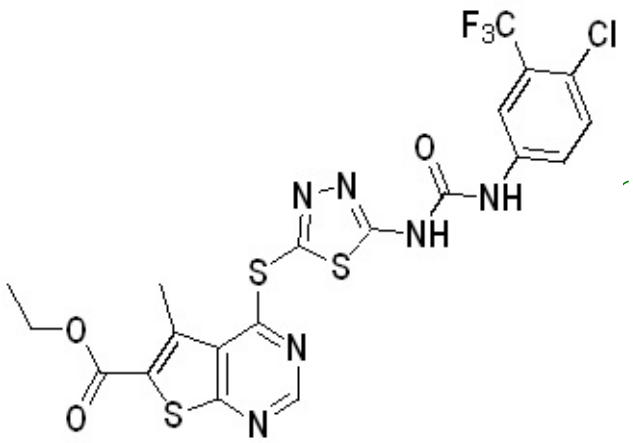
M2

11.39
9.23
8.86
7.40
7.38
6.86
6.86
6.85
4.35
4.33
4.32
4.30
3.71
3.68
3.30 H2O
2.99
2.48
2.46 DMSO-D6
2.46 DMSO-D6
2.46 DMSO-D6
2.45 DMSO-D6
2.45 DMSO-D6
1.31
1.29
1.28

0.70
0.86
1.00
2.06
2.09
2.06
3.09
3.02
3.01

f1 (ppm)

