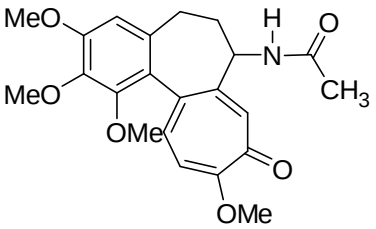
Research Article

Synthesis of New ١-(naphthalen-١-yl)-٥-(٣,٤,٥-trimethoxyphenyl)-١*H*-١,٢,٤-triazole-٣-carboxamide derivatives and study of their cytotoxicity

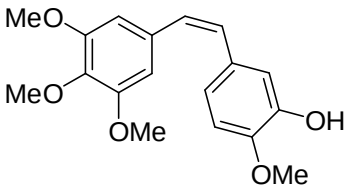
Islam Zaki*, Omar M. Aly, Mustafa H. Abdelrahman***, Mohamed Ramadan***,**
* Department of Organic Chemistry, Faculty of Pharmacy, Port Said University,
** Department of Medicinal Chemistry, Faculty of Pharmacy, Minia University,
*** Department of Pharmaceutical Organic Chemistry, Faculty of Pharmacy, Al-Azhar University.

Abstract
A new series of ١-(naphthalen-١-yl)-٥-(٣,٤,٥-trimethoxyphenyl)-١*H*-١,٢,٤-triazole-٣-amides **٤a-d** were synthesized by rearrangement of ٤-(naphthalen-١-yl)-hydrazono-٢-(٣,٤,٥-trimethoxyphenyl)-٢-oxazolin-٥-one **٣** in the presence of different benzylamines to afford compounds **٤a-d.** The structure of the prepared compounds was confirmed by ^{١}**H** and ^{١٣}**C NMR** along with High Resolution Mass Spectrometry (**HRMS**). The prepared compounds were evaluated for their *in vitro* cytotoxic activity against Hep-G٢ and leukemia HL-٦٠ cell lines. The result showed that compounds **٤a** and **٤d** exhibited remarkable results against Hep-G٢ cells with $IC_{٥٠}$. values of ٦٠ and ٩٠ □M, respectively compared to combretastatin A-٤ as a reference drug.
Keywords: Cytotoxicity / ١,٢,٤-Triazole / combretastatin

Introduction
The importance of tubulin and microtubules in chromosome segregation during cell division makes them an attractive targets for anticancer drug design, i.e. in the development of antimitotic agents.[١,٢] Tubulin is the building block of microtubules, which are important in cellular functions such as cell transport, movement, separation of chromosomes during mitosis in addition to the cytoskeleton Structure.[١, ٣] Several research studies have elucidated at least three distinct binding regions on tubulin, They are the vinca, taxane and colchicine binding sites. Antimitotic agents with the capability of binding at the colchicine site of tubulin have received much attention.[٤,٥] Among all the colchicine site agents, combretastatin A-٤ (CA-٤) has received special attention in the last few years. In addition to its potent cytotoxicity and inhibitory activity on tubulin polymerization, CA-٤ is one of the few anti-microtubule agents reported to have selective vascular disrupting activity. CA-٤ and its water-soluble prodrug, combretastatin A-٤ phosphate (CA-٤P), are selectively cytotoxic to rapidly proliferating tumor vasculature than normal blood vessels, resulting in reduced blood flow to tumor and eventual hemorrhagic necrosis.[٦]. Many analogs of CA-٤ have been designed to study the structure–activity relationship of the molecule in order to enhance both the cytotoxic and selective vascular disrupting activities.[٧,٨]



Colchicine



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Promoted with the above-mentioned studies, the present study is concerned with the synthesis of new ١-(naphthalene-١-yl)-٥-(٣,٤,٥-trimethoxyphenyl)-١*H*-١,٢,٤-triazole-٣-carboxylic acid derivatives with the objective of discovering novel and potent anti-tubulin agents. The cytotoxic activity of the prepared compounds was evaluated against two different cancer cell lines, Hep-G٢ and leukemia HL-٦٠ cell lines.

