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Enhancing colorectal cancer treatment with a triple therapy approach involving 5-fluorouracil, thymoquinone, *Polygonum minus*

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ABSTRACT

Colorectal cancer (CRC) remains a leading cause of cancer-related mortality globally, with chemoresistance to 5-fluorouracil (5-FU) and dose-limiting toxicities posing major therapeutic challenges. This study investigates the synergistic potential of 5-FU combined with bioactive compounds from *Polygonum minus* (PM) extract and thymoquinone (TQ) in free and calcium carbonate ($CaCO_3$)-nanoparticle-encapsulated forms (5-FU-$CaCO_3$, TQ-$CaCO_3$) against SW480 and SW620 CRC cell lines. Cytotoxicity (MTT assay), combination index (CI), and apoptosis/necrosis (Annexin V/PI flow cytometry) were evaluated at 24 –72 h. Both free-drug (FTP: 5-FU+TQ+PM) and $CaCO_3$-encapsulated (FT-NP/PM: 5-FU-$CaCO_3$+TQ-$CaCO_3$+PM) combinations exhibited dose- and time-dependent cytotoxicity in SW480 and SW620 cells. In SW620 cells, the encapsulated FT-NP/PM showed superior cytotoxicity compared to the free combination, with IC_{50} values decreasing from 11.75 μg/mL (24 h) to 6.70 μg/mL (72 h), whereas FTP declined from 17.75 μg/mL to 6.54 μg/mL. In SW480 cells, both formulations demonstrated comparable effects, maintaining cytotoxicity over time (FTP: 15.38–7.68 μg/mL; FT-NP/PM: 16.98–8.81 μg/mL). Combination index analysis confirmed strong synergism at lower concentrations (CI < 0.4) in both cell lines, with FT-NP/PM showing slightly greater synergy, particularly in SW620. Higher concentrations tended toward additive or antagonistic effects. Apoptosis and necrosis analyses further supported these results, where FT-NP/PM induced higher necrosis and late apoptosis compared to FTP, especially after 72 h in SW620 cells. In SW480, apoptosis remained the predominant mode of cell death, with nanoencapsulation sustaining the response over time. These findings underscore the therapeutic advantage of combining natural compounds with conventional chemotherapy. Overall, the synergistic interaction between 5-FU, TQ, and PM, enhanced by $CaCO_3$-mediated delivery, improved cytotoxic and apoptotic effects, especially in the chemo-resistant SW620 cells, suggesting the potential of FT-NP/PM as an optimized combination strategy for colorectal cancer treatment.

1. Introduction

Colorectal cancer (CRC) remains a devastating disease, responsible for a high mortality rate among patients living with CRC. It is the second leading cause of cancer-related deaths worldwide, following lung cancer [5]. Conventional treatments, such as 5-fluorouracil (5-FU), are

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frequently used in CRC management; however, the development of resistance to 5-FU presents a significant challenge, often leading to suboptimal therapeutic outcomes [17]. This resistance, coupled with the severe side effects associated with conventional treatments, has spurred significant interest in alternative therapies, particularly those based on natural products, which offer the potential for efficacy alongside reduced toxicity to healthy cells [18].

One promising approach to overcoming 5-FU resistance is the use of combination therapies, which enhance the drug's sensitivity in CRC cells. Combining 5-FU with natural products or other agents can enhance its efficacy and improve the sensitivity of CRC cells to the treatment, thereby overcoming drug resistance and leading to better outcomes. Studies have shown that curcumin and its analogue, dimethoxycurcumin, enhance the anticancer effect of 5-FU against CRC. The combination of 5-FU with either curcumin or dimethoxycurcumin enhances cytotoxic effects compared to 5-FU, curcumin, or dimethoxycurcumin alone, thereby generating a synergistic effect [30,32,33]. A similar effect was observed in recent studies, which showed that Protosappanin B enhances the chemosensitivity of 5-FU in colon adenocarcinoma tissues [16], while 17β-estradiol also increased the sensitivity in colon cancer cell lines [21]. Several natural products, including epigallocatechin-3-gallate [27] corilagin [19], quercetin [25] and resveratrol [6], have shown promise in enhancing the sensitivity of CRC cells to 5-FU. Other research has shown that combining 5-FU with other agents or drug delivery systems can improve the therapeutic outcomes of 5-FU by sensitizing cancer cells and overcoming resistance mechanisms. For instance, the encapsulation of 5-FU in calcium carbonate ($CaCO_3$) nanoparticles with thymoquinone (TQ) has been demonstrated to enhance drug delivery and increase the sensitivity of CT26 colon cancer cell lines to the treatment [10,11]. Furthermore, combining $CaCO_3$-encapsulated 5-FU, TQ, and additional agents in a triple therapy approach offers a promising strategy for overcoming resistance and improving treatment efficacy, further potentiating the anticancer effects of 5-FU [2].

