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Effect of a combination of newly synthesized two thiazole derivatives with curcumin and quercetin on the regulation of mTOR, Bcl-2, IL6, and Caspase3 genes Expression in HepG2 Cells

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Abstract

Background: Hepatocellular carcinoma (HCC) is characterized by limited treatment options and a high mortality rate. Thiazole derivatives represent a promising class of compounds with notable anticancer activity, particularly against HepG2 cells. HepG2 cells are widely used as a model for human liver cancer. Aim: This study aims to investigate the effect of a combination of newly synthesized two thiazole derivatives with curcumin and quercetin on multiple genes regulation in HepG2 cell lines. Materials and Methods: Two thiazole derivatives, derivative A and derivative B, were synthesized then tested on HepG2 cells alone and in combination with the flavonoid's curcumin and quercetin using MTT assay, aiming to enhance their anticancer efficacy. The activity of these compounds was assessed by examining the regulation of key genes involved in cell survival and apoptosis, including mTOR, Bcl-2, IL6, and Caspase-3 using rtPCR. Results: Derivative A was the most potent anti-HepG2 than derivative B. Notably, the combination of derivative A with curcumin demonstrated the most potent effect among all. This combination significantly downregulated mTOR, Bcl-2, and IL6, while upregulating Caspase-3, leading to apoptosis induction and inhibition of cell growth. Conclusion: These findings highlight thiazole derivatives as a promising class of anti-HCC agents whose efficacy can be further enhanced through combinatorial treatment with flavonoids such as curcumin and quercetin.

Keywords: Hepatocellular carcinoma, apoptosis, anticancer, flavonoids

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تأثير مزيج من مشتقين جديدين مُخلَّقين من الثيازول مع الكركمين والكيرسيتين على تنظيم تعبير جينات mTOR وBcl-2 وIL6 وCaspase3 في خلايا الكبد السرطانية البشرية HepG2

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مستخلص البحث: معلومات أساسية: يُعدّ سرطان الخلايا الكبدية (HCC) من أكثر أنواع السرطان فتكًا، مع خيارات علاجية محدودة ومعدلات وفاة مرتفعة. تُعتبر مشتقات الثيازول فئة واعدة من المركبات ذات نشاط مضاد للسرطان، خاصةً ضد خلايا HepG2 التي تُستخدم كنموذج شائع لدراسة سرطان الكبد البشري. الهدف من الدراسة: في هذه الدراسة، تمّ تخليق مشتقَّين جديدَين من الثيازول (المشتق A والمشتق B) وتقييم تأثيرهما على خلايا HepG2 سواء بشكل منفرد أو بالاشتراك مع الفلافونويدات الكركمين والكيرسيتين، بهدف تعزيز الفعالية المضادة للسرطان لهما. المواد والطرق المستخدمة: تمّ قياس نشاط هذه المركبات من خلال دراسة تنظيم التعبير الجيني للجينات الرئيسية المسؤولة عن بقاء الخلايا وموتها المبرمج (apoptosis) وهيmTOR و Bcl-2 وIL6 و Caspase-3 . النتائج: أظهرت النتائج أن المشتق A كان أكثر فعالية ضد خلايا HepG2 مقارنة بالمشتق B، وكان المزيج المشتق A والكركمين هو الأقوى بين جميع المعالجات. وقد أدت المعاملات إلى تثبيط ملحوظ لتعبير جينات mTOR وBcl-2 و IL6، مع زيادة في تعبير Caspase-3، مما ساهم في تحفيز الموت المبرمج للخلايا ومنع نموها وتكاثرها. الخلاصة: تشير هذه النتائج إلى أن مشتقات الثيازول تمثل فئة واعدة من العوامل المضادة لسرطان الكبد، ويمكن تعزيز فعاليتها من خلال الع لاج التكاملي مع فلافونويدات مثل الكركمين والكيرسيتين.

الكلمات المفتاحية: سرطان الخلايا الكبدية، موت الخلايا المبرمج، مضاد للسرطان، الفلافونويدات.

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1. Introduction

Hepatocellular carcinoma (HCC) ranks third in terms of cancer-related deaths globally and is the sixth most prevalent cancer (Ali et al. 2024). Although the incidence of HCC has decreased in several countries over recent decades, it is rising in other regions (Abruzzese et al. 2021). This complex disease is characterized by significant variations in its tumor biology and etiology. Chemotherapy and radiotherapy often prove ineffective or are not recommended for patients with cirrhosis (Zeng et al. 2024).

The discovery of novel drugs for HCC is crucial for addressing the limitations of conventional chemotherapy and reducing adverse effects. The thiazole nucleus is a key component of several clinically used anticancer agents, including dabrafenib, dasatinib, ixabepilone, and patellamide. Thiazole derivatives are characterized by their low toxicity, robust anticancer properties, and high efficacy (Sharma et al. 2020).

Numerous derivatives of thiazole demonstrate anticancer properties; certain thiazolidine-based compounds, such as the (RCT) compound, show promise against HCC. These compounds exhibit significant cytotoxic effects on the HepG2 cell line, impede cell motility, disrupt Akt/mTOR signaling pathways, inhibit cell proliferation, and induce apoptotic cell death (McCubrey et al. 2017; Bahadar et al. 2024; Abdelaziz et al. 2025; Abdu et al. 2022).

Numerous studies have explored the impact of thiazole-based compounds on significant genes related to apoptosis, autophagy, and inflammation, including *Caspase3, Bcl2, mTOR, and IL6*. Caspase-3, a protease with cysteine and aspartic acid. Caspase-3 has been identified as a vital facilitator of apoptosis. The relevant caspase is activated within the apoptotic cell through both extrinsic and intrinsic pathways (Bahadar et al. 2024; Hemdan et al. 2024).

