

Evaluation of Cytotoxicity and Apoptosis Induced by Coumarin Hydrazide-Hydrazone Derivatives in Human Hepatocellular Carcinoma Cell Line

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Abstract

Coumarin and aryl hydrazide-hydrazone have attracted our attention due to their vast biological properties. Previous studies suggested that coumarin-tethered aryl hydrazide-hydrazone showed potent activities against HepG2. In the present study, we investigated the cytotoxic potency of the coumarin derivatives 1 - 3 to compare with coumarin hydrazine-hydrazone hybrids 4 and 5 against hepatocellular carcinoma HepG2 and LH86 cell lines. Among the tested coumarins, hybrids 4 and 5 showed highly potent activity against HepG2 with IC₅₀ values of 17.82 ± 2.79 and 7.87 ± 0.88 $\mu\text{g/mL}$, respectively. The hybrid 4 also showed the strongest activity against LH86 cell line with IC₅₀ values of 48.32 ± 2.64 $\mu\text{g/mL}$. Further, we have studied the mechanism of action of the hybrid compounds 4 and 5 in HepG2 cells via the flow-cytometry analysis and the activation of the caspase-3 and caspase-7. The results showed that hybrids 4 and 5 obviously inhibited the proliferation of HepG2 cell line through inducing apoptosis.

Keywords: Coumarin, Hydrazide-hydrazone, Cytotoxicity, Apoptosis, Anticancer

Introduction

Liver cancer incidence and mortality are significantly increasing worldwide. The most common primary liver cancer is hepatocellular carcinoma (HCC), which is associated with cirrhosis caused by both alcohol and viral hepatitis [1]. By 2030, there will be an estimated > 1.14 million new liver cancer cases diagnosed and > 1.05 million liver cancer deaths annually [2].

Apoptosis, a process of programmed cell death, plays an important role in normal tissue development and homeostasis maintenance [3]. Accumulated evidence suggests that deficiencies at any stage of the apoptotic pathways and dysregulation of the controlling mechanisms can contribute to cancer. Caspases, a class of cysteinyl proteases, are responsible for inducing apoptosis by cleaving target proteins. The activation of caspases-3 and -7 is an essential event in apoptotic pathways [4]. Chemotherapy, a systemic cancer treatment, utilizes chemotherapeutic agents to activate cell death signaling pathways and promote apoptosis. However, most therapeutic agents are non-selective, affecting both cancer and normal cells, leading to significant harm to normal cells and severe toxic side effects. Additionally, resistance to cytotoxic agents related to resistance to apoptosis is a major challenge in cancer therapy [5]. Despite remarkable advancements in cancer treatment, researchers continue to devote significant efforts to discovering new anticancer molecules that target the apoptotic signaling pathway with high selectivity and minimal severe side effects. Understanding the molecular mechanisms of anticancer agents can greatly benefit cancer therapy.

Coumarins, also known as 1-benzopyran-2-one, are a class of natural products that contain oxygen-containing heterocyclic. Naturally occurring and synthetic coumarins possess a variety of biological activities including anticancer [5], anti-inflammatory [6], antiplatelet [7], antimicrobial [8], antioxidant [6], antiviral [9] and other activities [10]. The functionalization of the coumarin scaffold represents a significant challenge in the development of new potential lead compounds. Furthermore, the strategy of combining 2 or more pharmacophores within a single molecular scaffold, known as molecular hybridization, is employed to enhance the pharmacokinetic and pharmacodynamic properties while reducing toxicity [11]. Through this strategy, numerous researchers have designed and synthesized coumarin hybrids with various

bioactive molecules as anticancer agents [12-27] such as benzimidazole [12], benzothiazole [13], chalcones [14,15], dihydroartemisinin [16], dipyrromethene/porphyrin [17], isoxazoline [18], oxadiazole [19], piperazine [20], stilbene [21], thiazolyl-pyrazoline [22], uracil [23].