Chemistry

Melting points were determined on an electro thermal melting point apparatus [Stuart Scientific, model SMP٣, England, UK], and were uncorrected. A pre-coated silica gel plate (kieselgel ٠.٢٥ mm, ٦٠G F٢٥٤, Merck, Germany) was used for TLC monitoring of reactions. NMR spectra were taken using Varian Unity INOVA ٤٠٠ MHz spectrometers for proton and carbon at university of Aberdeen, United Kingdom. Chemical shifts are expressed in δ-value (ppm) relative to $CDCl_٣$ as internal standard. High resolution mass spectrometric data were obtained using the EPSRC mass spectrometry Centre in Swansea and Thermo Instruments MS system (LTQ XL/LTQ Orbitrap Discovery) coupled to a Thermo Instruments HPLC system (Accela PDA detector, Accela PDA autosampler and Pump) at university of Aberdeen, United kingdom. Reagents used for synthesis were purchased from *Sigma-Aldrich* and *Merck*. All solvents were obtained from commercial suppliers and used without further purification.

The starting materials ٣,٤,٥-trimethoxy-hippuricacid were synthesized according to reported procedures.[٩]

٤-(١-naphthyl)-hydrazono-٢-(٣,٤,٥-trimethoxyphenyl)-٢-oxazolin-٥-one٣

A mixture of ١ (٠.٠٢ mol) and acetic anhydride (٧٥ mL) was gently heated until a clear solution (A) was obtained. The resulting solution (A) was cooled to room temperature then in an ice bath before treating with a solution (B) of naphthalene daizonium chloride prepared as follows: ١-naphthyl-amine (٠.٠٢ mol, ٣.٢ g) was mixed with $H_٢O$ (٥ mL) and conc HCl (٦ mL). The mixture was cooled in an ice bath and an aqueous solution of $NaNO_٢$ (٠.٠٢ mol, g) was added in a dropwise manner and the resulting mixture was stirred for further ٣٠ min. Freshly prepared crude solution (A) was added in a dropwise manner in the presence of anhydrous sodium acetate (٠.٠٢ mol) and the stirring was continued for further ٢ h at ٠ °C. The resulting precipitate was filtered, washed with water, dried, and used without further purification for the next step.

١-(naphthalen-١-yl)-٥-(٣,٤,٥-trimethoxyphenyl)-١*H*-١,٢,٤-triazole-٣-carboxamides ٤a–d

A mixture of ٣ (٠.٠١ mol) and appropriate benzyl amine (٠.٠١ mol), was refluxed in methanol (٥٠ mL) for ١ h. after cooling the reaction mixture, the solvent was evaporated under reduced pressure and the obtained residue was recrystallized from ethylacetate /n- hexanes.

***N*-benzyl-١-(naphthalen-١-yl)-٥-(٣,٤,٥-trimethoxyphenyl)-١*H*-١,٢,٤-triazole-٣-carboxamide ٤a**

Brown crystals (٢٦٠ mg, ٧١٪ yield); m.p. ٨٦-٨٨ °C; ^{1}H NMR (٤٠٠ MHz, $CDCl_3$) δ ٨.٠٤ (d, *J* = ٨.١ Hz, ١H, Ar-H), ٧.٩٥ (d, *J* = ٨.٠ Hz, ١H, Ar-H), ٧.٧٠ (s, ١H, Ar-H), ٧.٦٠ – ٧.٥٢ (m, ٢H, Ar-H), ٧.٤٩ (s, ١H, Ar-H), ٧.٤٥ – ٧.٢١ (m, ٦H, Ar-H), ٦.٦٥ (s, ٢H, Ar-H), ٤.٧٣ (d, *J* = ٥.٧ Hz, ٢H, NHC$\underline{H}_2$), ٣.٧٥ (s, ٣H, OCH_3), ٣.٣٧ (s, ٦H, ٢OCH_3); ^{13}C NMR (١٠١ MHz, $CDCl_3$) δ ١٥٨.٩, ١٥٦.٤, ١٥٣.١, ١٣٩.٩, ١٣٧.٩, ١٣٤.٦, ١٣٤.٢, ١٣٠.٩, ١٢٩.٥, ١٢٨.٧, ١٢٨.٣, ١٢٨.٢, ١٢٧.٧, ١٢٧.٤, ١٢٥.٩, ١٢٥.٤, ١٢٢.٤, ١٢١.٠, ١٠٥.٧, ٦٠.٩, ٥٥.٨, ٤٣.٧; HRMS m/z calcd for $[M+H]^+$ $C_{٢٩}H_{٢٧}N_4O_4$: ٤٩٥.٢٠٢٧, found: ٤٩٥.٢٠٢١.