Research has demonstrated that curcumin can trigger apoptosis and suppress the growth of HepG2 cells by promoting programmed cell death, inhibiting cell division, and down-regulating *Bcl-2* gene expression levels, upregulating *Bax,* and *Caspase-3* gene expression levels. Curcumin exhibits multiple targets, pathways, and functions that are characterized by its broad availability, low toxicity, and high efficacy (Zeng et al. 2024; Salah et al. 2021).

Quercetin is a natural compound classified within the flavonoid family, known for its diverse medicinal benefits. These benefits encompass anti-allergic, anticancer, anti-inflammatory, and antiviral effects, in addition to providing cardiovascular protection (Bray et al. 2024). It induced apoptosis by regulating the p53-mediated signaling pathway, raising the Bax/Bcl-2 ratio. Moreover, quercetin inhibited cell viability and suppressed proliferation and migration. Quercetin influences apoptosis by upregulating the levels of *caspase-3, Bax*, and *p21*, while simultaneously downregulating the expression of *PLK-1, Akt, cyclin-B1, CDK-2, CDC-2,* and *Bcl-2*. Furthermore, it inhibits STAT3 activation. Quercetin holds promise for future applications in chemoprevention (Qian et al. 2022).

Furthermore, the combination of Curcumin and/or Quercetin with certain chemotherapeutic agents, such as 5-FU, has been shown to produce a synergistic effect that inhibits the proliferation of human hepatocellular carcinoma HepG2 (Sethi et al. 2023; Salah et al. 2024; Abd El-Moaty et al. 2025).

Therefore, this study aims to investigate the effect of the combination of newly synthesized two thiazole derivatives with curcumin and quercetin on hepatic cancers and test gene regulation of *TOR, Bcl-2, IL6*, and *Caspase3* in the HepG2 cell line.

2. Materials and Methods

2.1 Synthesis of nitrophenyl-triazolyl-thiazole derivatives

The chemicals were purchased from Sigma-Aldrich and Alfa Aesar and used without purification. The solvents were dried. A Bruker INVENIO-S FTIR spectrometer was used to measure Infrared spectra. The ^{1}H NMR spectra were obtained by JEOL (500 MHz) in DMSO-d6 as solvent. The elemental analysis of C, H, and N was recorded through the PerkinElmer 2400 analyzer (Narasimhamurthy, Swaroop, and Rangappa 2024).

2.1.1 Synthesis of 2-amino-4-(5-methyl-1-(4-nitrophenyl)-1H-1,2,3-triazol-4-yl) thiazole

Derivative (2)

In a dry RB flask (100mL) containing 35 mL of dry ethanol, add 4 mmol of each 2-bromo-1-(5-methyl-1-(4-nitrophenyl)-1H-1,2,3-triazol-4-yl) ethan-1-one (1) (1.30 g) and thiourea (0.30 g). The reaction mixture was refluxed for 8 h. The mixture was left to cool, poured into ice water, and then sodium carbonate was added. The obtained solid was filtered, dried, and crystallized with EtOH to afford the corresponding triazolyl-thiazol-2-amine derivative 2 (Abdel-Wahab et al. 2023).

Yellow crystals, yield = 63%, m.p. = 284-286°C. IR (ν/cm^{-1}): 3232 (NH_2), 1605 (C=N), 1529 (C=C). ^{1}H NMR (δ/ppm): 2.65 (s, 3H, CH3), 6.98 (s, 1H, thiazole-H5), 7.12 (s, 2H, NH_2), 7.97 (d, J = 8.50 Hz, 2H, Ar-H), 8.44 (d, J = 8.50 Hz, 2H, Ar-H). Analysis for $C_{12}H_{10}N_6O_2S$ (302.06): Calculated: C, 47.68; H, 3.33; N, 27.80%. Found: C, 47.43; H, 3.40; N, 27.91%.

2.1.2 Synthesis of (2-chloroacetamido-4-(1-(4-nitrophenyl)-5-methyl-1H-1,2,3-triazol-4-yl) thiazole (Compound A)

A solution of triazolyl-2-aminothiazole derivative 2 (1.20 g, 4 mmol) in 35 mL DMF containing Na2CO3 (0.55 g, 4 mmol) was stirred for 20 min. Then, chloroacetyl chloride (0.32 g, 4 mmol) was added and continued stirring for 30 h. Pour the mixture into ice-cold water. Finally, the solid underwent filtration, drying, and purification by heating in ethanol to give thiazole acetamide compound A.

Off-white crystals, yield = 76%, m.p. = 230-232°C. IR (ν/cm-1): 3295 (N-H), 1685 (C=O), 1591 (C=N), 1552 (C=C). ^{1}H NMR (δ/ppm): 2.69 (s, 3H, CH3), 4.42 (s, 2H, -CH2-Cl), 7.66 (s, 1H, thiazole-H5), 8.00 (d, J = 9.00 Hz, 2H, Ar-H), 8.46 (d, J = 9.00 Hz, 2H, Ar-H), 12.64 (s, 1H, N-H). Analysis for C14H11ClN6O3S (378.03): Calculated: C, 44.39; H, 2.93; N, 22.19%. Found: C, 44.19; H, 2.90; N, 22.28% r (Narasimhamurthy et al., 2024).

2.1.3 Synthesis of 2-((4-(5-methyl-1-(4-nitrophenyl)-1H-1,2,3-triazol-4-yl)thiazol-2 yl) amino) thiazolidin-4-one (Compound B)

A suspension of chloroacetamide compounds A (1.02 g, 0.005 mol) and ammonium thiocyanate (0.60 g, 0.005 mol) was heated under reflux in 30 mL of absolute ethanol for 4 hours. The solid product that formed on the hot surface was collected to produce compound B.