In our previous article, we described the synthesis of coumarin-tethered hydrazone-hydrazones derivatives, which exhibited potent cytotoxic activity against human hepatic carcinoma (HepG2) [27]. In continuation of our research, herein we have designed an evaluation to assess the cytotoxicity of coumarin derivatives 1 - 3 in comparison with coumarin hydrazine-hydrazones (CHH) 4 and 5 on hepatocellular carcinoma HepG2 and LH86 cell lines. Additionally, we investigated the potential molecular mechanisms of CHH 4 and 5 through Annexin V-FITC/propidium iodide apoptosis assay and caspase-3/7 activity assay to analyze cell apoptosis in the HepG2 cell line.

Materials and methods

Materials and instrumentation

The chemicals were purchased from commercial sources and used without further purification. Column chromatography was performed on silica gel (Kieselgel 60, 70 - 230 mesh, Merck) in common glass columns. Melting points (°C) were measured with the Gallenkamp melting point apparatus and were uncorrected. ¹H and ¹³C NMR spectra were obtained on a Bruker spectrometer. ESI mass spectra were recorded on a Thermo Finnigan LCQ Advantage Mass Spectrometer. High-Resolution Mass Spectrometry was measured with a MicroTOFLC, Bruker Daltonics. Infrared spectra were performed with a Perkin Elmer FT-IR Spectrum GX. The preparations of compounds 1 - 5 were achieved according to the previous work [27,28].

Synthesis of compounds 1 - 5

7-Hydroxy-4-methylcoumarin (1)

The mixture of resorcinol (30 mmol), ethyl acetoacetate (30 mmol) and iron (III) chloride hexahydrate (0.8 g, 10 mol %) in toluene (100 mL) was heated to reflux for 16 h. The reaction mixture was cooled to room temperature and then quenched with water (100 mL). The resulting reaction solution was extracted 3 times with 150 mL of ethyl acetate. The combined organic phases were dried over anhydrous Na₂SO₄ and concentrated by a rotary evaporator. The residue was purified by column chromatography on silica gel to give coumarin 1 (92 %) as a white solid.

Ethyl [(4-methyl-2-oxo-2H-chromen-7-yl)oxy]acetate (2)

The 7-Hydroxy-4-methylcoumarin (1, 10 mmol) was dissolved in dry THF (25 mL). Anhydrous potassium carbonate (20 mmol) and ethyl chloroacetate (20 mmol) were then added and the mixture was stirred under reflux for 24 h under the N₂ atmosphere. After cooling to room temperature, the precipitated solid was filtered off and washed with cold acetone. The resulting filtrate was evaporated in vacuo and recrystallized from ethanol to obtain 2 (75 %) as a faint yellow solid.

2-[(4-Methyl-2-oxo-2H-chromen-7-yl)oxy]acetohydrazide (3)

Hydrazine hydrate (1 mmol) was added dropwise to a solution of ethyl [(4-methyl-2-oxo-2H-chromen-7-yl)oxy] acetate (2, 1 mmol) in ethanol (6 mL). The reaction mixture was stirred at room temperature for 30 h and the resulting solid was filtered, washed with cold ethanol and recrystallized from chloroform/methanol to give 3 (92 %) as a white solid.

2-[(4-Methyl-2-oxo-2H-chromen-7-yl)oxy]-N'-(3-chlorobenzylidene) acetohydrazide (4)

A solution of 2-[(4-methyl-2-oxo-2H-chromen-7-yl)oxy] acetohydrazide (3, 0.5 mmol) and 3-chlorobenzaldehyde (0.5 mmol) in 1:1 methanol/chloroform (15 mL) and acetic acid (0.05 mL) was stirred at reflux for 5 h. After cooling to room temperature, the precipitated solid was filtered and recrystallized from methanol to obtain CHH 4 (91 %) as a white solid.

2-[(4-Methyl-2-oxo-2H-chromen-7-yl)oxy]-N'-[1-(4-chlorophenyl) ethylidene]aceto-hydrazide (5)

A solution of 2-[(4-methyl-2-oxo-2H-chromen-7-yl)oxy] acetohydrazide (3, 0.5 mmol) and 4'-chloroaceto-phenone (0.5 mmol) in acetic acid (10 mL) was stirred at room temperature for 18 h. After completion of the reaction, the precipitated solid was filtered and recrystallized from methanol to obtain CHH 5 (69 %) as a white solid.