Eman Nasr_SOVa
single_pulse



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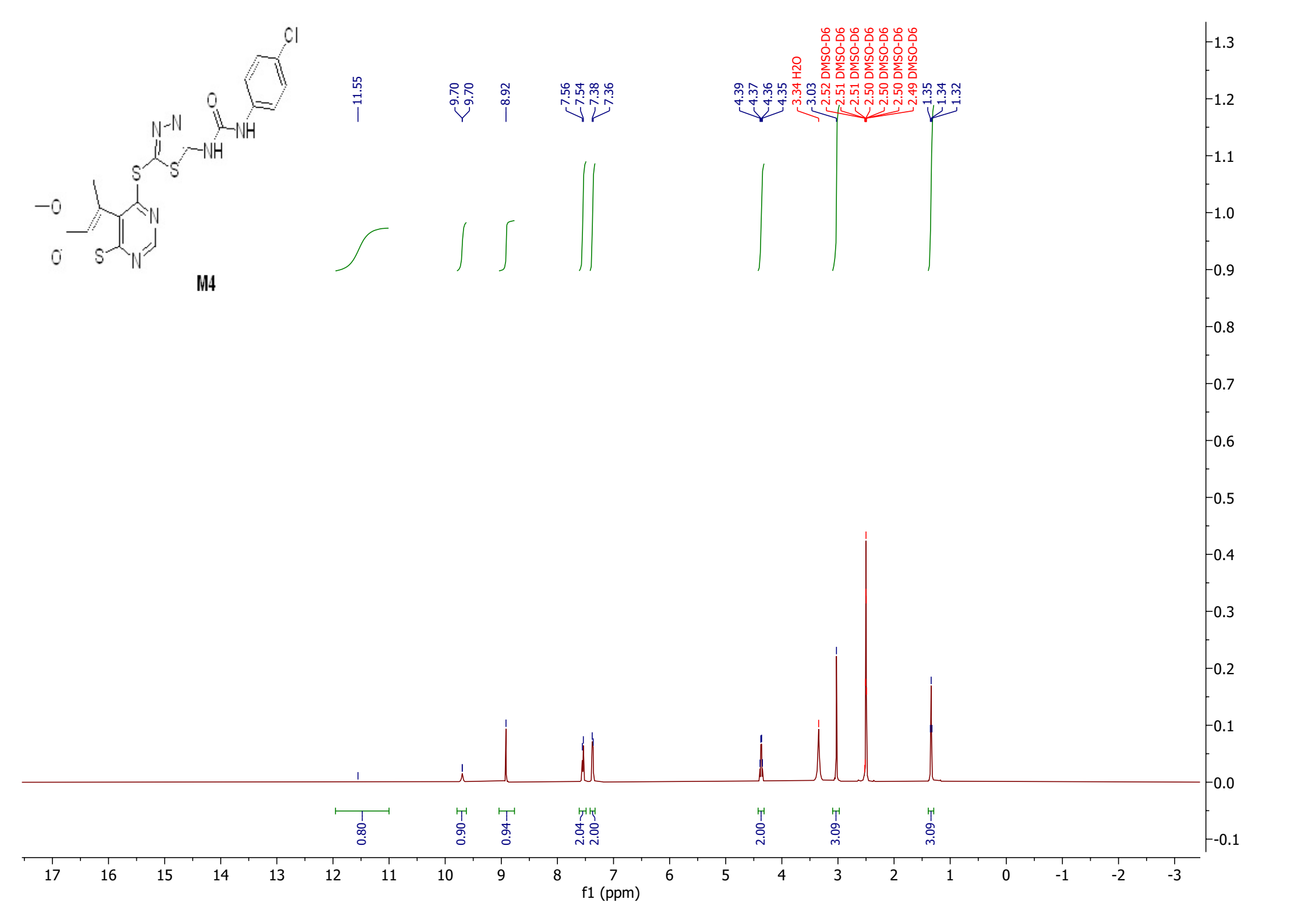
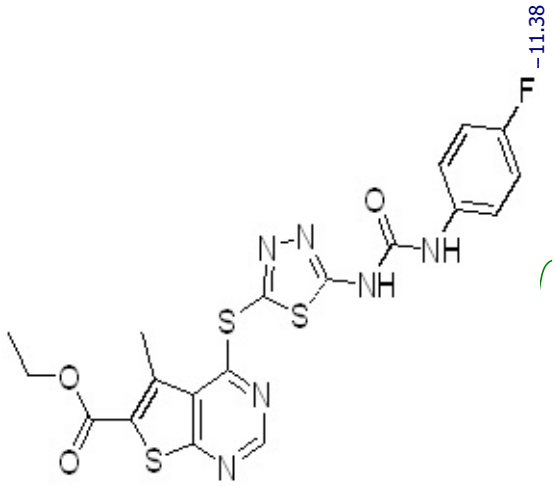
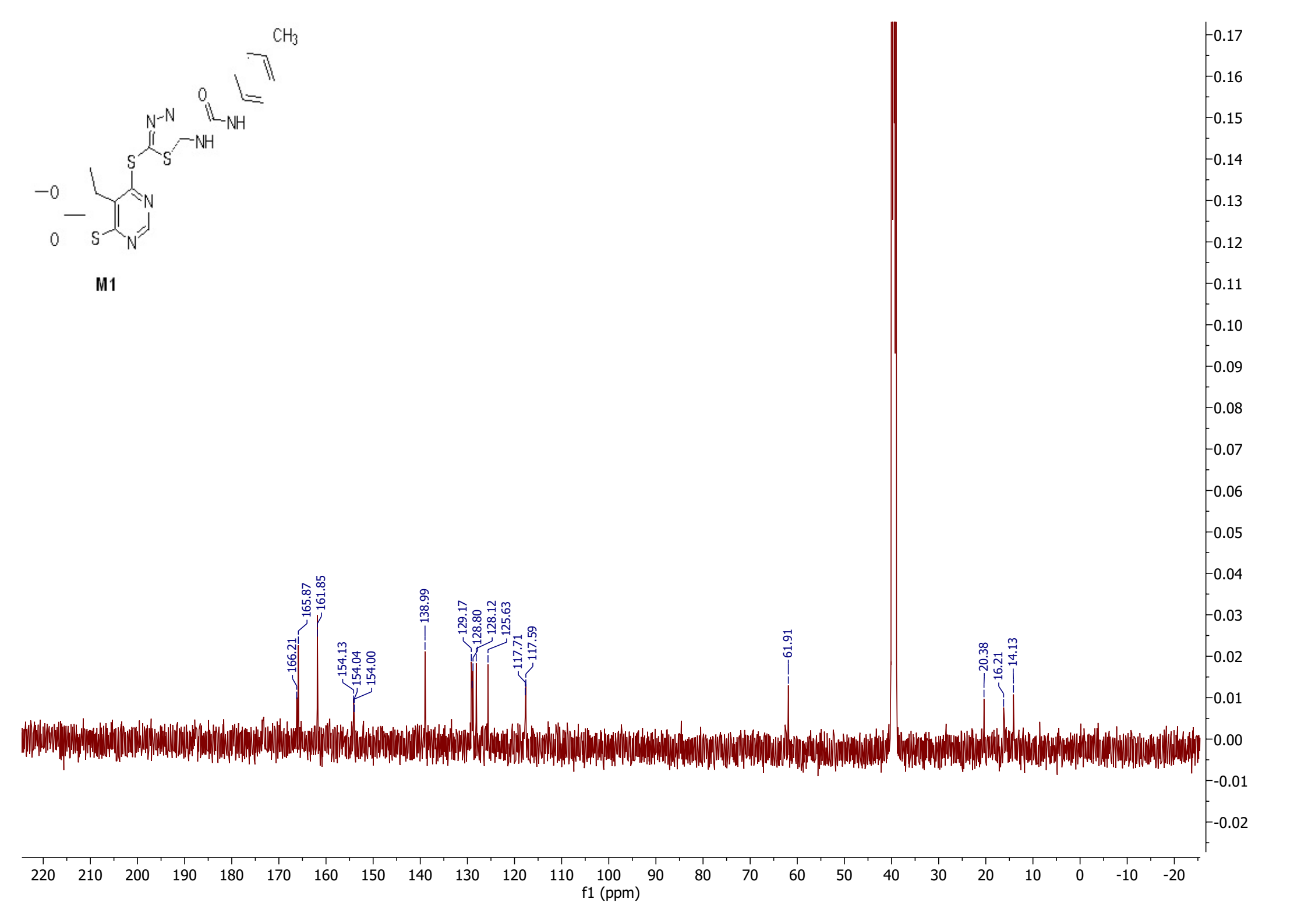

M4
11.55
9.70
9.70
8.92
7.56
7.54
7.38
7.36
4.39
4.37
4.36
4.35
3.34 H2O
3.03
2.52 DMSO-D6
2.51 DMSO-D6
2.51 DMSO-D6
2.50 DMSO-D6
2.50 DMSO-D6
2.50 DMSO-D6
2.49 DMSO-D6
1.35
1.34
1.32
0.80
0.90
0.94
2.04
2.00
2.00
3.09
3.09
f1 (ppm)