Pale orange crystals, yield = 73%, m.p. = above 300°C. IR (ν.cm^{-1}): 3119 (N-H), 1736 (C=O), 1614 (C=N). ^{1}H NMR (δ/ppm): 2.75 (s, 3H, CH3), 4.05 (s, 2H, thiazolidin-4-one-CH_2), 7.81 (s, 1H, thiazole-H5), 8.00 (d, J = 9.00 Hz, 2H, Ar-H), 8.46 (d, J = 9.00 Hz, 2H, Ar-H), 12.16 (s, 1H, N-H). Analysis for $C_{15}H_{11}N_7O_3S_2$ (401.04): Calculated: C, 44.88; H, 2.76; N, 24.43%. Found: C, 44.70; H, 2.71; N, 24.56%.

2.2 Cytotoxicity assessment of Compounds (A and B) on the HepG2

2.2.1 Cell line

For assessing the compounds' safety against normal cells, the Vero 2 cells were used. As well, the HepG2 cells, obtained from the National Cancer Institute (Cairo, Egypt) with ATCC HB-8065, were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and penicillin–streptomycin (50 U/mL penicillin and 50 µg.mL^{-1} streptomycin). Cells were maintained at 37 °C in 5% CO_2. For harvesting, cells were washed twice with 1× phosphate-buffered saline (PBS) and detached using 0.25% trypsin (Sigma-Aldrich, MO, USA) at 37 °C and 5% CO_2.

The 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

Cell lines' growth viability and IC_{50} detection were carried out using the MTT assay (Ebaid et al. 2024). Each experiment was done in triplicate.

2.2.2 Calculation of drug combination index

The evaluation of the drug combinations' synergism according to different reference models was carried out as reported previously by Duarte et al. (Duarte and Vale 2022) according to the following formula (Huang et al., 2017):

Formula: CI= D1/(Dx)1 + D2/(Dx)2

Where: D1 and D2 are the concentrations of drugs in the combination that gives IC_{50}

(Dx)1 and (Dx)2 are the concentrations of each drug alone that give IC_{50}.

Interpretation of CI: CI < 1: Synergism

CI=1: Additive effect

CI >1: Antagonism

2.2.3 Detection of selected HepG2, 4 key genes regulation by quantitative real-time PCR

The HepG2 cells' total RNA extraction and cDNA synthesis were carried out according to previous studies (Qian et al. 2022; Dawood et al. 2023). In detail, the cDNA was amplified using the real-time PCR (qPCR) is frequently employed. Using fluorescent dyes or probes to monitor DNA synthesis in real time provides both qualitative and quantitative insights into gene expression. Amplification curves generated during qPCR reflect the relative abundance of the target transcript, and the threshold cycle serves as an indicator of expression levels.

Result analysis involved confirming the specificity and accuracy of amplification. Agarose gel electrophoresis is often applied to visualize PCR products, ensuring that the amplified fragments correspond to the expected size and that non-specific products are absent. In qPCR, melt curve analysis can further verify the specificity by assessing the dissociation characteristics of amplified products. Data interpretation may include fold-change calculations and RQ values calculations (Noori et al. 2017; Zeng et al. 2024).

The quantitative real-time PCR was utilized as the primers of selected genes (Beta-actin as control, housekeeping gene) and 4 key HepG2 genes (*Caspace3, mTOR, IL6 and Bcl2*) were designed according to the genes' sequence obtained from the NCBI GenBank, the primer design was carried out using Primer Blast Program software and their G and C content were evaluated with their suspected annealing temperatures (Mohamed et al. 2024).

The following table (Table 1) illustrates the primers of the tested genes that were reported in this study:

Table 1. The designed primers (forward and reverse) are used for the QPCR procedure.

Gene	Forward primer	Reverse primer
Beta-actin	GTGGCCGAGGACTTTGATTG	GTGGGGTGGCTTTTAGGATG
Caspase 3	GGAAGCGAATCAATGGACTCTGG	GCATCGACATCTGTACCAGACC
mTOR	AGTGGACCAGTGGAAACAGG	TTCAGCGATGTCTTGTGAGG
IL6	AGACAGCCACTCACCTCTTCAG	TTCTGCCAGTGCCTCTTTGCTG
Bcl2	TGTGTGTGGAGAGCGTCAAC	CTACCCAGCCTCCGTTATCC

2.2.4 Relative quantification (RQ)

Relative quantification (RQ) in qPCR measures how much a target gene's expression level changes in comparison to a reference sample (e.g., control) and a housekeeping gene (such as GAPDH or ACTB). In terms of the fold change in expression.

The RQ is calculated as previously reported (Livak and Schmittgen 2001).

Ct value is the cycle at which fluorescence surpasses background, reflecting the abundance of the target, shown as the Lower Ct = higher gene expression.

A double delta Ct was analyzed to calculate the relative quantitative PCR (RQ).

RQ calculates a fold change in gene expression in samples (drug-treated cells) compared to a control (non-treated cells).

