Design and Synthesis of Coumarin Derivatives as Cytotoxic Agents through PI3K/AKT Signaling Pathway Inhibition in HL60 and HepG2 Cancer Cells

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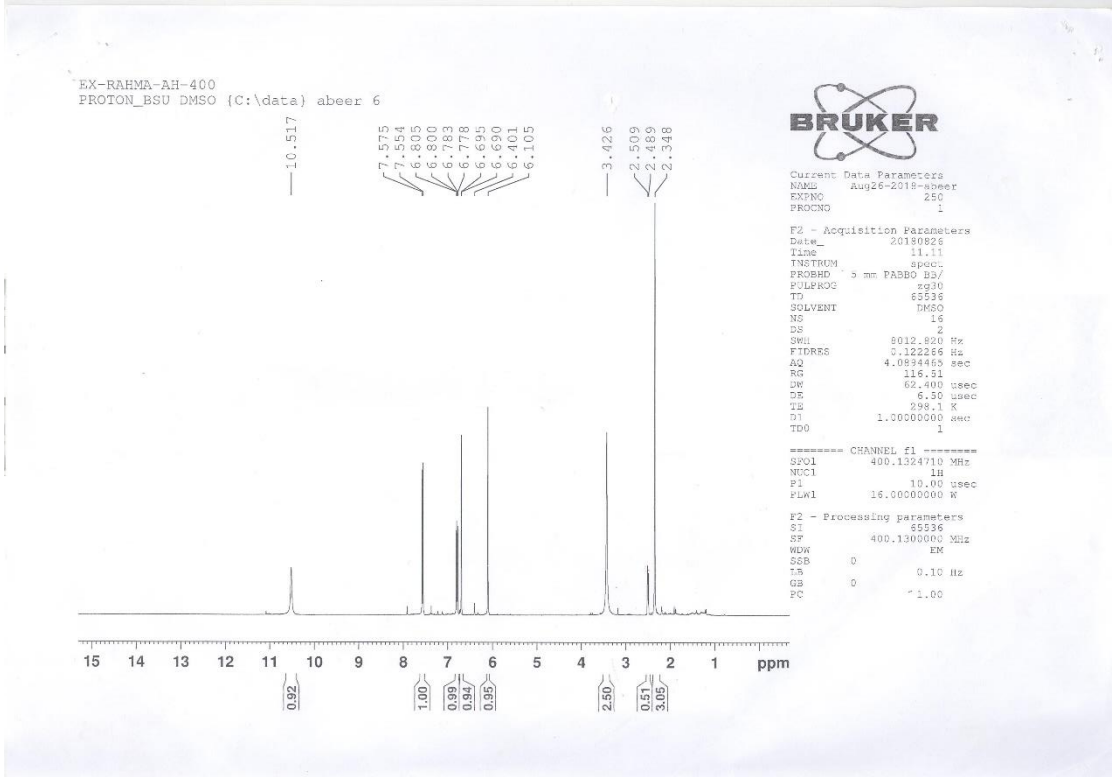

Fig. S1. ^{1}H NMR of Compound (3)

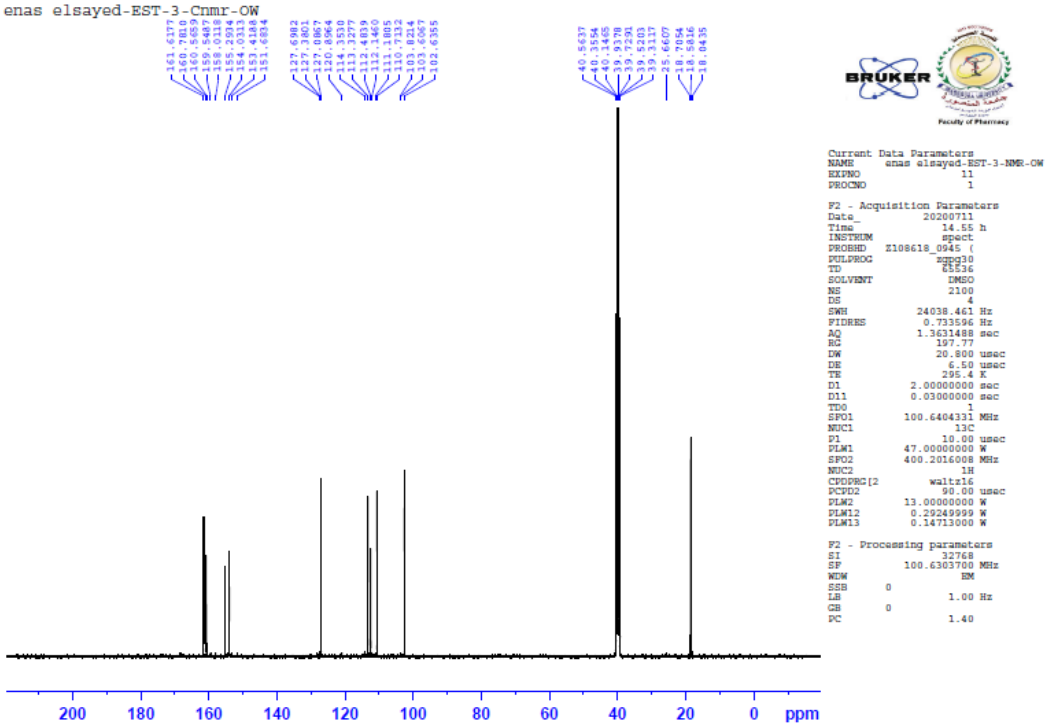

Fig. S2. ^{13}C NMR of Compound (3)