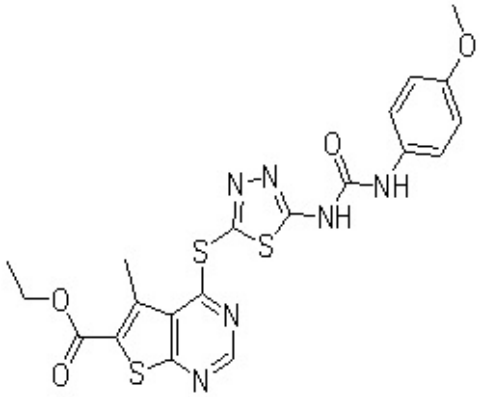
Eman Nasr_F
single_pulse



M5

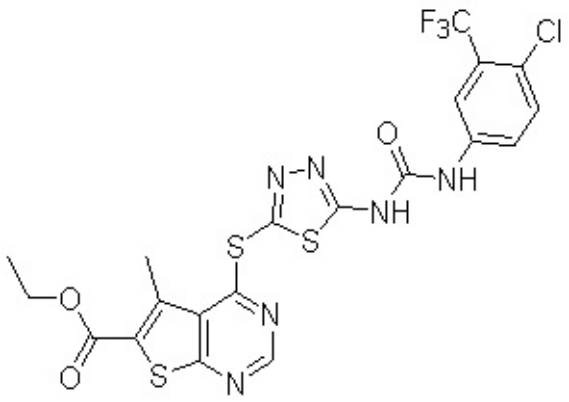

M1
166.21
165.87
161.85
154.13
154.04
154.00
138.99
129.17
128.80
128.12
125.63
117.71
117.59
61.91
20.38
16.21
14.13
f1 (ppm)