***N*-(٢-methoxybenzyl)-١-(naphthalen-١-yl)-٥-(٣,٤,٥-trimethoxyphenyl)-١*H*-١,٢,٤-triazole-٣-carboxamide ٤b**

Brown crystals (٢٠٥ mg, ٥٣٪ yield); m.p. ٧٠-٧١ °C; ^{1}H NMR (٤٠٠ MHz, $CDCl_3$) δ ٨.٠٤ (d, *J* = ٨.٣ Hz, ١H, Ar-H), ٧.٩٥ (d, *J* = ٨.٠ Hz, ١H, Ar-H), ٧.٨١ (s, ١H, Ar-H), ٧.٦٥ – ٧.٤٨ (m, ٤H, Ar-H), ٧.٤٣ - ٧.٣٧ (m, ٢H, Ar-H), ٧.٣١ – ٧.٢٥ (m, ١H, Ar-H), ٦.٩٩ – ٦.٨٤ (m, ١H, Ar-H), ٦.٦٧ (s, ٢H, Ar-H), ٤.٧٤ (d, *J* = ٦.٠ Hz, ٢H, NHC$\underline{H}_2$), ٣.٨٧ (s, ٣H, OCH_3), ٣.٧٥ (s, ٣H, OCH_3), ٣.٣٨ (s, ٦H, ٢OCH_3); ^{13}C NMR (١٠١ MHz, $CDCl_3$) δ ١٥٨.٧, ١٥٧.٨, ١٥٣.١, ١٣٩.٩, ١٣٦.٦, ١٣٤.٧, ١٣٤.٢, ١٣٠.٩, ١٣٠.١, ١٢٩.٧, ١٢٩.١, ١٢٨.٥, ١٢٨.٤, ١٢٧.٥, ١٢٥.٩, ١٢٥.٤, ١٢٢.٤, ١٢٠.٨, ١١٠.٥, ١٠٥.٨, ٦٠.٩, ٥٥.٨, ٥٥.٥, ٣٩.٢; HRMS m/z calcd for $[M+H]^+$ $C_{٣٠}H_{٢٩}N_4O_5$: ٥٢٥.٢١٣٢, found: ٥٢٥.٢١٢٧.

***N*-(٣-Methoxybenzyl)-١-(naphthalen-١-yl)-٥-(٣,٤,٥-trimethoxyphenyl)-١*H*-١,٢,٤-triazole-٣-carboxamide ٤c**

Brown crystals (٢٣٢ mg, ٦٠٪ yield); m.p. ٨٩-٩٠ °C; ^{1}H NMR (٤٠٠ MHz, $CDCl_3$) δ ٨.٠٥ (d, *J* = ٨.١ Hz, ١H, Ar-H), ٧.٩٦ (d, *J* = ٨.١ Hz, ١H, Ar-H,), ٧.٦٠–٧.٤٩ (m, ٤H, Ar-H), ٧.٤٠ (d, *J* = ٨.٢ Hz, ١H, Ar-H), ٧.٣٣–٧.٢٧ (m, ١H, Ar-H), ٦.٩٩ -٧.٠٩ (m, ٢H, Ar-H), ٦.٨٥ - ٦.٨٠ (m, ١H, Ar-H), ٦.٦٦ (s, ٢H, Ar-H), ٤.٧١ (d, *J* = ٥.٩ Hz, ٢H, NHC$\underline{H}_2$), ٣.٨١ (s, ٣H, OCH_3), ٣.٧٦ (s, ٣H, OCH_3), ٣.٣٩ (s, ٦H, ٢OCH_3); ^{13}C NMR (١٠١ MHz, $CDCl_3$) δ ١٥٣.١, ١٤٧.٦, ١٣٤.٥, ١٣٤.٢, ١٣٣.٦, ١٣٠.٩, ١٢٩.٩, ١٢٩.٧, ١٢٨.٥, ١٢٨.٤, ١٢٧.٥, ١٢٥.٩, ١٢٥.٤, ١٢٤.٣, ١٢٢.٥, ١٢١.٣, ١٢٠.٥, ١١٣.٩, ١١٣.٣, ١٠٥.٧, ٦٠.٩, ٥٥.٨, ٥٥.٥, ٤٣.٧; HRMS m/z calcd for $[M+H]^+$ $C_{٣٠}H_{٢٩}N_4O_5$: ٥٢٥.٢١٣٢, found: ٥٢٥.٢١٢٨.