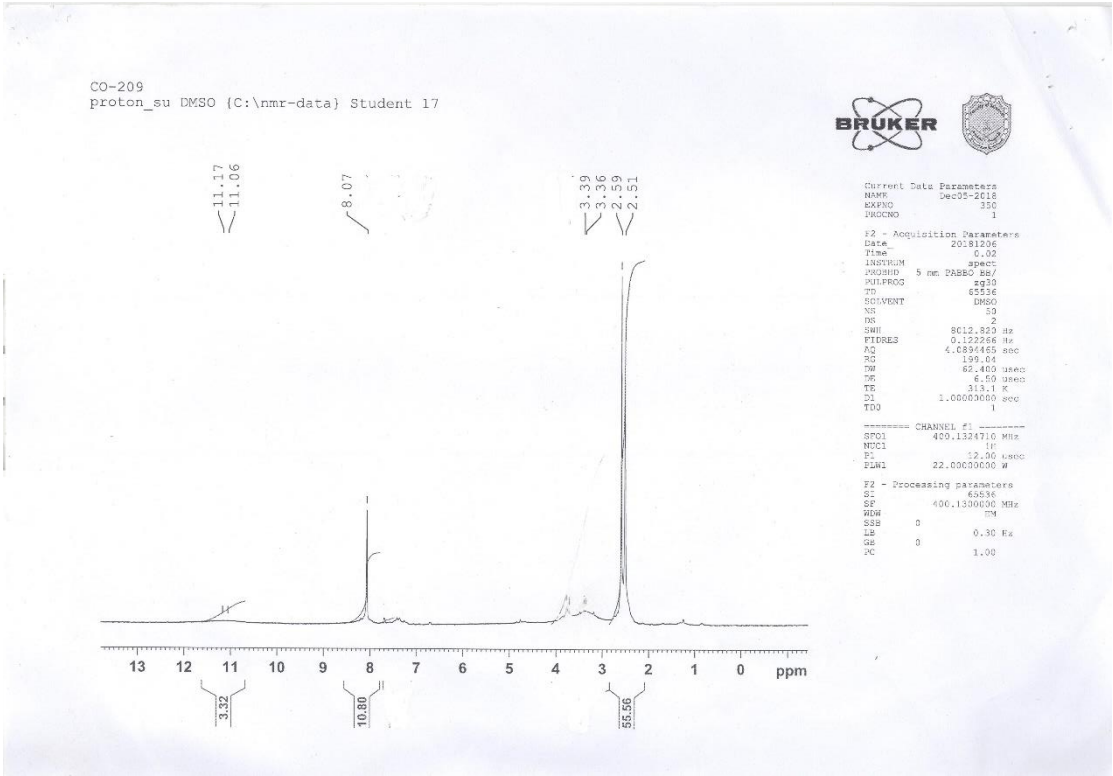

Fig. S3. ^{1}H NMR of Compound (4)

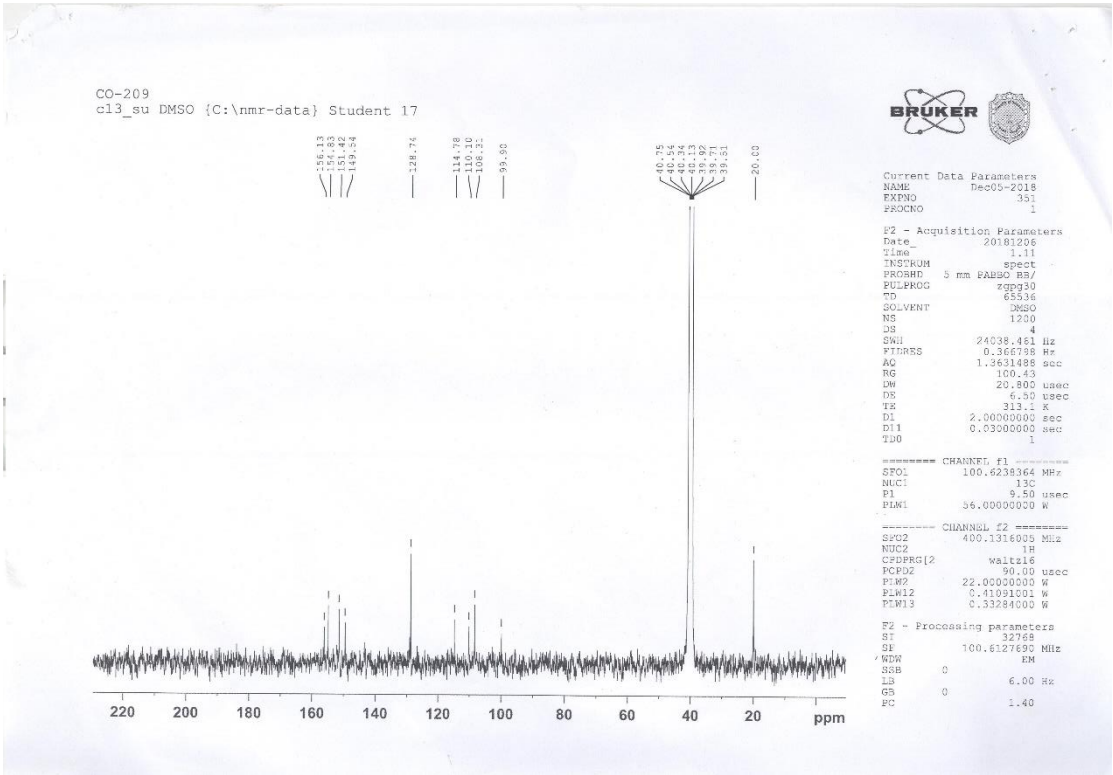

Fig. S4. ^{13}C NMR of Compound (4)

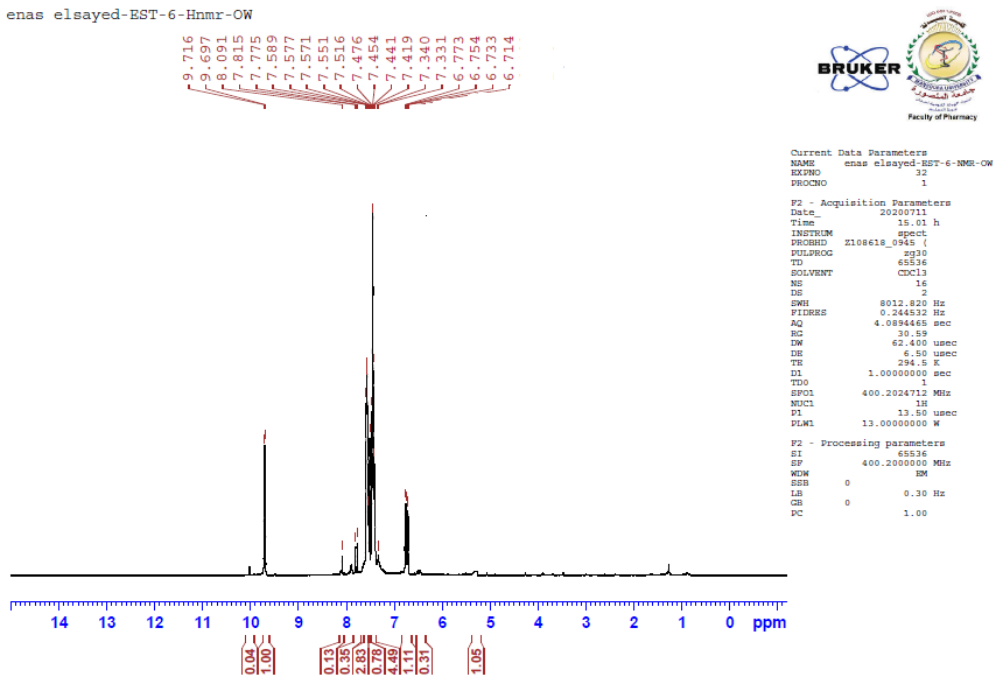

Fig. S5. ^{1}H NMR of Cinnamaldehyde (5)