3. Results

3.1 Synthesis and characterization of compounds A and B

This work aims to synthesize drug's core structure, nitrophenyl-triazolyl-thiazole compound 2, and two new derivatives, A and B, as shown in (Figure 1) Bromoacetyl-triazole compound **1** was refluxed with thiourea in ethyl alcohol to give the conforming 2-amino-4-nitrophenyl-triazolyl-thiazole compound 2. The molecular structure of 2-aminothiazole compound 2 was assured by the infrared frequencies (IR) and ^{1}H NMR spectra. The IR spectrum demonstrated the absorption of the amino group (NH_2) at 3232 cm^{-1}. Moreover, the substitution reaction of aminothiazole **2** with chloroacetic chloride in dry *N, N*-dimethylformamide containing K_2CO_3 yielded 2-chloroacetamido-thiazole compound **A**. The skeleton of compound **A** was affirmed by the IR spectrum, which displayed distinctive absorption peaks at 3295 cm^{-1} for the (N-H) group and 1685 cm^{-1} for the (C=O) group. The ^{1}H NMR spectrum lacked the singlet signal of the amino group (NH_2) and showed the two singlet signals of methylene protons and the proton of the function (NHCO) at δ 4.42 and 12.64 ppm, respectively. The proton of the thiazole moiety and four protons of the phenylene ring were detected as a singlet and doublet in the region from δ 7.66 to 8.46 ppm.

Furthermore, 2-chloroacetamido-thiazole compound **A** reacted with ammonium thiocyanate (NH_4SCN) in dry ethanol to afford the thiazolidin-4-one in conjugated compound B. In the IR spectrum of conjugate compound B, the absorption of the amino and carbonyl groups of the five-member ring (thiazolidin-4-one) was exhibited at 3119 and 1736 cm^{-1}, respectively. The ^{1}H NMR showed singlet signals in the high field region for methyl protons and methylene protons at δ 2.75 and 4.05 ppm, respectively. In contrast, the proton of the thiazole unit (C5) and the proton of the amino group were observed in a low field area at δ 7.81 and 12.16 ppm, respectively. Further, four protons of the phenyl ring showed as two doublet signals at δ 8.00 and 8.46 ppm (Figure 2,3,4, and 5).

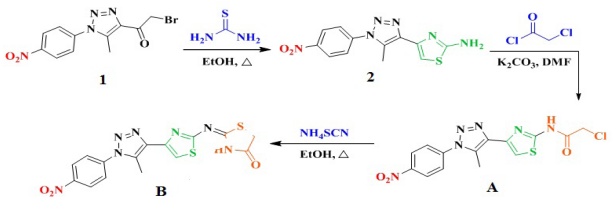


Figure1. Synthetic route of Compound A and B.

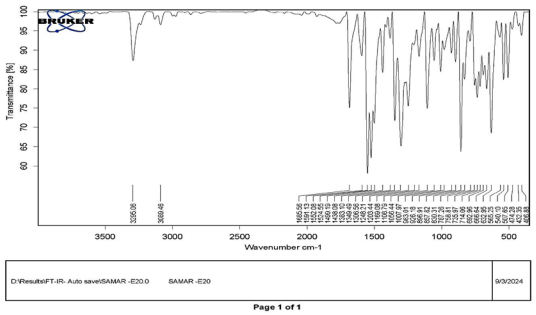


Figure 2. IR spectrum of compound A.

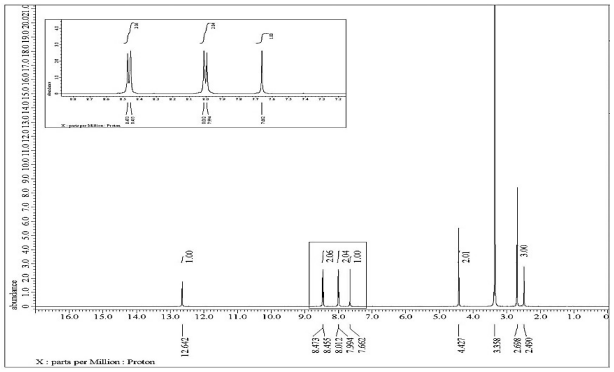


Figure 3. [1]HNMR spectrum of compound A.

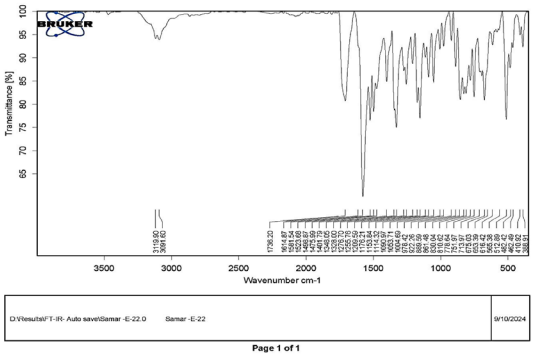


Figure 4. IR spectrum of compound B.

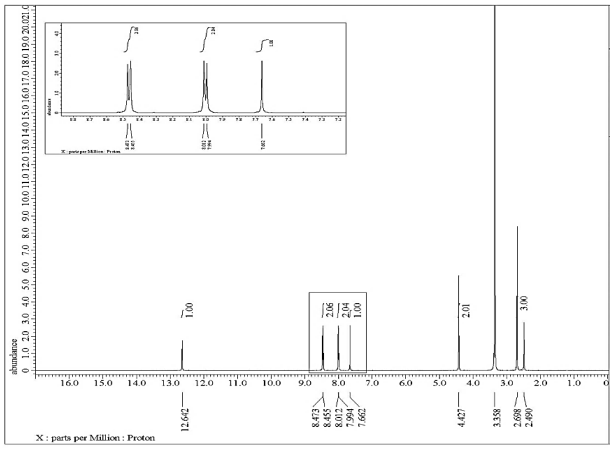


Figure 5. 1HNMR spectrum of compound B

3.2 Anticancer Activity

After treating Vero 2 cells with the thiazoles, curcumin, quercetin, and their combinations, the IC_{50} values were reported after an incubation period of 48 h (Table 2). The safety of all compounds was reported.