Cell culture

The hepatocellular carcinoma HepG2 and LH86 cell lines were obtained from ATCC (MD, USA). The HepG2 was cultured in Eagle's Minimum Essential Medium (EMEM) medium whereas LH86 was cultured in Dulbecco's modified Eagle medium (DMEM) medium. All media (Gibco, Langley, VA, USA) were supplemented with 10 % fetal bovine serum (Gibco) and 1 % Antibiotic-antimycotic which contains 10,000 units/mL of penicillin, 10,000 µg/mL of streptomycin and 25 µg/mL of Gibco Amphotericin B, respectively: Gibco, USA). Cell cultures were maintained under standard conditions: incubation at 37 °C, 95 % relative humidity with 5 % CO₂ atmosphere.

Cytotoxic assay

The HepG2 and LH86 cell lines were treated with varying concentrations (200, 100, 50, 25, 12.5, 6.25, 3.13 and 1.57 µg/mL) of coumarins 1 - 3, CHH 4 and CHH 5 and doxorubicin (positive control) in a 96-well microtiter plate (Corning, NY, USA) for 48 h. Effects of all compounds on the survival of all cell lines were determined using MTT assay. In brief, the cells (HepG2 and LH86) were seeded into each well of the 96-well microtiter plate (15,000 cells/well) and incubated for 24 h at 37 °C, 5 % CO₂ atmosphere and 95 % humidity before exposing to coumarins 1 - 3, CHH 4 and 5 or doxorubicin. Following 48 h incubation, the MTT reagent (Sigma Co. Ltd., MO, USA) was added to each well and the plate was further incubated for 3 h. After that, the supernatants were removed and the precipitated formazan was dissolved by adding 100 µL of DMSO. Absorbance at 570 nm was determined using a microplate reader (Varioskan™ Flash Multimode Reader; Thermo Scientific™). Results were calculated by subtracting blank readings. Cell viability and corresponding IC₅₀ (concentration of each compound that produces a 50 % inhibitory effect on cell growth relative to control) were determined using CalcuSyn™ v. 2.11 software (Biosoft, Cambridge, UK).

Apoptosis investigation

The HepG2 cells (5×10⁵ cells) were grown overnight in a 25 cm² filter flask. Cells were harvested by trypsinization after exposure to CHH 4, CHH 5 and doxorubicin at the IC₅₀ (7.80, 17.8 and 2.9 µg/mL, respectively) and IC₇₅ (28.0, 30.0 and 21 µg/mL, respectively) concentrations for 24 and 48 h. The apoptotic protein marker phosphatidylserine and intracellular DNA content were stained by fluorescence dye according to the manufacturer's protocol (BD Pharmingen™ FITC Annexin V Apoptosis Detection Kit, BD Biosciences, CA, USA). The numbers of both living and apoptotic (early and late) cells were determined by FACSVerse flow cytometer (BD Biosciences, CA, USA) at 281 and 278 energy voltages for FITC and PI, respectively. The experiment was performed in triplicate.

The apoptotic effect of CHH 4, 5 and doxorubicin on the HepG2 cell line was also investigated based on caspase 3/7 expression [29]. The cells (2.0×10⁵ cells/well) were seeded in 24 well culture plates (Corning, NY, USA) and incubated overnight at 37 °C under 5 % CO₂ atmosphere before exposing them to compounds at IC₂₅, IC₅₀ and IC₇₅ concentrations for 24 h. The apoptotic proteins caspase 3/7 were stained with fluorescence CellEvent™ Caspase-3/7 Green detection assay (Thermo Fisher Scientific, NY, USA) and cells with caspase-3/7 expression were examined under DeltaVision™ Elite Cell Imaging System (GE HealthCare, IL, USA) using 40x objective lens.