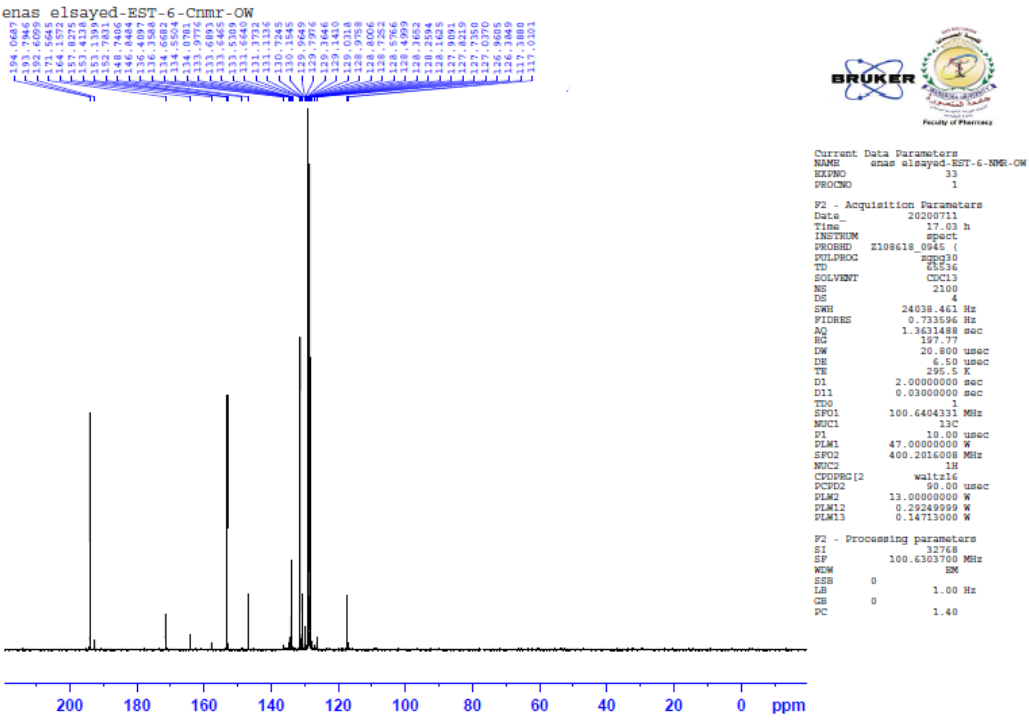

Fig. S6. ^{13}C NMR of Cinnamaldehyde (5)

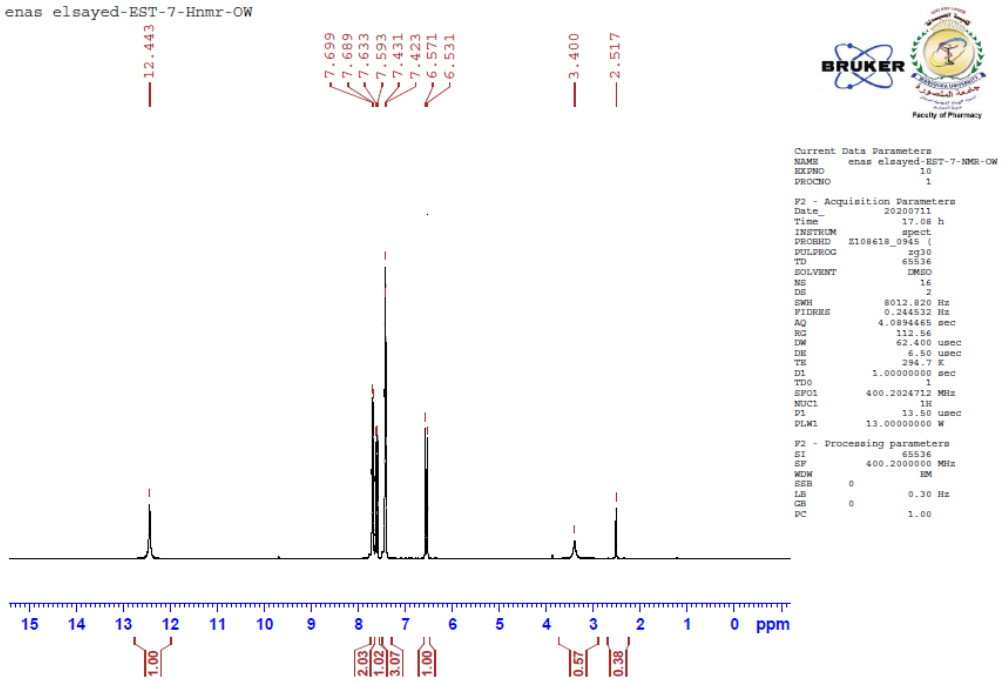

Fig. S7. ^{1}H NMR of Cinnamic Acid (6)

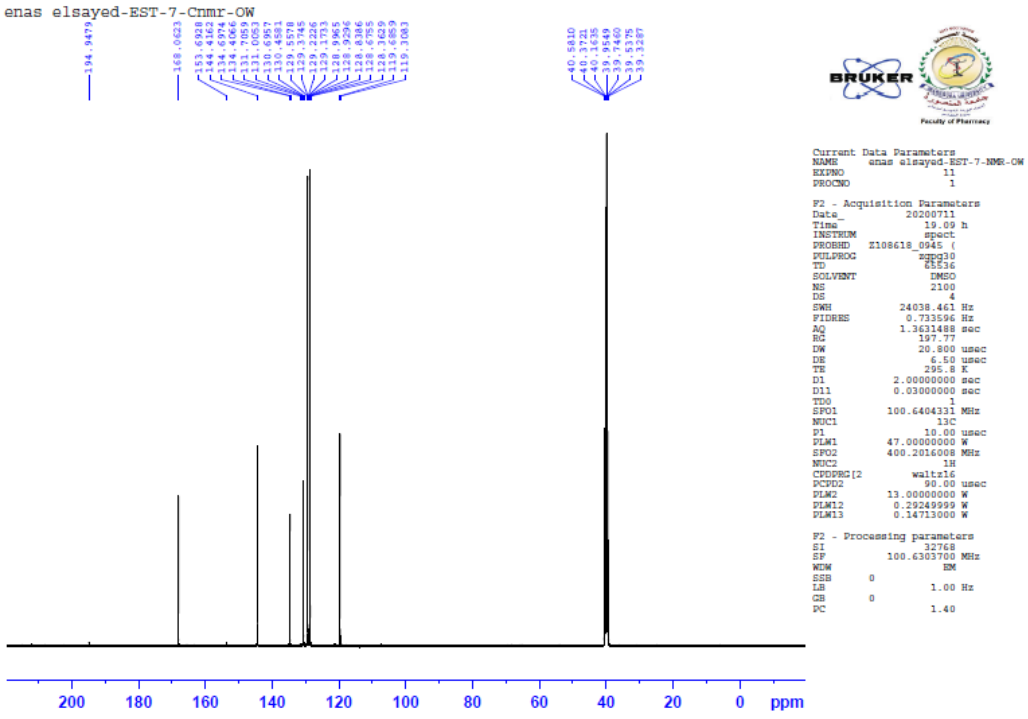

Fig. S8. ^{13}C NMR of Cinnamic Acid (6)