***N*-(٣,٤-Dimethoxybenzyl)-١-(naphthalen-١-yl)-٥-(٣,٤,٥-trimethoxyphenyl)-١*H*-١,٢,٤-triazole-٣-carboxamide ٤d**

Pale brown crystals (٢٥٤ mg, ٦٢٪ yield); m.p. ٨٩-٩٠ °C; ^{1}H NMR (٤٠٠ MHz, $CDCl_3$) δ ٨.٠٦ (d, *J* = ٨.١ Hz, ١H, Ar-H), ٧.٩٨–٧.٩٢ (m, ١H, Ar-H), ٧.٨٥ – ٧.٧٦ (m, ١H, Ar-H), ٧.٦٢ – ٧.٤٧ (m, ٤H, Ar-H), ٧.٣٩ (t, *J* = ٤.٤ Hz, ١H, Ar-H), ٦.٩٩ – ٦.٩٤ (m, ١H, Ar-H), ٦.٨٤ (d, *J* = ٨.١ Hz, ١H, Ar-H), ٦.٦٨ (s, ٢H, Ar-H), ٤.٦٧ (d, *J* = ٥.٨ Hz, ٢H, NHC$\underline{H}_2$), ٣.٩٠ (s, ٣H, OCH_3), ٣.٨٧ (s, ٣H, OCH_3), ٣.٧٦ (s, ٣H, OCH_3), ٣.٣٩ (s, ٦H, ٢OCH_3); ^{13}C NMR (١٠١ MHz, $CDCl_3$) δ ١٥٣.١, ١٤٩.٣, ١٤٨.٨, ١٣٤.٢, ١٣١.١, ١٣٠.٥, ١٢٩.٦, ١٢٨.٦, ١٢٨.٥, ١٢٧.٦, ١٢٥.٩, ١٢٥.٤, ١٢٢.٣, ١٢٠.٨, ١١١.٩, ١١١.٣, ١٠٥.٩, ٦٠.٩, ٥٦.١, ٥٦.١, ٥٥.٩, ٤٣.٦; HRMS m/z calcd for $[M+H]^+$ $C_{٣١}H_{٣١}N_4O_6$: ٥٥٥.٢٢٣٨, found: ٥٥٥.٢٢٣٢.

Cell culture

Human hepatocarcinoma cell line (HepG٢) and leukemia(HL-٦٠), which were purchased from ATCC, USA, were used to evaluate the cytotoxic effect of the tested samples. Cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM). Media was supplemented with ١٠٪ fetal bovine serum (FBS), ٢ mML-glutamine, containing ١٠٠ units/mL of penicillin G sodium, ١٠٠ units/mL of streptomycin sulfate, and ٢٥٠ ng/mL amphotericin B. Cells were maintained at sub-confluence at ٣٧°C in humidified air containing ٥٪ CO_2. For sub-culturing, monolayer cells were harvested after trypsin/ EDTA treatment at ٣٧°C. Cells were used when confluence had reached ٧٥٪. Tested samples were dissolved in dimethyl sulfoxide (DMSO), and then diluted thousand times in the assay to begin with the indicated concentration. All cell culture material was

obtained from Cambrex BioScience (Copenhagen,Denmark). All chemicals were from Sigma/Aldrich (USA), except mentioned. All experiments were repeated three times, unless mentioned.

Anti-tumor activity

Cytotoxicity of tested samples was measured against each cell line using the MTT cell viability assay. MTT (٣-[٤,٥–dimethylthiazole-٢-yl]-٢,٥-diphenyltetrazolium bromide) assay is based on the ability of active mitochondrial dehydrogenase enzyme of living cells to cleave the tetrazolium rings of the yellow MTT and form a dark blue, insoluble formazan crystal, which is largely impermeable to cell membranes, resulting in its accumulation within healthy cells. Solubilization of the cells results in the liberation of crystals, which are then solubilized. The number of viable cells is directly proportional to the level of soluble formazan dark blue color. The extent of the reduction of MTT was quantified by measuring the absorbance at ٥٧٠ nm.