Statistical analysis

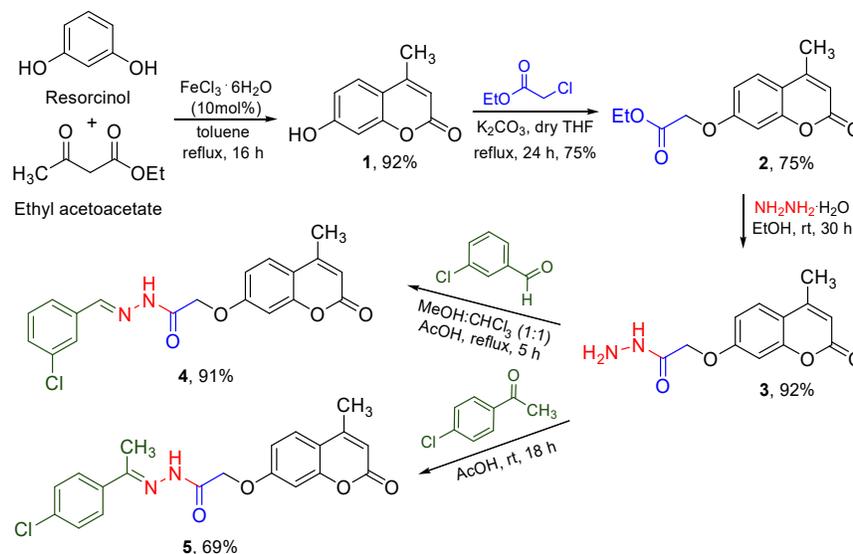
All experiments were carried out in triplicates. The results were expressed as mean ± standard error values. Statistical significance was calculated using one-way analysis of variance (ANOVA). A value of *p* < 0.05 and *p* < 0.001 were considered statistically significant.

Results and discussion

The synthesis of coumarins 1 - 3 and CHH 4, 5

The synthesis of coumarins 1 - 3 and CHH 4, 5 is illustrated in **Scheme 1**. 7-Hydroxy-4-methylcoumarin (1) was prepared via the Pechmann reaction by reacting resorcinol with ethyl acetoacetate in the presence of 10 mol % FeCl₃·6H₂O in refluxing toluene for 16 h. The introduction of the ethyl acetate moiety into coumarin 1 was achieved by adding ethyl chloroacetate to the oxygen atom of the hydroxyl group in the presence of potassium carbonate under reflux conditions in dry THF for 24 h, resulting in the formation of compound 2 with a yield of 75 %. The substitution reaction of coumarin-ethyl ester 2 with hydrazine hydrate was carried out in ethanol at room temperature for 30 h, leading to the synthesis of coumarin-hydrazide 3. Next, the condensation reaction of hydrazide 3 with 3-chlorobenzaldehyde was conducted in the presence of a catalytic amount of glacial acetic acid in a mixture of methanol and

chloroform (1:1) under reflux conditions for 5 h, resulting in the formation of CHH 4 with a yield of 91 %. Likewise, the condensation reaction of compound 3 with 4'-chloroacetophenone in acetic acid at room temperature for 18 h led to the CHH 5 in 69 % yield.



Scheme 1 Synthesis of coumarins 1 - 3 and CHH 4, 5.

Cytotoxic activity of coumarins 1 - 3 and CHH 4, 5 against HepG2 and LH86 cell lines

This study assessed the efficacy of coumarins 1 - 3 and coumarins substituted hydrazone-hydrazone 4, 5 in suppressing human hepatocellular carcinoma cell growth, in comparison with the activity of the known anticancer reference drug doxorubicin (Dox). **Figure 1** illustrates the antiproliferative activity of coumarins 1 - 3, CHH 4, 5 and Dox on 2 studied cell lines, i.e. HepG2 and LH86 cell lines. The results revealed a concentration-dependent decrease in the percentage of cell viability for both HepG2 and LH86 cell lines when exposed to a concentration range of 1.56 - 200 $\mu\text{g}/\text{mL}$ of compounds 1 - 5 and Dox for 48 h.