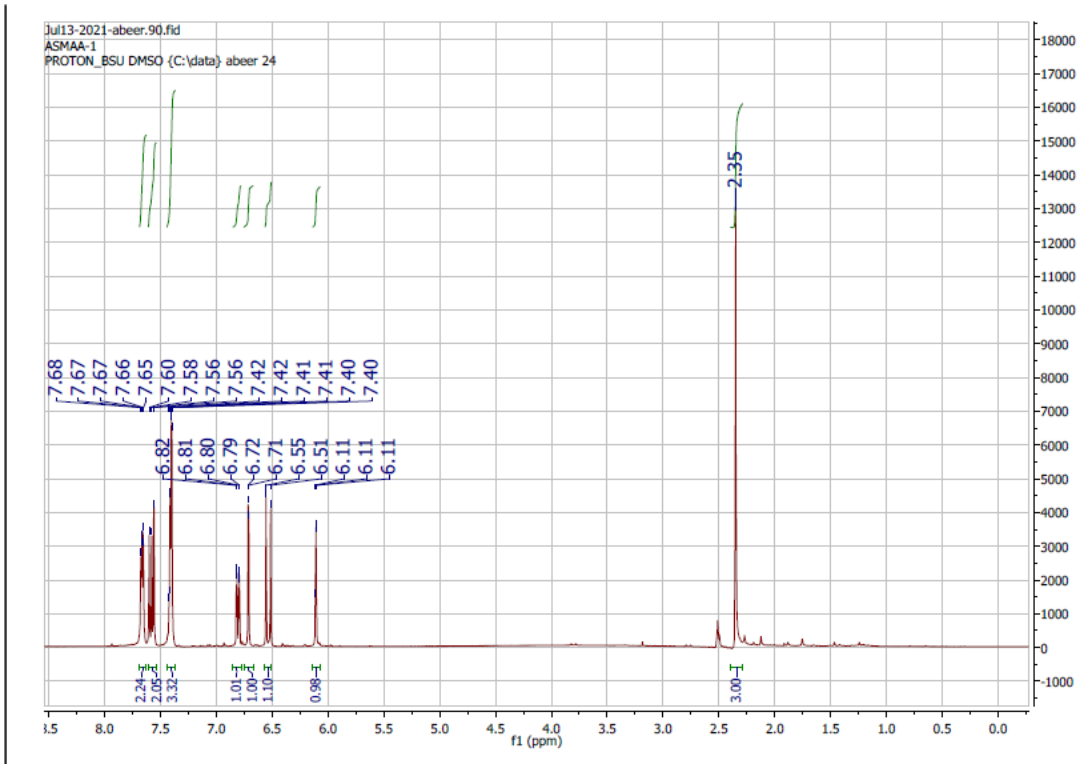

Fig. S9. ^{1}H NMR of Compound (8a)

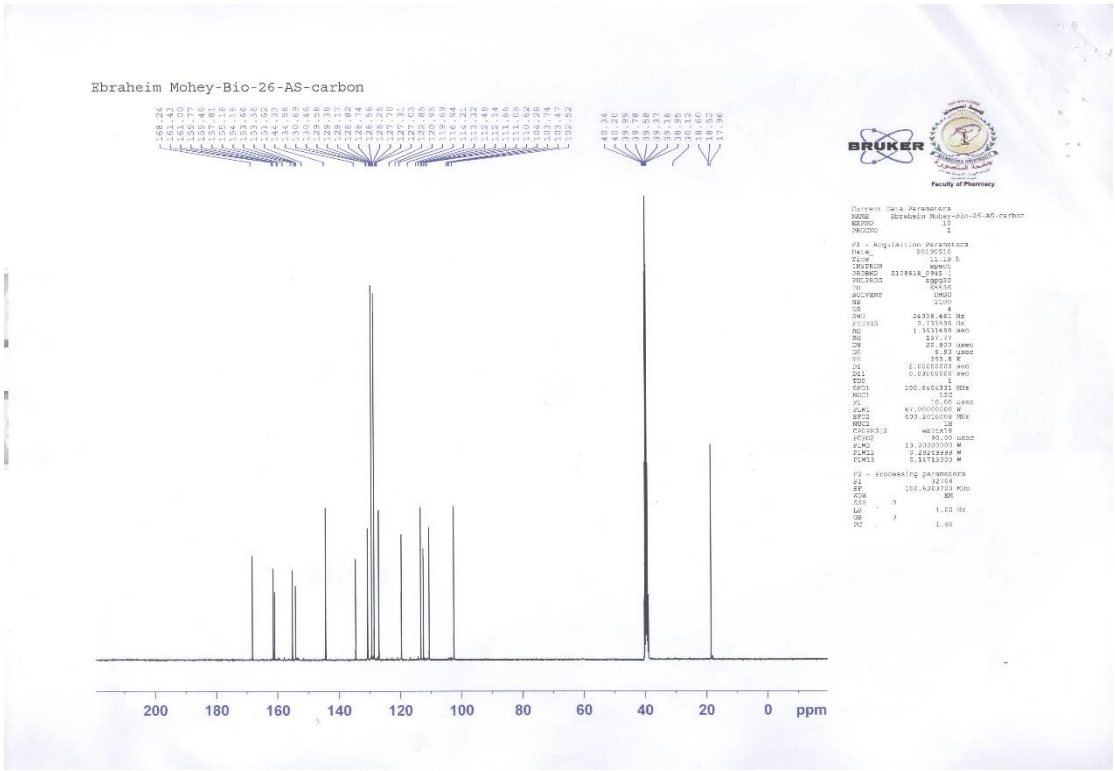

Fig. S10. ^{13}C NMR of Compound (8a)