Briefly, cells (٠.٥x١٠^{٥} cells/well), in serum-free media, were plated in a flat bottom ٩٦-well microplate, and treated with ٢٠mL of serial concentrations of the tested samples for ٤٨h at ٣٧°C, in a humidified ٥٪ $CO_٢$ atmosphere. After incubation, media were removed and ٤٠mLof MTT solution (٥ mg/mL of MTT in ٠.٩٪ NaCl) in each well was added and incubated for an additional ٤ h. MTT crystals were solubilized by adding ١٨٠mL of acidified isopropanol/well and the plate was shaken at room temperature, followed by photometric determination of the absorbance at ٥٧٠ nm using microplate ELISA reader. Triplicate repeats were performed for each concentration and the average was calculated. Data were expressed as the percentage of relative viability compared with the untreated cells compared with the vehicle control, with cytotoxicity indicated by <١٠٠٪ relative viability. Percentage of relative viability was calculated using the following equation: [absorbance of treated cells/absorbance of control cells)] x١٠٠. Then the half maximal inhibitory concentration ($IC_{٥٠}$) was calculated from the equation of the dose–response curve (linear regression).

$$Y = a * +b$$

$$IC_{٥٠} = \frac{(٠.٥ - b)}{a}$$

Where drug concentrations $x_١, x_٢, \ldots, x_n$ and growth inhibition $y_١, y_٢, \ldots, y_n$

Results and discussion

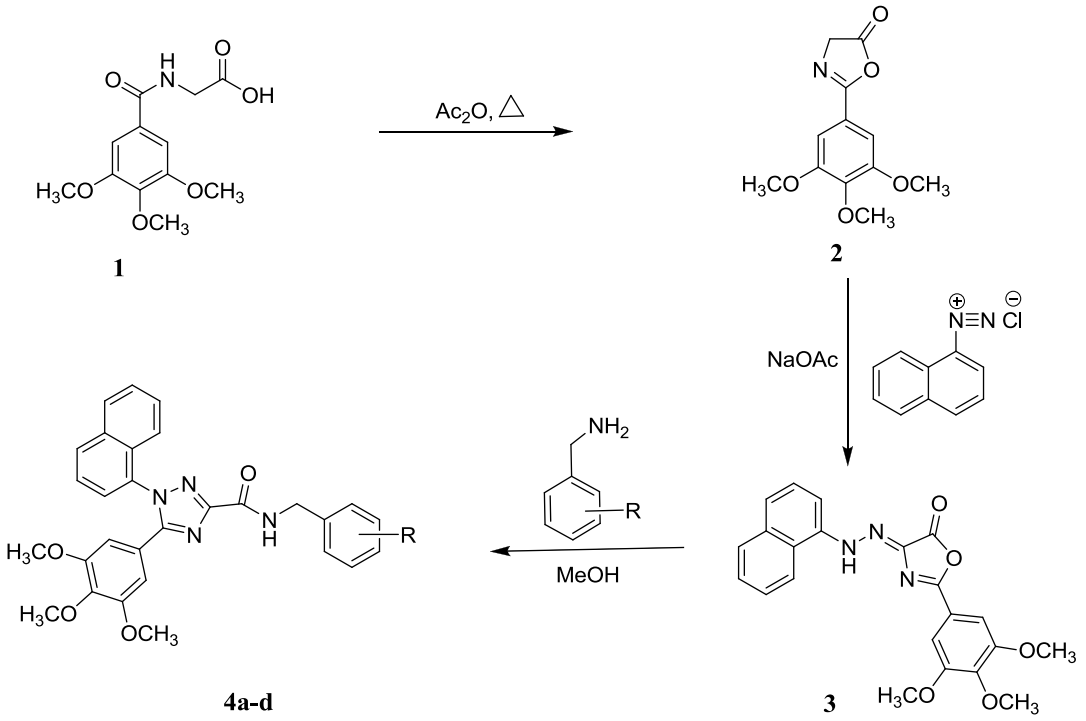
A new series of triazole derivatives were design, synthesized, and structurally elucidated using ^{١}H NMR and ^{١٣}C NMR as well as high resolution mass analysis. All compounds were tested for their possible anti-tumor activities and they shows a significance results especially compound **٤a**, Only unsubstituted anilides showed the highest activity while the substitution showed a decrease in the activity. In conclusion, the prepared ١-(naphthalen-١-yl)-٥-(٣,٤,٥-trimethoxyphenyl)-١*H*-١,٢,٤-triazole-٣-carboxylic-acid and compound ٤a showed the highest activity.