Eman Nasr_OCH3
single pulse decoupled gated NOE

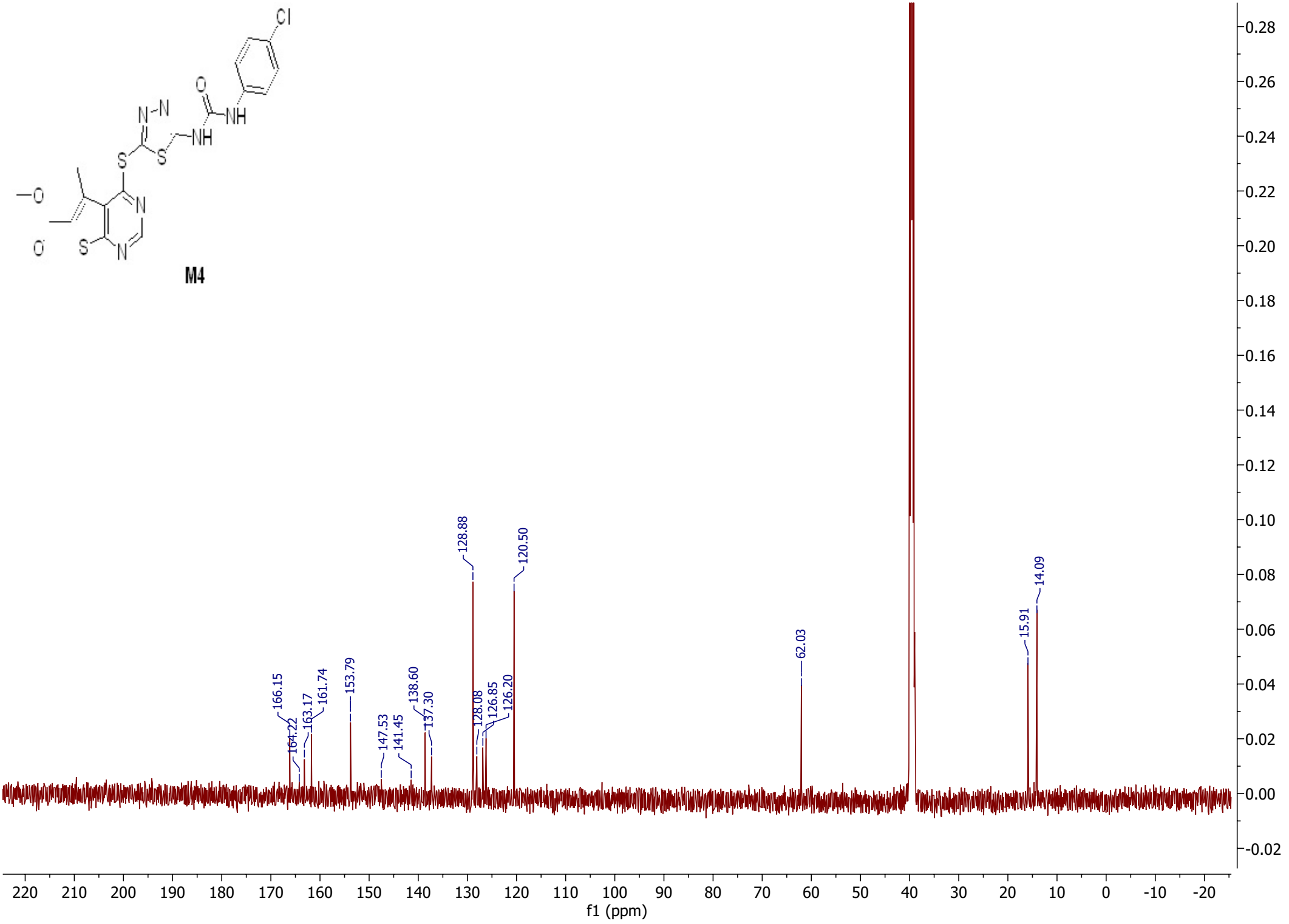


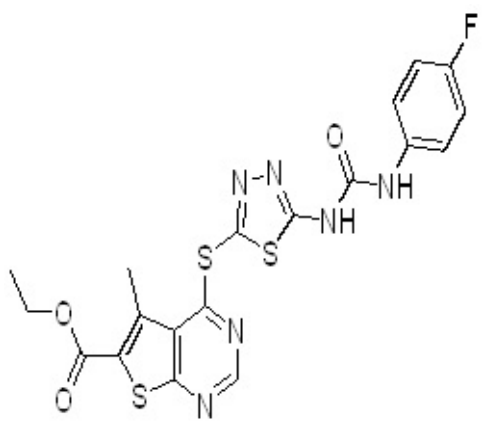
M2

Eman Nasr_SOVa
single pulse decoupled gated NOE



M3


Cl
O
NH
N-N
NH
S
S
N
O
O
S
N
M4
166.15
164.22
163.17
161.74
153.79
147.53
141.45
138.60
137.30
128.88
128.08
126.85
126.20
120.50
62.03
15.91
14.09
0.28
0.26
0.24
0.22
0.20
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
-0.02
220 210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10 -20
f1 (ppm)


F
O
NH
N-N
NH
S
S
O
O
N
S
N

M5

39.99
38.99
166.10
163.18
161.67
159.08
157.17
153.73
138.52
134.51
128.02
126.17
121.04
120.97
115.60
115.43
61.97
15.86
14.04
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
220 210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10 -20
f1 (ppm)