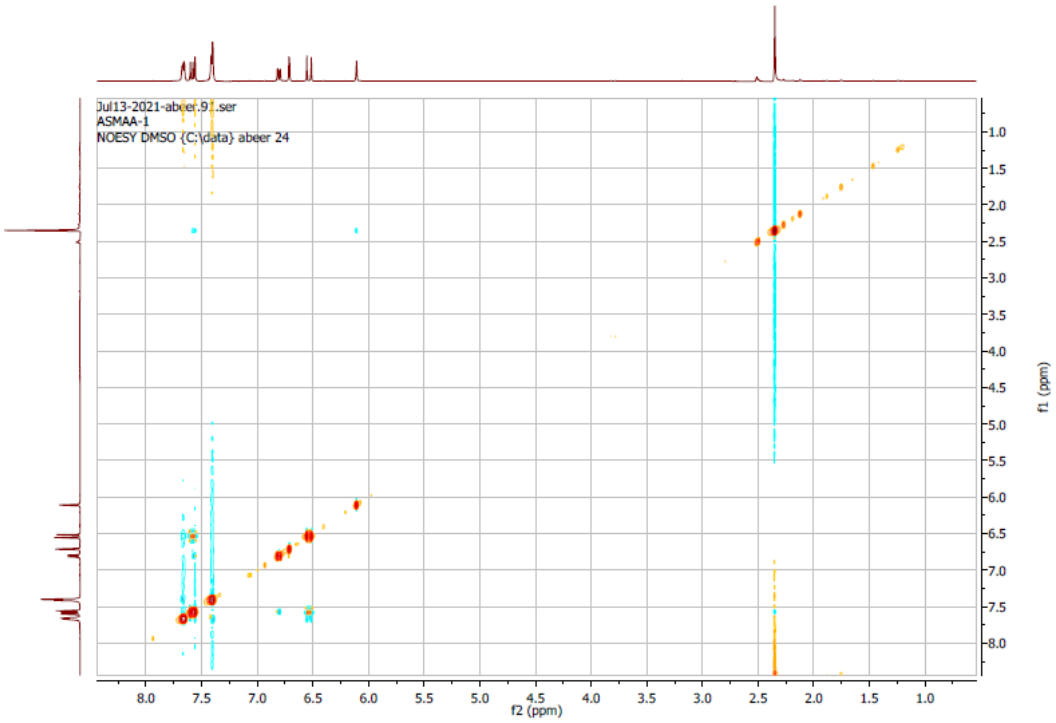

Fig. S11. NOSY of Compound (8a)

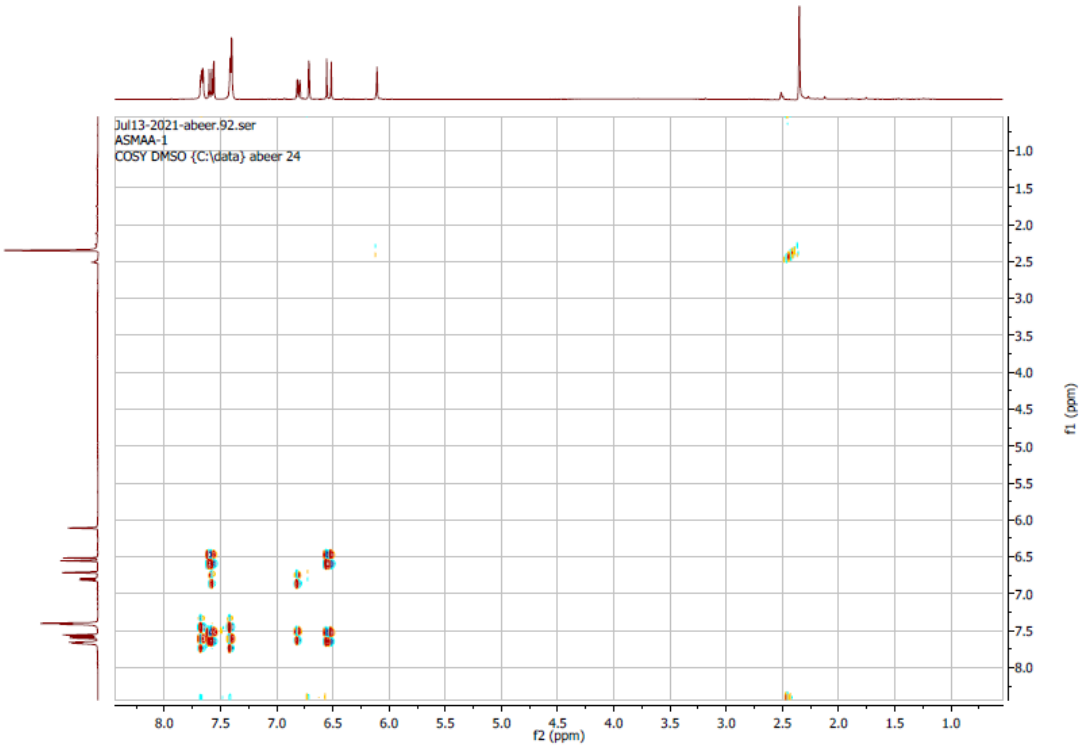

Fig. S12. COSY of Compound (8a)

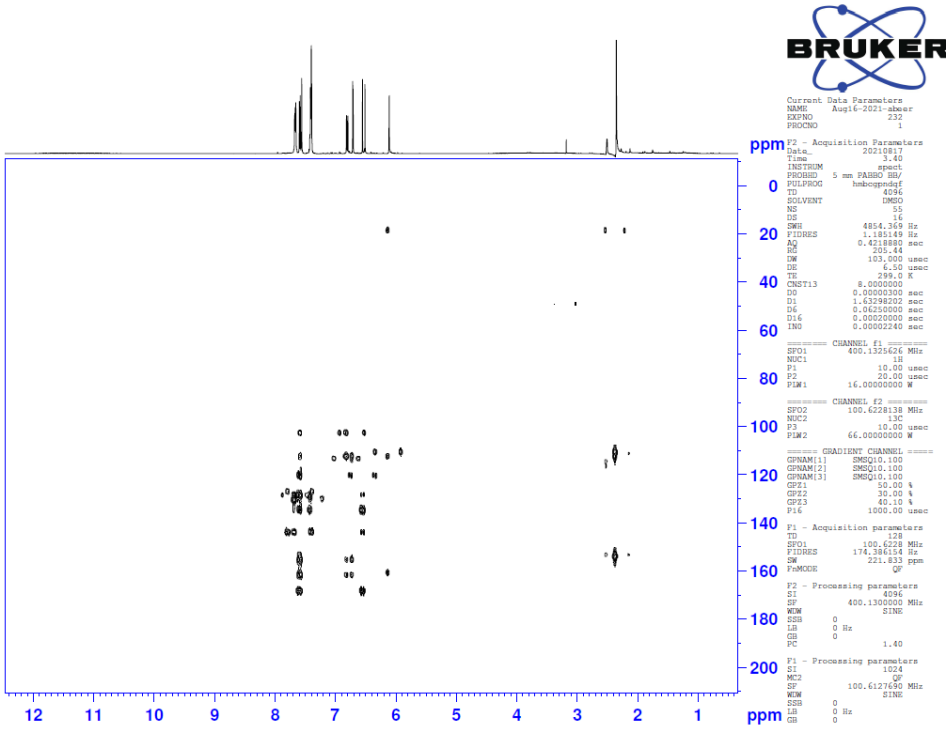

Fig. S13. HMBC of Compound (8a)