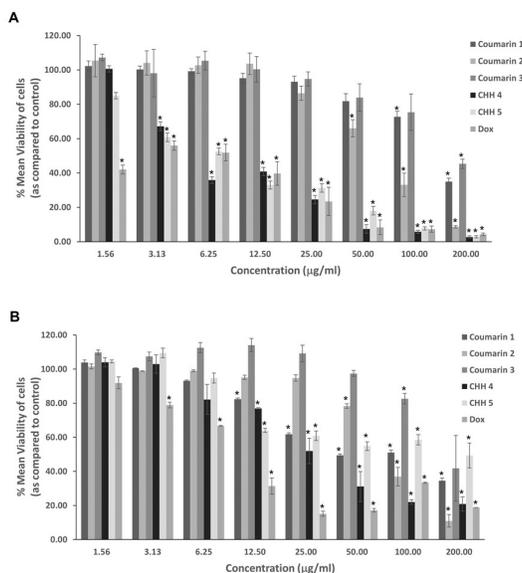


Figure 1 The cytotoxic activity of coumarins 1 - 3, CHH 4, 5 and doxorubicin (Dox) on HepG2 and LH86 cell lines. The percentage viability of HepG2 (A) and LH86 (B) cell lines after 24 h of compounds 1 - 5 and doxorubicin treatment. Data were expressed as mean \pm SEM values of 3 independent tests ($n = 3$), with each experiment completed in triplicate. * $p < 0.001$ compared to the control.

The growth inhibitory effects of coumarins 1 - 3, CHH 4, 5 and doxorubicin on HepG2 and LH86, expressed as IC_{50} values, are summarized in **Table 1**. Notably, among the tested compounds, CHH 5 exhibited the strongest cytotoxic activity against the HepG2 cell line, with an IC_{50} value of $7.87 \pm 0.88 \mu\text{g/mL}$ (approximately 37 % of the positive control, doxorubicin, with an IC_{50} value of $2.90 \pm 0.79 \mu\text{g/mL}$). Conversely, CHH 4 exhibited the highest activity against the LH86 cell line, with an IC_{50} value of $48.32 \pm 2.64 \mu\text{g/mL}$, representing approximately 28 % of the positive control (with an IC_{50} value of $13.35 \pm 1.49 \mu\text{g/mL}$). It is noteworthy that HepG2 cancer cells displayed greater sensitivity to CHH 4 and 5, as evidenced by their lower IC_{50} values.

Table 1 Half-maximal inhibitory concentrations (IC_{50}) of coumarins 1 - 3, CHH 4, 5 and doxorubicin (Dox) against HepG2 and LH86 cell lines.

Compound	HepG2 ($\mu\text{g/mL}$)	LH86 ($\mu\text{g/mL}$)
Coumarin 1	$104.88 \pm 3.36^*$	65.12 ± 1.12
Coumarin 2	$80.87 \pm 4.04^*$	$81.45 \pm 2.88^*$
Coumarin 3	$139.03 \pm 5.57^*$	$> 200^*$
CHH 4	17.82 ± 2.79	48.32 ± 2.64
CHH 5	7.87 ± 0.88	70.87 ± 7.45
Dox	2.90 ± 0.79	13.35 ± 1.49

Results are presented as mean \pm SEM of 3 independent experiments. *To indicate the significant difference at $p < 0.001$ from the positive control (doxorubicin).

Apoptosis assessment induced by CHH 4 and 5 in HepG2 cells

To assess the mechanism of cell death triggered by CHH 4 and 5 compared with Dox in HepG2 cells, an apoptosis assay was conducted utilizing flow cytometric analysis. Externalized phosphatidylserine on the cell membrane is a remarkable feature of apoptotic cells. Annexin V, a recombinant protein, exhibits a high affinity for this externalized moiety, which is employed to assess apoptosis [30]. The results obtained from flow cytometry facilitated the categorization of cell populations into living, early apoptotic and late apoptotic cells based on Annexin V-FITC/PI staining (**Figures 2 and 3**).