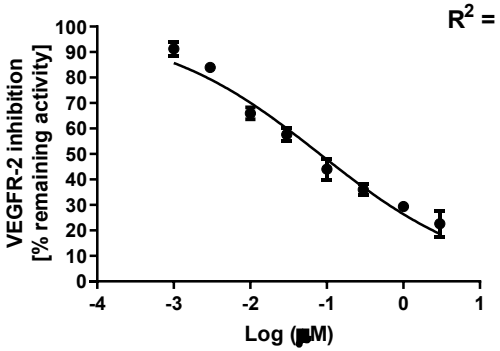
VEGFR-2

Best-fit values	
LogIC50	-1.092
HillSlope	-0.4078
IC50	0.08098

M1

IC_{50} = 0.08098

R^2 = 0.9639

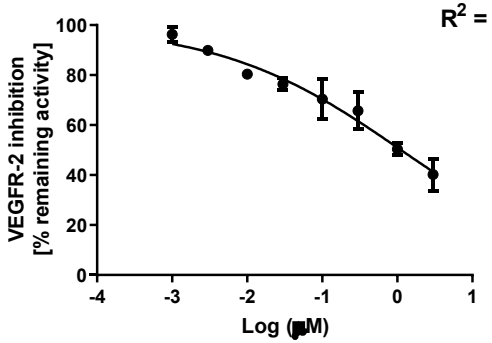


Best-fit values	
LogIC50	0.04985
HillSlope	-0.3571
IC50	1.122

M2

IC_{50} = 1.122

R^2 = 0.9327

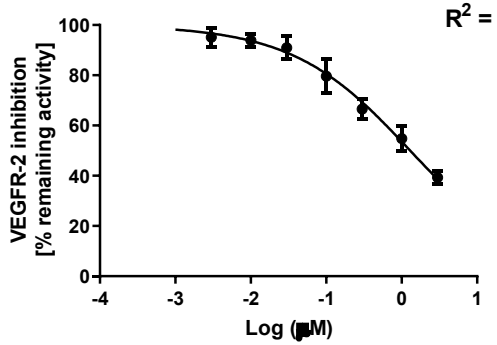


Best-fit values	
LogIC50	0.1133
HillSlope	-0.5549
IC50	1.298

M3

IC_{50} = 1.298

R^2 = 0.9579

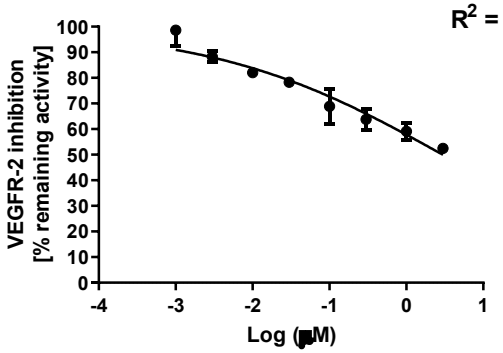


Best-fit values	
LogIC50	0.4741
HillSlope	-0.2885
IC50	2.979

M4

IC_{50} = 2.979

R^2 = 0.9019

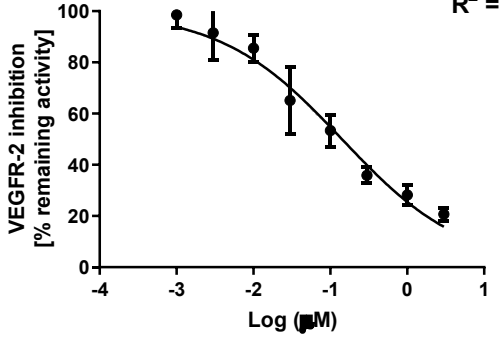


Best-fit values	
LogIC50	-0.8485
HillSlope	-0.5487
IC50	0.1417

M5

IC_{50} = 0.1417

R^2 = 0.9409

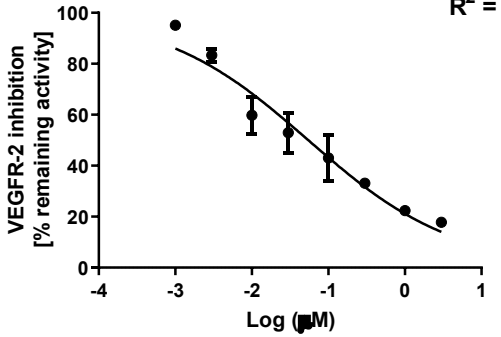


Best-fit values	
LogIC50	-1.262
HillSlope	-0.4530
IC50	0.05473

Sorafenib

IC_{50} = 0.05473

R^2 = 0.9368



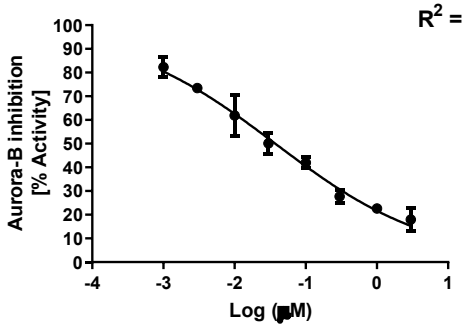
Aurora-B

Best-fit values	
LogIC50	-1.428
HillSlope	-0.3906
IC50	0.03730

M1

IC_{50} = 0.03730

R^2 = 0.9673

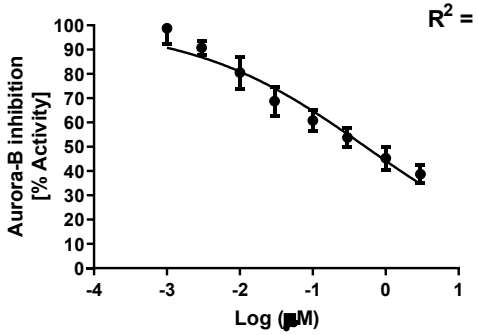


Best-fit values	
LogIC50	-0.2766
HillSlope	-0.3646
IC50	0.5290

M2

IC_{50} = 0.5290

R^2 = 0.9179

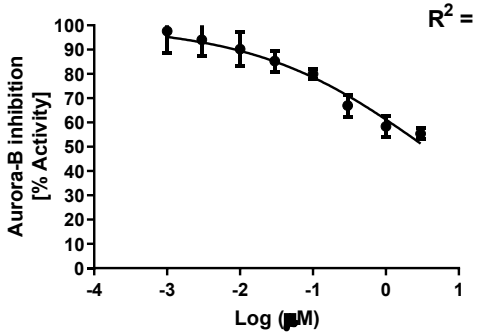


Best-fit values	
LogIC50	0.5403
HillSlope	-0.3664
IC50	3.469

M3

IC_{50} = 3.469

R^2 = 0.8960

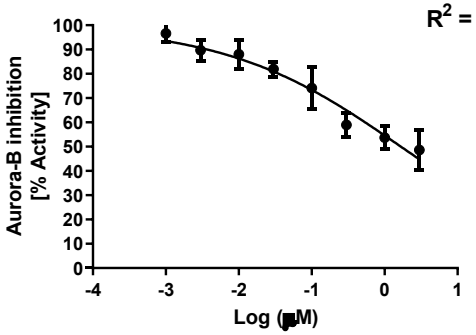


Best-fit values	
LogIC50	0.2236
HillSlope	-0.3593
IC50	1.673

M4

IC_{50} = 1.673

R^2 = 0.9026

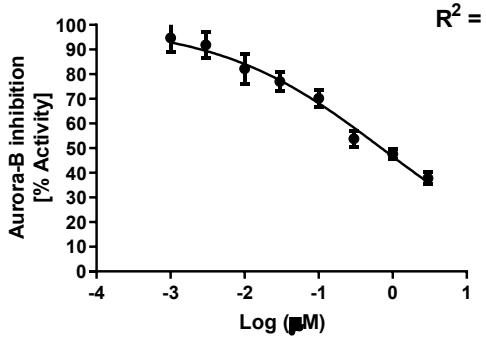


Best-fit values	
LogIC50	-0.1518
HillSlope	-0.3916
IC50	0.7050

M5

IC_{50} = 0.7050

R^2 = 0.9570

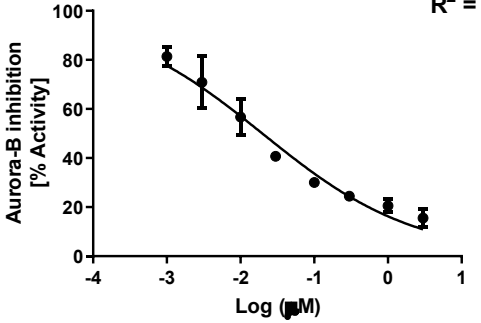


Best-fit values	
LogIC50	-1.704
HillSlope	-0.4177
IC50	0.01978

SP-96

IC_{50} = 0.01978

R^2 = 0.9469



***In vivo* anti-breast cancer activity assay of compound M1 and its effect on the apoptotic marker (caspase-3) level**

Induction of breast cancer in experimental animals:

Healthy 8-week-old Swiss albino female mice with a weight range (20 to 25 g) were provided by VACSERA, Cairo, Egypt. The mice were housed in a temperature-controlled (~20–22 ◦C) specific pathogen-free animal room with a 12 h light/12 h dark cycle and received food and water *ad libitum*. The experimental design was approved by Mansoura University Animal Care and Use Committee (MU-ACUC) in accordance with ARRIVE guidelines for animal experimentation. Breast cancer was induced by a single dose of 7,12-dimethylbenz[a]anthracene, intraperitoneally daily for 20 days. Mice tumor volume and body weight were monitored in the 2^{nd} to 5^{th} groups every five days. The tumor volume was measured via a digital vernier caliper.