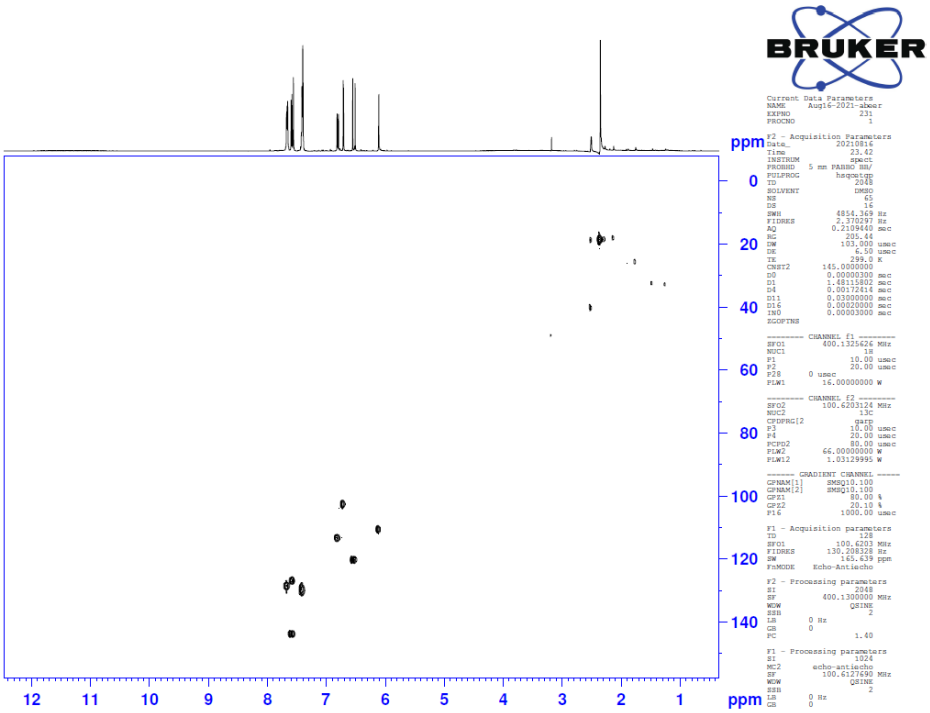

Fig. S14. HSQC of Compound (8a)

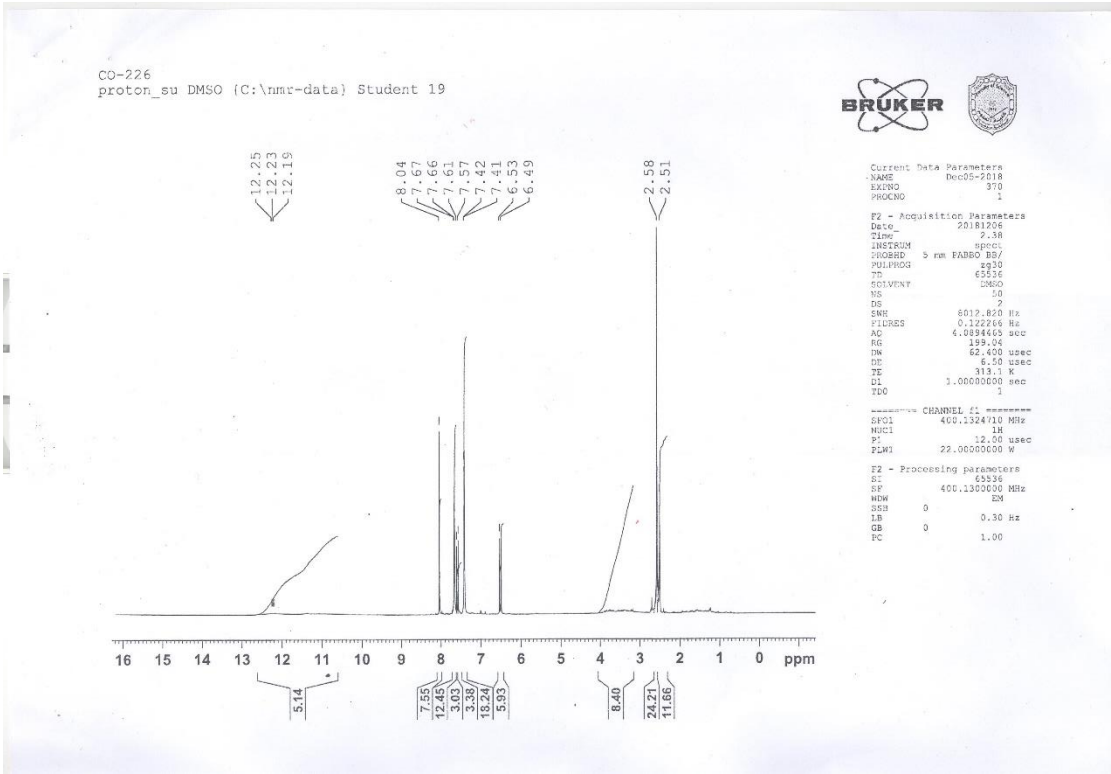

Fig. S15. ^{1}H NMR of compound (8b)