Table 2. a) ANOVA results and IC_{50} values of A, B, Curcumin, Quercetin, and Doxorubicin against Vero 2 cells

Compound	A	B	Curcumin	Quercetin	Doxorubicin
IC50(mean± S.D)	9.97±0.86	19.2±1.2*	14.6±1.5*	21.5±1.6*	4.1±0.07**

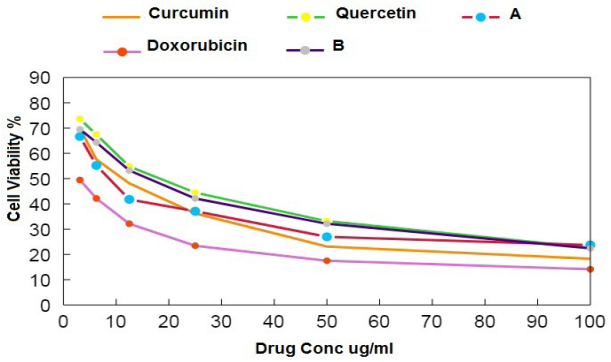
*** there is a significant difference between this group and A (p<0.05).**
**** there is a significant difference between this group and A, Curcumin, and Quercetin groups (p<0.05).**

Table 2. b) ANOVA results and IC_{50} values of Curcumin+ A, Curcumin+ B, Quercetin+ A, and Quercetin+ B against Vero 2 cells

Compound	Curcumin+ A	Curcumin+ B	Quercetin+ A	Quercetin+ B
IC50(mean± S.D)	7.9±1.4	12.8±1.67*	9.4±0.97	17.5±4.16**

*** there is a significant difference between combination A and B (p<0.05).**
**** there is a significant difference between combination group B and other combinations (p<0.05).**

The cytotoxic potential of the newly synthesized compounds (A, B, curcumin, and quercetin) in the presence of a reference drug, Doxorubicin, was examined against HepG2 cells using the MTT assay after 48 hrs. of incubation. The test includes each drug alone and combinations between two drugs, as shown in (Figure 6).



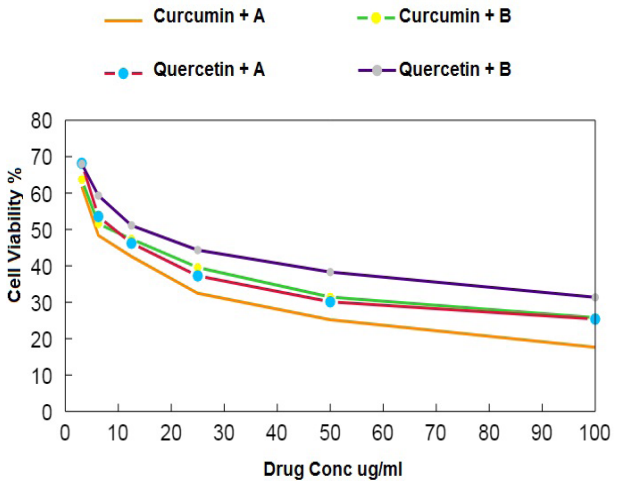


Figure 6. Cell viability % for HepG2 cell line after treating with curcumin, quercetin, and Doxorubicin, (Curcumin+ A, Curcumin+ B, Quercetin+ A, and Quercetin+ B).

3.3 Determination of the IC_{50} of drugs, combinations against HepG2 cell lines

As compared to the drugs alone, the combination of curcumin with compounds A and B reduced the IC_{50} values from 8.67 to 5.87 $\mu g.mL^{-1}$ and 16.2 to 10.5 μgmL^{-1}, respectively. While Quercetin, combined with compound A and B, decreased the IC50 values from 8.67 to 8.53 µg/mL and 16.2 to 14.6 $\mu g.mL^{-1}$ (Table 3) (Figure 8 and 9).

Table 3a. IC_{50} of single drugs (A, B, Curcumin, Quercetin, and Doxorubicin)

Drug	A	B	Curcumin	Quercetin	Doxorubicin
IC50 ug/ml	8.67	16.20*	11.26*	18.3*	2.95**

*** there is a significant difference between this group and A ($p<0.05$).**
**** there is a significant difference between this group and A, Curcumin, and Quercetin groups ($p<0.05$).**

Table 3b. IC_{50} of drug combinations (Curcumin+ A, Curcumin+ B, Quercetin+ A, and Quercetin+ B)

Drug	Curcumin+ A	Curcumin+ B	Quercetin +A	Quercetin +B
IC50 µg/ml	5.87	10.50*	8.53	14.6**

*** there is a significant difference between combination A and B ($p<0.05$).**
**** there is a significant difference between combination group B and other combinations ($p<0.05$).**

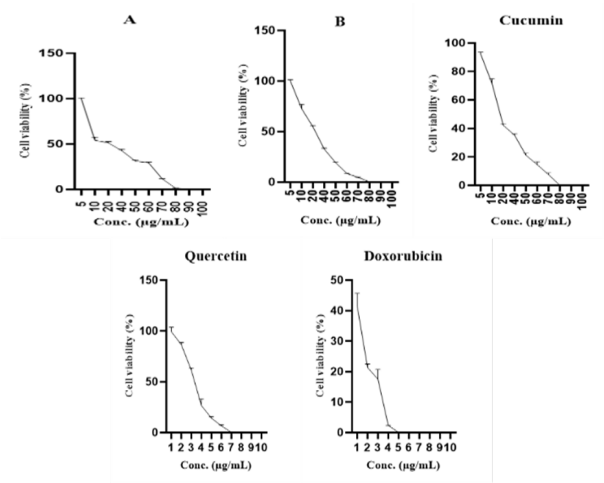


Figure 8. The cell viability % of the tested compound against Vero 2 cells (A, B, Curcumin, Quercetin, and Doxorubicin).