Chemistry

The reaction sequences employed for synthesis of the target compounds are shown in scheme. ١. Key starting compound, ٢-[٣,٤,٥-trimethoxybenzamido] acetic acids **٢**, was prepared in good yield (٨٥٪) by the reaction of glycine with ٣,٤,٥-trimethoxybenzoyl chloride in ١٠٪ NaOH. The key intermediate, ٤-(١-naphthyl)-hydrazono-٢-(٣,٤,٥-trimethoxyphenyl)-٢-oxazolin-٥-one **٣**

was prepared by Diazotization of ١-naphthylamine followed by reaction with the active methylene compounds [٢-(٣,٤,٥-trimethoxyphenyl)-٢-oxazolin-٥-one] **٣.** IR spectrum of **٣a** showed a weak and broad band at ٣٦١٥–٣٣٠٠ cm^{-1}. The broadening of the NH stretching band indicates the effect of possible intramolecular hydrogen bonding with nitrogen atom of oxazolinone. Strong stretching bands occurred at ١٧٩٤ (C=O) of the lactone, bands at ١٦٢٦ (C=N), ١٢٢٩ (C–O– C), and ١٦٠٠ – ١٥٨٥ (C=C) cm^{-1}.

Scheme 1

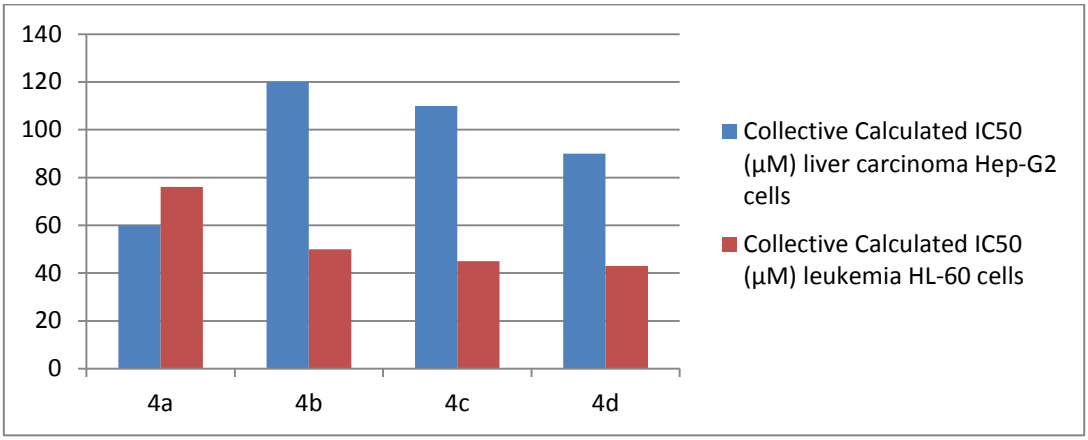


Scheme 1: Synthetic route of compounds 4a-d

٤a,R = H, ٤b, R = ٢-OCH_3, ٤c, R = ٣-OCH_3, ٤d, R= ٣,٤-di-OCH_3

Table ١: Collective Calculated $IC_{٥٠}$ (μM) from linear equation of dose response curve for each tested sample against **liver carcinoma Hep-G٢ cells and leukemia HL-٦٠ cells.**

Compound No.	Collective Calculated $IC_{٥٠}$ (μM)	
	liver carcinoma Hep-G٢ cells	leukemia HL-٦٠ cells
٤a	٦٠	٧٦
٤b	١٢٠	٥٠
٤c	١١٠	٤٥
٤d	٩٠	٤٣



Refluxing compound ٣ with different benzyl amines afforded compounds **٤a-d**. The structure of prepared compounds **٤a-d** was confirmed by ^{1}H, and ^{13}C NMR as well as high resolution mass spectroscopy.

Biological investigations

Anti-tumor activity

The triazole series **٤a-d** were tested for their anti-tumor activity on two different cancer cell lines, HepG-٢ and leukemia HL-٦٠ cell lines, using MTT assay compared to CA-٤ as a reference compound, the effect of the tested compounds on the viability of different human cancer cell lines were studied after ٤٨ h of incubation. The treatment of hepatocellular carcinoma HepG٢ cells, and leukemia HL-٦٠ cells with gradual concentrations of different compounds revealed that compound **٤a** possessed the highest promising cytotoxic effect against HepG-٢ cells, while compound **٤c** and **٤d** demonstrated the highest promising cytotoxic effect against HL-٦٠ cells as concluded from their $IC_{٥٠}$ values.

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