Experimental design

Twenty 8-week-old Swiss albino female mice were randomly divided into five groups (five mice/group) as follows:

- Control group (received an equivalent volume of physiological saline);
- DMBA-induced group (received an equivalent volume of physiological saline)
- M1-treated group (injected intraperitoneally with ethyl 5-methyl-4-((5-(3-(p-tolyl) ureido)-1,3,4-thiadiazol-2-yl) thio) thieno [2,3-d] pyrimidine-6-carboxylate **(M1)** (10 mg/kg)).
- Dox-treated group (injected intraperitoneally with DOX (2 mg/kg)
- At the end of the experiment, all mice were anaesthetized and euthanized. Breast tissue tumor from all groups and corresponding control was dissected out, washed, and fixed in 10% formalin buffered and neutral solution for 72 hrs and processed for histopathological and immunohistochemical examination.

Histopathological examination

Formalin-fixed breast tissue was washed and processed in ascending graded ethanol, then processed using the paraffin embedding technique. Four µm-thick paraffin sections were further

processed to be stained with hematoxylin and eosin (H&E) stain for general histopathological evaluation [1].

Immunohistochemical analysis

According to manufacturers' reconstructions and following [2]. Four µm-thick tissue sections were processed in xylene, antigenic retrieval in citrate buffer (10 mM at pH 6.0), endogenous peroxidase blocking in 3% hydrogen peroxide, and nonspecific reactions blocked in 10% normal goat serum. Incubation with primary antibody of against active caspase 3 Cleaved Caspase-3 (Asp175) (5A1E) rabbit mAb (Catalog No# 9664; Cell Signaling Technology, Danvers, Massachusetts, USA) at 4°C overnight was followed by polyvalent and HRP labeled secondary antibodies and DAB immunoreaction was labelled with 3, 30-diaminobenzidine tetrahydrochloride chromogen, counterstained with Mayer's hematoxylin were doubled blinded examined and captures with Olympus BX51 microscope under the same exposure setting conditions.

Histomorphometric analysis: We used a Leica microscope (Dm 3000, Leica, Germany) and the computer package suite Leica LAS EZ (LAS EZ V 3.4.0, Germany) to scan and capture images of the breast tissue sections. Morphometrical analysis of active caspase-positive cells (in 18 high-power fields, at the magnification of x400) using the image's J analyzer computer software (IMAGE J/FIJI 1.46r, National Institutes of Health).

Statistical analysis: Statistical comparison (Analysis of variance, ANOVA) among groups was performed using GraphPad Prism 8 analysis software (Graph-

Pad, San Diego, CA, USA) for Windows. The values were defined as statistically significant as $p<0.05$ and were represented by mean ±SE for each group of the recordings reported by two observers.

Western Blot Protocol for Bax, Bcl-2, and β-actin in MDA-MB-231 Cells

I. Experimental Design & Sample Preparation

Cell Culture & Treatment

- **Cell Line:** MDA-MB-231 (triple-negative breast cancer cells)
- **Culture Conditions:** DMEM + 10% FBS + 1% penicillin/streptomycin at 37°C, 5% CO_2
- **Treatment:** Seed cells at 2×10^6 cells per 100 mm dish. Treat with compound at optimized concentration (include dose-response pilot). Include vehicle control (e.g.,

DMSO ≤0.1%). Harvest after 24-48 hours (time-course dependent on compound mechanism).

- **Replicates:** Minimum n=3 biological replicates per group.

II. Reagents & Commercial Kits

Lysis Buffer Kit

- **Recommended: RIPA Lysis Buffer Kit** (Thermo Fisher Scientific, #89900)
    - Includes: RIPA buffer, protease inhibitor cocktail (add 1:100), phosphatase inhibitors (if studying phosphorylation).

Protein Quantification

- **Kit: Pierce™ BCA Protein Assay Kit** (Thermo Fisher Scientific, #23225)

Electrophoresis & Transfer

- **Precast Gels: 4-20% Mini-PROTEAN® TGX™ Precast Gels** (Bio-Rad, #456109)
- **Transfer System: iBlot™ 2 Transfer System** (Thermo Fisher Scientific) with iBlot™ 2 Transfer Stacks, PVDF, regular size (0.2 μm, #IB24002)

Blocking & Detection

- **Blocking Buffer: EveryBlot Blocking Buffer** (Bio-Rad, #12010020) – reduces background with phospho-proteins.
- **Chemiluminescent Substrate: Clarity™ or Clarity Max™ ECL Western Blotting Substrate** (Bio-Rad, #1705060 or #1705062)

III. Antibodies

Primary Antibodies

Target	Host	Clone	Supplier & Catalog #	Dilution	Expected Band Size
Bax	Rabbit	D2E11	Cell Signaling, #5023	1:1000	20 kDa
Bcl-2	Mouse	100	Santa Cruz, sc-7382	1:500	26 kDa
β-actin	Mouse	AC-15	Sigma-Aldrich, A5441	1:5000	42 kDa

Secondary Antibodies

- **Anti-Rabbit IgG HRP-linked** (Cell Signaling, #7074) – 1:2000 for Bax.
- **Anti-Mouse IgG HRP-linked** (Cell Signaling, #7076) – 1:2000 for Bcl-2 and β-actin.