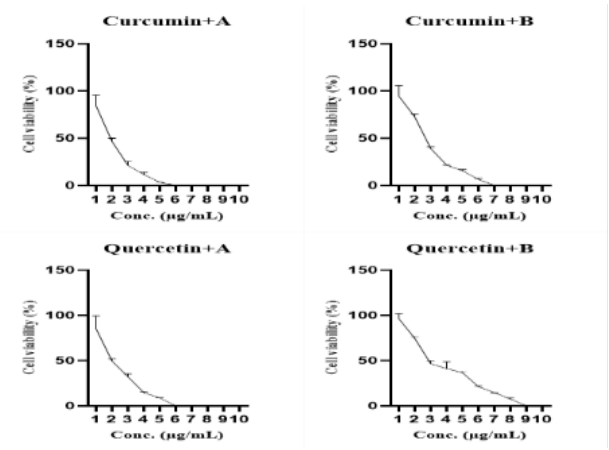


Figure 9 . The cell viability % of the tested compound against Vero 2 cells

(Curcumin+ A, Curcumin+ B, Quercetin+ A, and Quercetin+ B).

3.3.1 Calculation of drug combination index

The combination index (CI) was calculated and illustrated in Table 4. All combinations showed a synergistic effect as referred to the CI values of all combinations, but the combination that gives the lowest IC_{50} and best synergistic effect is (Curcumin+ A).

Table 4. The combination index (CI) for the tested combinations

Combination	CI	Result
Curcumin+ A	0.57	Synergism
Curcumin+ B	0.79	Synergism
Quercetin+ A	0.72	Synergism
Quercetin+ B	0.84	Synergism

3.4 HepG2 viability for the most potent combination (Curcumin + A)

The viability degree of the selected most potent drugs that give lower IC50 on HepG2 cells, Curcumin, Drug A, and (Curcumin +Drug A).

According to image analysis and IC_{50} of each drug A, curcumin, and the combination compared to the control groups, it can arrange the groups according to the degree of potency that causes more inhibition for HepG2 cells viability as follows:

Curcumin, as a lone natural drug used, is less potent than Drug A solely. In comparison, Curcumin+ A is the most powerful, which gives the highest inhibitory effect than Drug A or curcumin. The HepG2 cells viability is shown to be lower in curcumin+ A than in curcumin and Drug A alone. The number of viable cells is shown in (Figure 10).

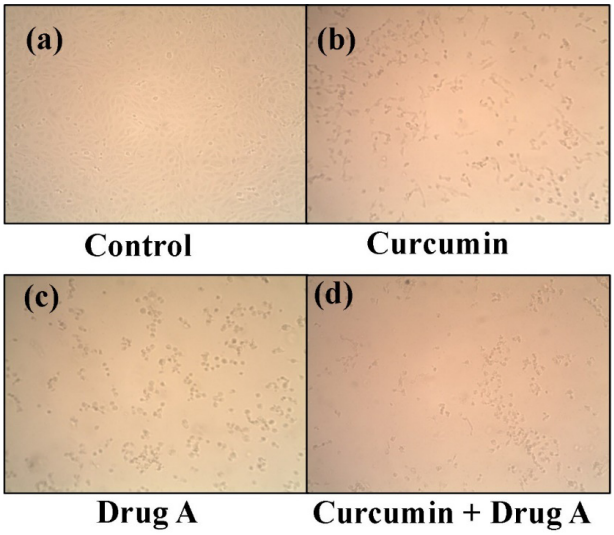


Figure 10. Morphological changes in HepG2 cells exposed to the IC_{50} concentration of compound A, curcumin, and (Curcumin+ drug A) for 48 hrs. (A) HepG2 cell viability for control. (B) HepG2 cell viability for curcumin. (C) HepG2 cell viability for drug A. (d) HepG2 cell viability for (Curcumin+ drug A).

3.5 Regulation of 4 Key genes (mTOR, Bcl2, IL6, and Caspase3)

To detect the effect of the most potent combination (Curcumin+ A) that gives the lower and significant IC_{50} at HepG2 cells, quantitative gene expression was performed for several genes, including *mTOR, Bcl2, IL6,* and *Caspase3*.

The significant result of gene quantitation is expressed when the RQ value shows a more than two-fold change upregulation (RQ > 2) or less than 0.5-fold downregulation (RQ < 0.5), which is considered a biologically relevant difference above experimental noise (Noori et al. 2017). The results of RQ for genes in each group are plotted in (Table 5).

Table 5. The RQ results for the tested genes obtained by QPCR for the tested genes

Genes RQ	Curcumin	A	Curcumin+ A
Caspase3	8.8 ±2.4	14.9 ± 5.1	18.2 ± 4.7
mTOR	0.56 ± 0.08 *	0.07 ± 0.03	0.02 ± 0.00
IL-6	0.1 ± 0.02	0.6 ± 0.08 *	0.08 ± 0.02
Bcl-2	0.18 ± 0.01	0.19 ± 0.05	0.04 ± 0.02