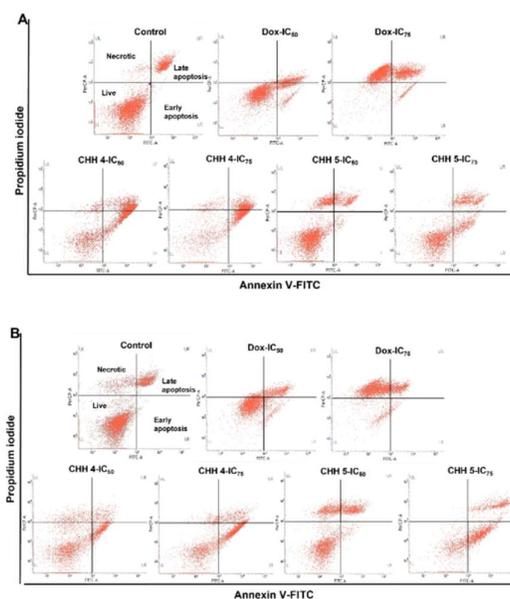


Figure 2 Flow cytometry analysis was performed to assess apoptosis in HepG2 cells following exposure to different concentrations of CHH 4 and 5, along with doxorubicin. The scatter plots illustrate the populations of live cells, early apoptotic cells, late apoptotic cells and necrotic cells after exposure at 24 h (A) and 48 h (B).

The scatter plot data (**Figure 2**), illustrating the fluorescence resulting from FITC and PI staining, provide clear evidence from flow cytometry analysis that CHH 4 and 5, along with doxorubicin, primarily induce cell death through both early and late apoptotic pathways, ultimately leading to necrotic cell death.

Furthermore, as illustrated in **Figure 3**, exposure to CHH 4 at IC_{50} concentrations for 24 h led to a significant increase in the proportion of late apoptotic cells compared to the control (Mean \pm SD: 45.18 ± 19.75 vs. 17.94 ± 12.07 %) ($p < 0.05$). Similarly, exposure to CHH 4 at IC_{75} concentrations for 24 h significantly increased the percentage of early apoptotic cells compared to the control (Mean \pm SD: 36.78 ± 2.90 vs. 0.42 ± 0.12 %) ($p < 0.05$). For CHH 5, exposure at IC_{75} concentrations for both 24 and 48 h significantly increased the percentage of early apoptotic cells compared to the control (Mean \pm SD: 16.93 ± 2.06 vs. 0.42 ± 0.12 % (24 h) and 32.82 ± 17.90 vs. 0.51 ± 0.56 % (48 h)) ($p < 0.05$).

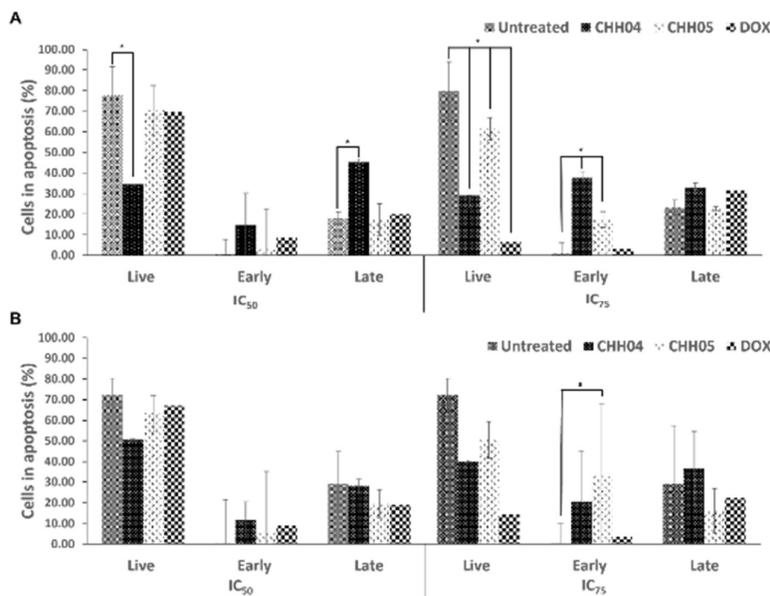


Figure 3 Apoptosis degree of HepG2 cell population following CHH 4, 5 and doxorubicin exposure at IC_{50} and IC_{75} for 24 and 48 h. Data are presented as Mean \pm SD and (*) showed a significant increase in cell population after the CHH compounds exposure compared to untreated control at 24 h (A) and 48 h (B).

Apoptosis, in contrast to necrosis, serves as a beneficial somatic defense mechanism against cancer cells. Annexin V-FITC/PI staining demonstrated a significant increase in early and late apoptotic populations in HepG2 cells for CHH 4 and 5 incubated overtime points and doses. Annexin V-FITC/PI considered as live cells, while Annexin V-FITC⁺/PI⁻ staining patterns showed early apoptotic cells; whereas Annexin V-FITC⁺/PI⁺ exhibited late apoptotic cells due to a loss of plasma membrane integrity and Annexin V-FITC⁻/PI⁺ was considered as necrotic cells [31]. This study is worth noting that CHH 4 and 5 did not promote necrosis at a high dose (IC_{75}) compared with a positive drug (Dox).