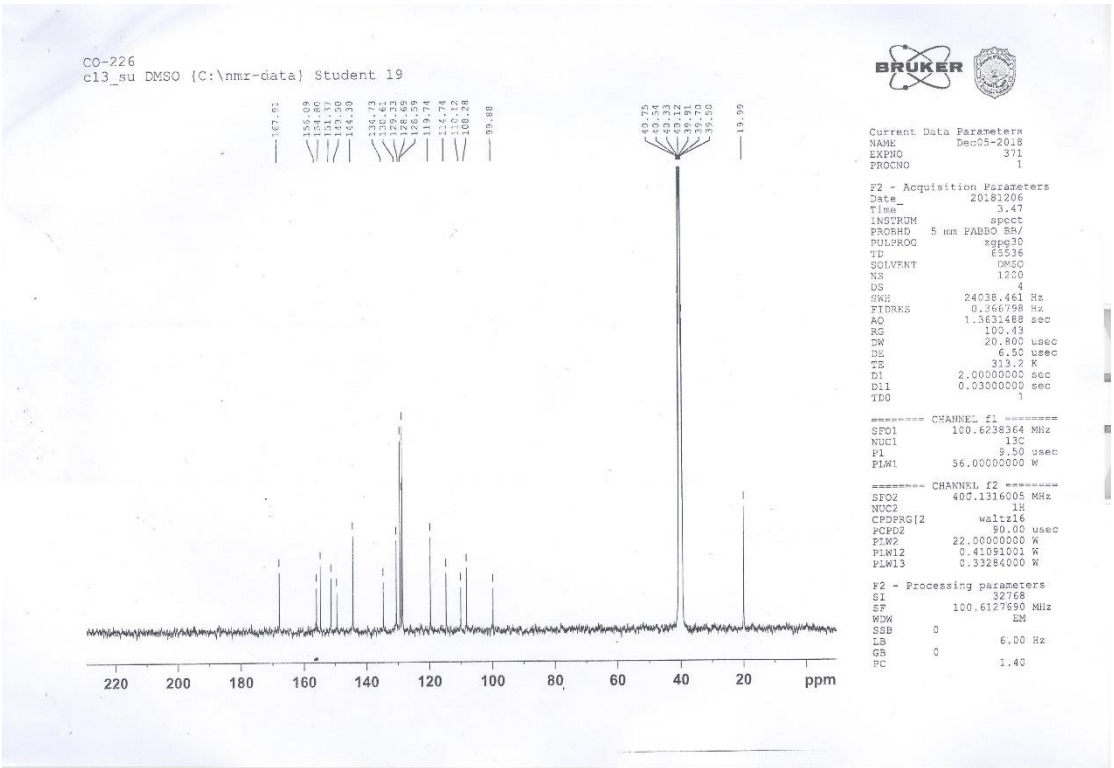

Fig. S16. ^{13}C NMR of compound (8b)

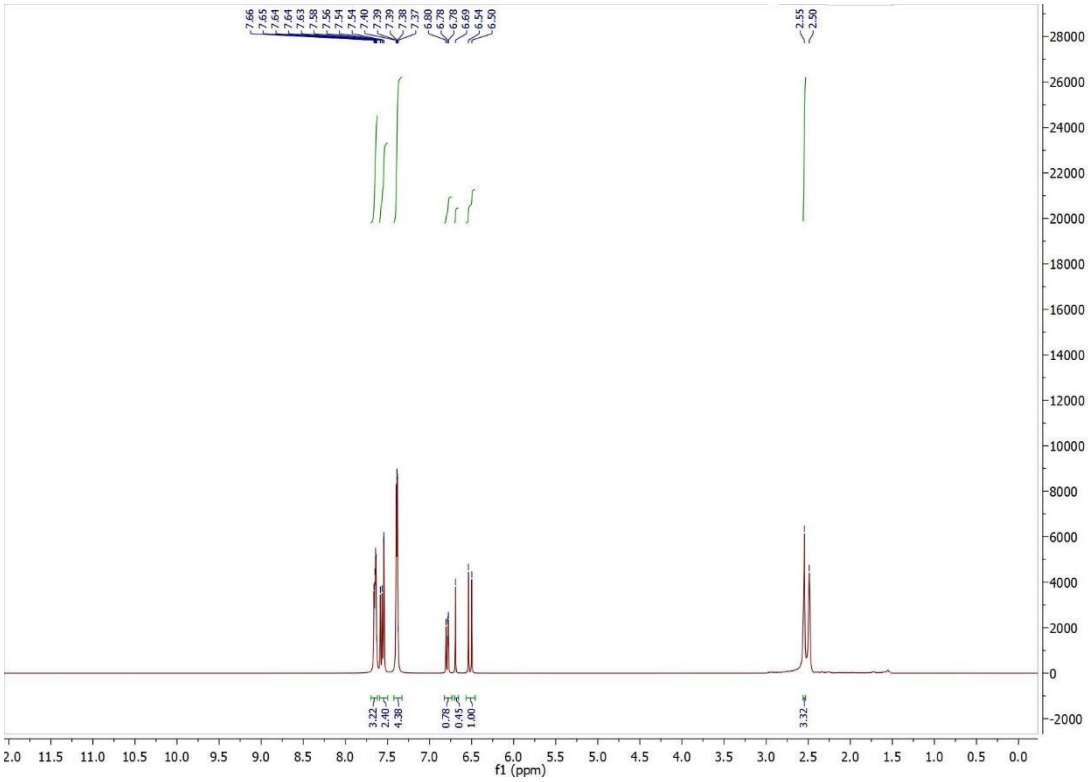

Fig. S17. ^{1}H NMR of compound (8c)

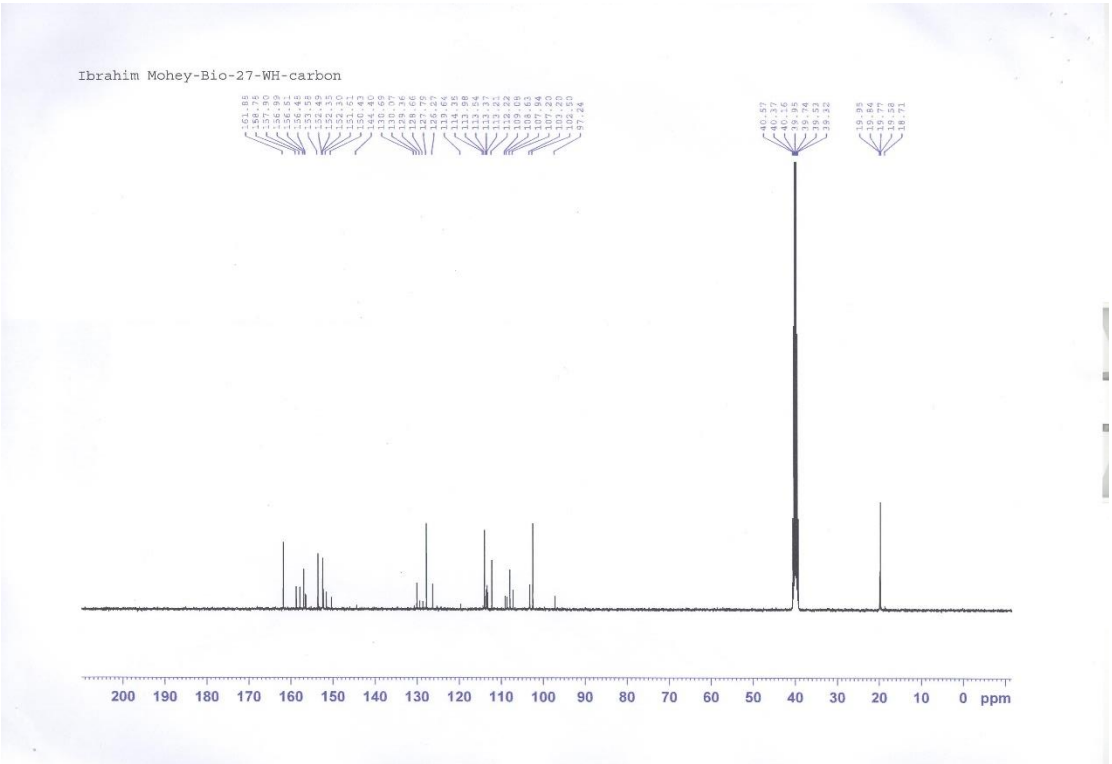

Fig. S18. ^{13}C NMR of compound (8c)