***Non-significant downregulation**

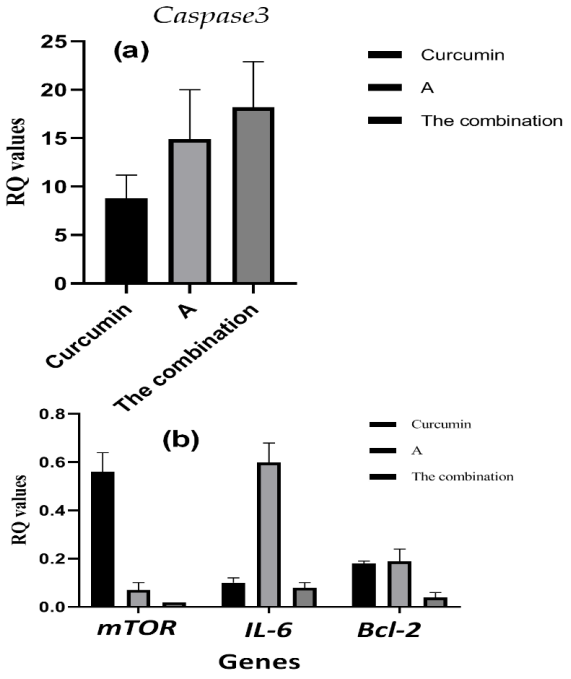


Figure 11. The RQ results for the tested genes obtained by QPCR. (a) The RQ analysis for *Caspase3 gene* on treated cells with (curcumin, drug A, the combination (curcumin+ A). (b) The RQ analysis for *mTOR, IL-6, and Bcl-2* genes on treated cells with (curcumin, drug A, the combination (curcumin+ A).

Furthermore, RQ of gene regulation in drug-treated groups was obtained to show that the *Bcl-2, Caspase3, and mTOR* genes had a significant up-regulation in all groups. While the *mTOR* gene reported a significant down-regulation in (A and curcumin+ A) groups, but non-significant downregulation in (curcumin) group, and *IL-6* gene showed a significant down-regulation in (curcumin and curcumin+ A) groups but non-significant down-regulation in (A) group (Figure 11).

4. Discussion

The most common type of primary liver cancer is HCC, which is characterized by a significant incidence of morbidity and mortality worldwide. There exists an urgent need to develop new therapeutic agents capable of circumventing multidrug resistance mechanisms, thereby providing a more sustainable clinical response (Zou et al. 2025; Song et al. 2023).

Anti-HepG2 activity of synthetic compounds A and B

Many compounds with this scaffold induce apoptosis and cause cell-cycle arrest through multiple mechanisms (Dawood et al. 2023). Some derivatives also reduce EGFR kinase activity, inhibiting cancer cell metastasis (Bray et al. 2024). In this study, the safety index was measured by calculating the IC_{50} and observing the cell viability of the tested compounds against the Vero 2 cell. TO affirm the suspected compounds' safety or low level of toxicity (Abdu et al. 2022; Marquardt et al. 2015).

After that, compounds A and B (both bearing a nitrophenyl-triazolyl-thiazole moiety) were synthesized to assess anticancer activity. Against HepG2 cells, compound B (thiazolidine-4-one side chain) showed moderate activity, whereas compound A (chloroacetamide side chain) was more potent, likely due to the chloroacetamide group (Marquardt et al. 2015).

The chloroacetamide group is a promising anticancer motif because it forms stable, irreversible covalent bonds with nucleophilic protein sites, such as cysteine thiols and histidine imidazoles (Duarte and Vale 2022). Incorporating this group into a suitable scaffold enables targeted engagement of cancer-associated proteins and can be effective against treatment-resistant cancers (Xu and Liu 2025; Zhou et al. 2017).

By comparison, the thiazolidine-4-one side chain generally confers lower potency than the chloroacetamide chain against HepG2 cells. Thiazolidine-4-one derivatives act mainly via reversible, non-covalent interactions (hydrogen bonding and hydrophobic forces), functioning as enzyme inhibitors or receptor modulators (Huang, Xu, and Zhuang 2025).

Anti-HepG2 activity of curcumin and quercetin

To enhance the anti-HepG2 activity of the A and B, they combined them with two well-known flavonoids as anticancer products, curcumin and quercetin (Sharma et al. 2020).

Curcumin is a potent therapy against HepG2 cells; it has anticancer efficacy against several malignancies, including breast, lung, gastric, liver, and prostate cancers, through many mechanisms (Auti et al. 2024). Studies have indicated that curcumin exerts significant inhibitory effects on the proliferation and migration of HepG2 cells. This is achieved through arresting the cell cycle at the S phase, induction of apoptosis in cancer cells, and interfering with the expression of key oncogenic signaling pathways such as VEGF, STAT3, and HIF-1α (Olszewska et al. 2016; Othman et al. 2023).

Quercetin strongly inhibits some multidrug-resistance regulatory proteins and transporters associated with tumor activity, such as proliferation, invasion, migration, and metastasis, which reflects tumor aggressiveness (Guan et al. 2023; El-Hddad et al. 2024), with an inhibitory effect on HepG2 cells proliferation as it induces apoptosis and diminishes their viability.

The power of drug combination

A combination of curcumin with drugs like ABT-737 induces apoptosis significantly in HepG2 cells by activating caspase-9 and caspase-3 enzymes (Bahadar et al. 2024).

In the current study, 4 combinations were designed as follows: (Curcumin+ A), (Curcumin+ B), (Quercetin+ A), and (Quercetin+ B). It was found to be a synergistic activity in all combinations with varying degrees (Dawood et al. 2023; El-Hddad et al. 2024). Moreover, It was found that the most potent and active combination was (Curcumin+ A), which gives a lower IC50 and significant anticancer activity against HepG2, and this may be due to interfering with multiple targets and pathways in HepG2 cells.