To investigate the involvement of caspase activation in the cell death induced by CHH 4 and 5, we performed staining on HepG2 cells incubated with these compounds at IC_{25} , IC_{50} and IC_{75} concentrations for 24 h using a caspase 3/7-specific fluorochrome detection dye. The staining results demonstrated a concentration-dependent increase in the proportion of caspase 3/7-positive cells (stained green) in CHH 4 and CHH 5-treated HepG2 cells compared to the untreated controls (**Figures 4(A) - 4(B)**). Apoptosis is the ultimate outcome of caspases 3 and 7, cysteine proteases, which can be triggered through either the extrinsic pathway, involving initiator caspase 8 activated by death receptors, or the intrinsic pathway, initiated by caspase 9 in response to DNA damage [32]. Caspase 3 and caspase 7 share similar functions in executing the final stages of apoptosis, including cleaving various cellular substrates that lead to cell dismantling and DNA fragmentation. However, as you mentioned, caspase 3 is specifically associated with plasma membrane blebbing, which is a characteristic morphological change in apoptotic cells. Caspase 7, in addition to its role in apoptosis, has been reported to be involved in the inflammation process, which can be important in immune responses [33,34]. These results suggest that samples-induced apoptosis was, in part, due to activation of caspases 3/7.

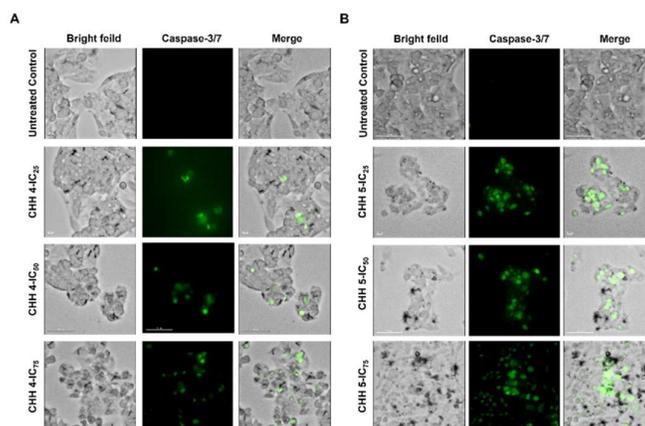


Figure 4 Effect of CHH 4 (A) and CHH 5 (B) on caspase 3/7 (Casp3/7+) activity in HepG2 cells. Detection of Casp3/7+-positive (green) cells after treatment for 24 h with IC₂₅, IC₅₀ and IC₇₅ concentrations of CHH 4 and 5.

Conclusions

In summary, the present study revealed the cytotoxic activity of coumarin derivatives 1 - 3 and coumarin hydrazine-hydrazone hybrids 4 and 5 against 2 cancer cell lines, HepG2 and LH86. Hybrids 4 and 5 exhibited the highest cytotoxic activity against the HepG2 cell line, with IC₅₀ values of 17.82 ± 2.79 and $7.87 \pm 0.88 \mu\text{g/mL}$, respectively. They also showed moderate cytotoxic activities against the LH86 cell line, with IC₅₀ values of 48.32 ± 2.64 and $70.87 \pm 7.45 \mu\text{g/mL}$, respectively. Furthermore, the apoptosis-inducing mechanism of hybrids 4 and 5 against the HepG2 cell line was investigated using Annexin V-FITC/PI assay. The flow cytometry results revealed a significant increase in the percentage of total apoptotic cells (early and late apoptotic cells) upon treatment with hybrids 4 and 5. Additionally, the activation of caspase-3 and -7, known markers of apoptosis, was measured. The observed elevation in the expression of caspase-3/7 indicated the occurrence of apoptosis. These results suggest that coumarin hydrazide-hydrazone hybrids are attractive as lead compounds and that additional structural alterations could result in the creation of novel anticancer drugs.

Acknowledgments

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