IV. Step-by-Step Protocol

Day 1: Cell Lysis & Protein Quantification

1. **Wash cells** with cold PBS.
2. **Lyse cells** with 300 µL ice-cold RIPA buffer + inhibitors per dish, scraped, collected, and vortexed.
3. **Centrifuge** at 14,000×g for 15 min at 4°C.
4. **Collect supernatant**, aliquot, and store at -80°C.
5. **BCA Assay:**
    - Prepare standards (0-2000 µg/mL).
    - Mix 10 µL sample with 200 µL working reagent in 96-well plate.
    - Incubate 30 min at 37°C, measure absorbance at 562 nm.
6. **Adjust all samples to equal concentration** (2 µg/µL) with RIPA buffer + Laemmli sample buffer (4X, Bio-Rad, #1610747) + 5% β-mercaptoethanol.
7. **Denature** at 95°C for 5 min, cool on ice.

Day 1: SDS-PAGE & Transfer

8. **Load 20-30 µg protein** per lane alongside pre-stained ladder (e.g., Precision Plus Protein™ Dual Color, Bio-Rad, #1610374).
9. **Run gel** at 80 V (stacking) then 120 V (resolving) until dye front reaches bottom.
10. **Activate PVDF membrane** in methanol for 1 min, rinse in transfer buffer.
11. **Transfer** using iBlot™ 2: Program P0 (20 V, 1 min; 23 V, 4 min; 25 V, 2 min).
12. **Block membrane** in EveryBlot Blocking Buffer for 5 min at RT.

Day 1: Incubation with Primary Antibodies

13. **Incubate with primary antibody** diluted in blocking buffer **overnight at 4°C** with gentle shaking.

- *Optional:* Cut membrane horizontally using ladder markers:
    - **Top half (26-42 kDa):** Bcl-2 & β-actin (mouse primaries).
    - **Bottom half (20 kDa):** Bax (rabbit primary).

Day 2: Secondary Incubation & Detection

14. **Wash** 3×10 min with TBST.
15. **Incubate with species-appropriate HRP-secondary** in blocking buffer for 1 h at RT.
16. **Wash** 3×10 min with TBST.
17. **Develop:**

- Mix Clarity Max ECL reagents 1:1, apply to membrane.
- Image with **ChemiDoc™ MP Imaging System** (Bio-Rad).

V. Equipment List

Device	**Example Model/Supplier**
Cell culture hood	Class II, Thermo Scientific
CO_2 incubator	Thermo Forma Series
Centrifuge	Microcentrifuge, Eppendorf 5424R
Nanodrop/Spectrophotometer	Thermo NanoDrop™ One
Gel electrophoresis chamber	Mini-PROTEAN Tetra Cell, Bio-Rad
Power supply	PowerPac™ Basic, Bio-Rad
Transfer system	iBlot™ 2, Thermo Fisher
Orbital shaker	VWR® Standard
Imaging system	ChemiDoc™ MP, Bio-Rad
Software for analysis	Image Lab™ (Bio-Rad) or ImageJ

VI. Expected Results & Analysis

- **Bax:** Increased band intensity in compound-treated vs. untreated (pro-apoptotic).
- **Bcl-2:** Decreased band intensity in treated vs. untreated (anti-apoptotic).
- **β-actin:** Equal loading control across all lanes.
- **Calculate ratio:** (Bax or Bcl-2 band density) / (β-actin band density) for each sample.

VII. Troubleshooting Tips

- **High background:** Increase TBST washes, optimize antibody concentration.

- **Non-specific bands:** Include negative control (no primary antibody).
- **No signal:** Check antibody expiration, ensure HRP substrate is fresh.
- **β-actin uneven:** Re-run samples, ensure equal protein loading.

Note: Validate antibodies using knockdown/knockout controls if available. Optimize exposure times to avoid saturation. Always include molecular weight ladder for accurate band identification.

Detailed Results:

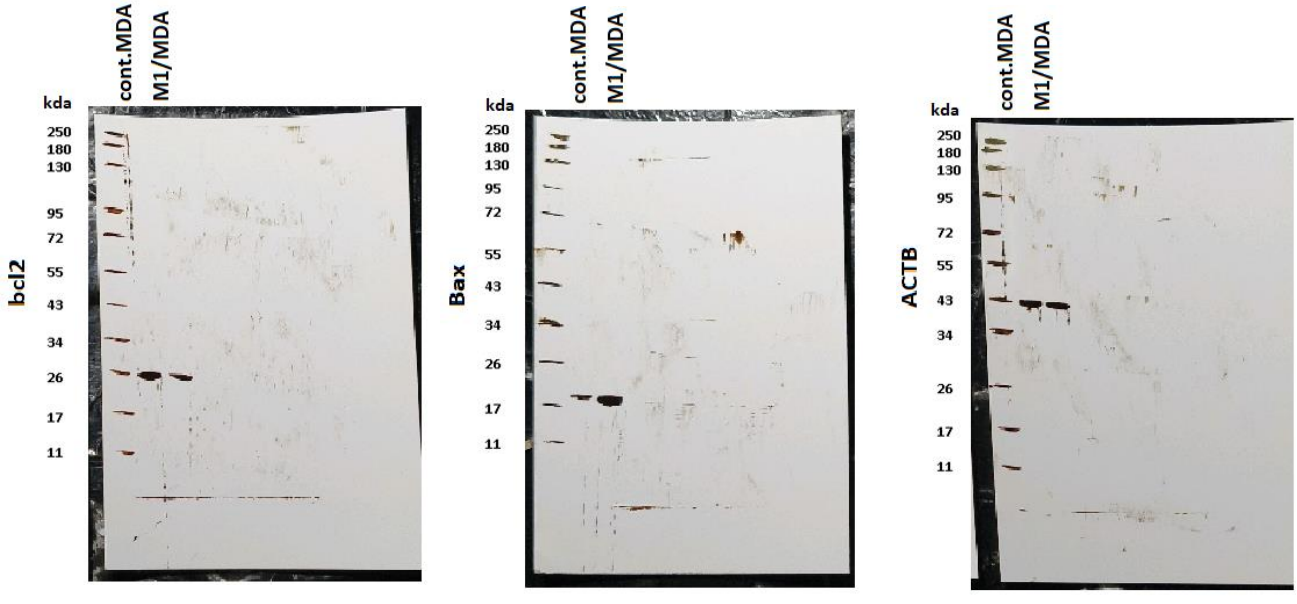
MDA

Bax	20 kda			
sample band	area	%	Rel.Density	adj. Density
Standared	1762	17.62	1	1
M1/MDA	6554	65.54	3.719636776	5.951418842
cont.MDA	2116	21.16	1.200908059	1.921452894

[redacted]

Bcl2	26 kda			
sample band	area	%	Rel.Density	adj. Density
Standared	2193	21.93	1	1
M1/MDA	2911	29.11	1.32740538	2.12384861
cont.MDA	5433	54.33	2.47742818	3.96388509

[redacted]



1- Solubility in PBS (pH = 7.4) for 2 hours of bath sonication

1.5 mg = 1500 ug with PBS (pH = 7.4) to 10 ml in a measuring flask

Not soluble after 2 hours	Highly turbid with visible floating particles

Filtered by 0.45 um Syringe nylon filter — **Absorbance of the filtrate represents solubility in PBS (pH=7.4)**

A at 295 nm against suitable blanking	**0.035**	**From calibration equation**	**C = A/slope**	**1.11 ± 0.06 ug/ml**

2- Calibration curve

1.9 mg = 1900 ug dissolved in a 10 ml mixture of ethanol and chloroform in a 2:3 ratio respectively

Clear stock solution

C stock = 1900/10 = 190ug/ml

Spectrum against the mixture blanking

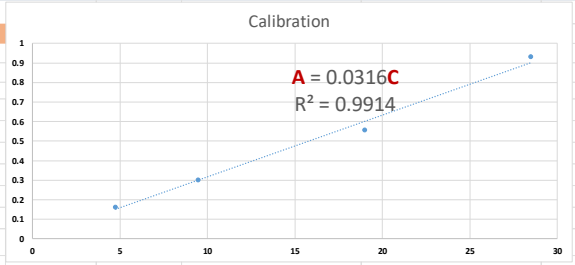
295 nm was selected for calibration curve

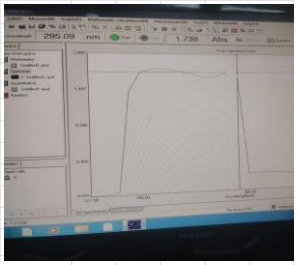
Serial dilutions from the stock with the mixture to a volume of 10 ml.

Volume taken (ml)	final volume (ml)	C (ug/ml)	Absorbance
0.25	10	4.75	0.16
0.5	10	9.5	0.3
1	10	19	0.556
1.5	10	28.5	0.93

Spectrum of 1.5 ml dilution was superimposed on that of stock

Average Slope **0.0316 ± 0.002 ml/ug**





3- Partitioning Co-efficient (log P)

2 mg = 2000 ug with 20 ml chloroform- bath sonication (turbid suspension with no visible particles or sedimentation)

Filtered by 0.45 um Syringe nylon filter — **10 ml of clear filtrate (saturated) vigorously mixed with 10 ml water**

Separating funnel (lower chloroform phase and upper water phase)

log p = log (concentration in chloroform phase/concentration in water phase)

log p = log (Absorbance of chloroform phase/Absorbance of water phase)

A at 295nm of chloroform phase against suitable blanking	**0.713**	**log p**	**1.309**
A at 295nm of water phase against suitable blanking	**0.035**		

As water phase could possibly have chloroform droplets during separation

Maximum absorbance of saturated water with formulation at 295 nm against suitable blanking was taken into consideration (most near to separating funnel method)

Absorbance of saturated water		**0.008**	
	log p	**1.95**	
Average log p	**1.63**	**SD**	**0.453**

Average log p ± SD = 1.63 ± 0.45

[1] K.S. Suvarna, C. Layton, J.D. Bancroft, Bancroft's theory and practice of histological techniques E-Book, Elsevier health sciences, 2018.
[2] G.A. Abdel-Latif, A.S. Al-Kashef, M.U. Nooman, A.E.-N.A. Khattab, S.M. Gebril, N.F. Elmongy, S.S. Abbas, The mechanistic interplay between Nrf-2, NF-κB/MAPK, caspase-dependent apoptosis, and autophagy in the hepatoprotective effects of Sophorolipids produced by microbial conversion of banana peels using Saccharomyces cerevisiae against doxorubicin-induced hepatotoxicity in rats, Food and Chemical Toxicology, 182 (2023) 114119.