A significant reduction in HepG2 cells' proliferation and migration was observed when curcumin was combined with anticancer drugs, such as celecoxib (Yang and Chan 2009; Zou et al. 2025; Zhou et al. 2017). The same, when quercetin is combined with anti-HepG2 drugs, such as gemcitabine or doxorubicin, a synergistic effect is found, causing cell growth inhibition and apoptosis. A combination of quercetin with cisplatin induces p16-mediated cell cycle arrest and apoptosis (Hassan et al. 2020; Zheng et al. 2016).

Regulation of HepG2 key genes

To confirm these results and suggest the mechanism, we examined the regulation of four key genes (*mTOR, Bcl-2, IL-6, and caspase3*) in HepG2 cells, that affected by the most potent combination, curcumin + compound A. it found that the first three genes *(mTOR, Bcl-2, and IL-6*) are all downregulated. In contrast, *caspase3* upregulated which reflected the potency of the combination in inhibition of HepG2 cells and selectively in induced apoptosis (Qian et al. 2022; Zeng et al. 2024).

***mTOR* Downregulation**

Inhibition of mammalian target of rapamycin (*mTOR*) by combination therapy is a significant therapeutic achievement. A direct mechanistic link exists between the structure of compound A and this effect, as a chloroacetamide derivative has been shown to inhibit the AKT/mTOR signaling pathway, which is a key pro-survival cascade. This provides a direct causal link between the drug's proposed mechanism of action and the observed cellular response (Guertin and Sabatini 2007; Abruzzese et al. 2021).

On the other hand, curcumin may directly repress the transcriptional regulation of the *mTOR* gene, leading to lower mRNA levels, and induce promoter hypermethylation of the *mTOR* gene, which is a mechanism that silences gene expression and reduces the synthesis of its mRNA (Liu et al. 2017). It interferes with the PI3K/Akt/mTOR pathway, leading to inhibition of proliferative signaling and enhancing apoptotic responses and cancer cells' autophagy (Zheng et al. 2016; Hassan et al. 2020; Akter, Gul, and Mumtaz 2025).

***Bcl-2* Downregulation**

Bcl-2 is an anti-apoptotic protein. It is overexpressed in many types of cancers, as it facilitates tumor survival and resistance to chemotherapy, because it suppresses programmed cell death (apoptosis) (Sharma et al. 2020). In HepG2 cells, upregulation of *Bcl-2* causes multidrug resistance and is linked to factors like insulin resistance (Zeng et al. 2024)

The curcumin + compound A combination causes a downregulation of this gene, so it is suggested that the combination directly targets and reverses a pre-existing mechanism of drug resistance, thereby leading to the cancer cells to death (Sherif et al. 2018; Shama and Savaliya 2025). It was found that some derivatives containing thiazole and triazole structures can inhibit the NF-κB pathway, suppress the anti-apoptotic *Bcl-2* gene regulator, and induce apoptosis in cancer cells by downregulating Bcl2 gene expression (Sethi et al. 2023).

Other thiazole derivatives cause inhibition of *Bcl2* in cancer cells, leading to promote apoptosis (Sharma et al. 2020). Curcumin inhibits Bcl2 proteins in HepG2 cells and induces apoptosis. Studies show curcumin reduces NF-κB's impact on cancer cells, ultimately inhibiting tumor growth and potentially acting as a therapeutic agent for HCC (Shama and Savaliya 2025; Marquardt et al. 2015; Liu et al. 2017).

***IL-6* Downregulation**

Interleukin-6 (IL-6) helps to sustain a pro-tumorigenic microenvironment. In liver cancer patients, the IL-6, if found in high concentration in serum, is associated with a poor prognostic indicator, so therapy needs to disrupt this pathway (Song et al. 2023; Nenu et al. 2023; Mustafa et al. 2019).

It was found that some thiazole derivatives inhibit IL-6 by reducing the DNA binding of the transcription factor NF-κB. Leading to inhibit *IL-6* mRNA in some cancer cell lines, which is the other part of the combination, has an inhibition effect on *IL-6* transcription in HepG2 cells. (Moawad et al. 2023; Noori et al. 2017).

***Caspase3* Upregulation**

Caspase-3 is a cysteine protease and key executioner enzyme in the apoptosis pathway, responsible for dismantling cells during programmed cell death. Caspase-3 is at the end of the caspase cascade, meaning it is a critical and final effector in the apoptotic process (Padhariya et al. 2020). The upregulation and activation of Caspase-3 is the definitive molecular marker of a successful proapoptotic therapeutic intervention (Sayed et al. 2020). Many studies have reported the role of thiazole and triazole compounds in upregulation of caspase-3 gene transcription in a lot of cancer cell lines, such as HepG2 (Ali et al. 2024). Curcumin induces apoptosis in HepG2 cells through activation of proapoptotic mediators such as caspase3. Curcumin nano capsules induce apoptotic processes through upregulation of *caspase-3* gene mRNA (Moawad et al. 2023; Abd El-Moaty et al. 2025).

The most attractive finding is, this activity increases significantly when combined with other flavonoids, especially curcumin. The combination gives a lower IC50, nearing the reference drug used, doxorubicin, which reflects the unique character of this formula, suggesting being promising formula against one of the most aggressive forms of cancer in the world (Mustafa et al. 2019; Marquardt et al. 2015).

5. Conclusions

In conclusion, the biological activity of these compounds against HepG2 cells is significantly influenced by their side-chain structure. The combination of compound A and curcumin led to the downregulation of *mTOR*, a central regulator of cell growth, and *IL6*, a pro-inflammatory cytokine. This study identified the synergistic combination of compound A and curcumin as a promising therapeutic formula for HCC. For future direction more in-depth research is recommended about this combination in other cancer types, and further development as a promising novel agent in cancer therapy.

Ethical approval: no human subjects were involved.

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