Supplementary materials for detailed methodology

2.2.2.1. Flow cytometric analysis

2.2.2.1.1. *FITC/Annexin-V-FITC/PI Differential Apoptosis/Necrosis Assessment*

Apoptosis and necrosis cell populations are determined using Annexin V-FITC apoptosis detection kit (Abcam Inc., Cambridge Science Park, Cambridge, UK) coupled with 2 fluorescent channels flowcytometry. After treatment with test compounds for 48 h, cells (10^5 cells) are collected by trypsinization and washed twice with ice-cold PBS (pH 7.4). Then, cells are incubated in dark with 0.5 ml of Annexin V-FITC/PI solution for 30 min in dark at room temperature according to manufacturer protocol. After staining, cells are injected via ACEA Novocyte™ flowcytometer (ACEA Biosciences Inc., San Diego, CA, USA) and analyzed for FITC and PI fluorescent signals using FL1 and FL2 signal detector, respectively (λex/em 488/530 nm for FITC and λex/em 535/617 nm for PI). For each sample, 12,000 events are acquired and positive FITC and/or PI cells are quantified by quadrant analysis and calculated using ACEA NovoExpress™ software (ACEA Biosciences Inc.,San Diego, CA, USA).

2.2.2.1.2. *DNA Content-Flow Cytometry Aided Cell Cycle Analysis*

After treatment with test compounds for 48 h, cells (10^5 cells) are collected by trypsinization and washed twice with ice-cold PBS (pH 7.4). Cells are re-suspended in two milliliters of 60% ice-cold ethanol and incubated at 4ºC for 1 h for fixation. Fixed cells are washed twice again with PBS (pH 7.4) and re-suspended in 1 mL of PBS containing 50 µg/mL RNAase A and 10 µg/mL propidium iodide (PI). After 20 min of incubation in dark at 37 C, cells are analyzed for DNA contents using flow cytometry analysis using FL2 (λex/em 535/617 nm) signal detector (ACEA Novocyte™ flowcytometer, ACEA Biosciences Inc., San Diego, CA, USA). For each sample, 12,000 events are acquired. Cell cycle distribution is calculated using ACEA NovoExpress™ software (ACEA Biosciences Inc., San Diego, CA, USA).

2.2.2.1.3. *Caspase- 3/7 green flow cytometry assay*

The ability of compounds **4** and **9** to induce the enzymatic activities of caspase 3/7 in HL60 and HepG2 respectively were assayed using caspase 3/7 green flow cytometry assay method. Briefly, control and the tested compounds treated cells ($2.5x10^5$/mL) were rinsed with ice cold PBS, cell lysates were prepared and subsequently protein

concentration was measured. Lysates were combined with reaction buffer and incubated with Caspase 3/7 Detection Reagent (specific colorimetric substrates) at 37 °C for 6 h. The samples were estimated at λ 488 nm in BD FACS Calibur flow cytometer.

2.2.2.3. Immunoblotting (western plot) for PI3K and AKT

Untreated and treated HL-60 and HepG2 cancer cells were treated with compound **4** (IC_{50}=8.09 μM, 48 h) and compound **9** (IC_{50}=13.14 μM, 48 h), respectively were washed in PBS and lysed in boiling sample buffer (62.5 mM Tris-HCl pH 6.8, 1% SDS, 10% glycerol, and 5% β-mercaptoethanol) for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The lysates were boiled for 5 min with lamellae buffer, and the proteins will be separated by SDS-PAGE and transferred to an Immobilon membrane (Millipore). The antibodies used will be antigen affinity-purified polyclonal sheep IgG anti-human annexin V (R&D Systems). After incubation in 5% non-fat dry milk, Tris-HCL, 0.1% Tween 20 for 1 hr, p-PI3K and p-AKT primary antibodies were added to one of the membranes containing specimen samples and incubated at 4℃ overnight. Appropriate secondary antibodies were incubated for 2 hr at room temperature. After being washed twice n 1 x TBS-T, densitometric analysis of the immunoblots was performed to quantify the amounts of p-PI3K and p-AKT against control sample by total protein normalization by Image analysis software on the ChemiDoc MP imaging system (version 3) produced by Bio-Rad (Hercules, CA). (Tantawy et al 2020)

Table S1. Docked interaction analysis of synthesized ligands screened with the two proteins (PI3K and AKT1).

Ligands	Receptor (Protein)	binding free energy (kcal/mol)	Number of H-bonds	Active site residues	Bonds length (Å)
3	PI3K	-9.13	2	Val882 Lys833	2.06 2.32
	AKT1	-9.60	4	Asp292 Ala230 Met227 Lys179	1.77 2.13 2.76 2.49
4	PI3K	-11.80	3	Lys833 Asp841 Val882	1.98 2.60 1.98
	AKT1	-13.27	6	Lys179 Ala230 Thr211	1.98 2.45 2.65

				Glu228 Asp202 Asp274	2.90 3.00 3.03
6	PI3K	-9.18	2	Lys833 Val882	2.04 - 2.52 3.17
	AKT1	-10.02	3	Lys179 Ala230 Met227	2.29 1.88 3.78
8a	PI3K	-12.30	2	Val882 Lys833	2.08 2.05
	AKT1	-10.06	3	Ala230 Lys276 Gly157	3.20 2.30 2.33
8b	PI3K	-13.00	4	Asp964 Cys869 Lys833 Val882	3.38 3.72 2.28 2.19
	AKT1	-10.89	6	Lys179 Asn279 Asp292 Gly157 Met281 Glu228 Ala230	2.63 3.44 2.35 3.57 2.96 3.01 3.65
8c	PI3K	-12.76	1	Val882	2.05
	AKT1	-10.16	2	Lys179 Glu228	1.83 2.16
Staurosporine	PI3K	-14.07	2	Val882 Lys833	1.89 1.76
Spirochromane derivative	AKT1	-14.90	6	Asp292 Glu228 Ala230 Phe161	1.60 2.04 2.58 2.12 2.10 2.40