Polygonum minus (PM), commonly known as Kesum, has attracted considerable attention as a natural product with diverse pharmacological properties, including antioxidant, anti-inflammatory, and antimicrobial activities [28]. Native to Southeast Asia, PM is not only utilized in culinary applications but also recognized by the Malaysian government as an essential crop within the Herbal Product Blueprint, reflecting its economic and medicinal significance [9]. A recent study has shown that the solvent fractions of PM demonstrate inhibitory effects in CT26 colorectal cancer cells [31]. Its constituent polygonumins A have been reported to have anti-proliferative activities against various cancer cells, including CRC cells [1].

To further improve CRC treatment, it is crucial to investigate how PM, in combination with other therapeutic agents, can enhance the efficacy of established 5-FU treatments. This study aims to fill this gap by evaluating the cytotoxic effects of PM extract on the SW480 and SW620 CRC cell lines and exploring the potential of a triple therapy approach involving 5-FU, TQ, and PM. Furthermore, through the use of $CaCO_3$ nanoparticles to deliver the effects of 5-FU and TQ, and a combination of these agents with non-encapsulated PM, the study seeks to uncover novel therapeutic strategies that can not only overcome 5-FU resistance but also improve overall therapeutic outcomes in CRC through synergistic interactions between 5-FU, TQ, and PM.

2. Materials and methods

2.1. Chemicals, reagents, and plant material

Methanol, hexane, and dichloromethane (DCM) were obtained from Fisher Scientific (USA). Additionally, MTT powder (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ), sodium carbonate, and phosphate-buffered saline (PBS, pH 7.4) were also sourced from Fisher Scientific. All other chemicals used were of analytical grade, and deionized water (dH_2O) was used throughout the experiments to maintain high purity standards. Plant material was acquired from the Seri Kembangan Wet Market (Pasar Borong Selangor, Malaysia). The plant material was authenticated by a certified botanist at Universiti Putra Malaysia, where a voucher specimen was deposited for reference.

2.2. Cell line and culture conditions

Human colorectal cancer cell lines SW620 and SW480 were obtained from the American Type Culture Collection (ATCC, USA). Cells were maintained in RPMI-1640 medium supplemented with 10 % fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were incubated at 37°C in a humidified atmosphere containing 5 % CO_2 incubator and routinely subcultured by trypsinization upon reaching 70–80 % confluence.

2.3. Plant preparation and extraction

Fresh PM leaves were thoroughly washed with deionized water and dried in an oven at 45°C for 24 h to preserve thermolabile compounds while ensuring complete dehydration. The dried leaves were then ground into a fine powder using a mechanical grinder. Sequential extraction was performed using solvents of increasing polarity: hexane, dichloromethane, methanol, and water. For each extraction, 50 g of leaf powder was macerated at room temperature in an incubator shaker set to 150 rpm for three extraction cycles, each lasting 1 h. The extracts were then filtered through Whatman No. 1 filter paper, and the solvents were removed under reduced pressure using a Büchi rotary evaporator (Model R-300, Switzerland) set at 40°C. The concentrated extracts were stored at −80°C until further use. For the present study, only the water extract of PM was used in all biological assays.

2.4. Drug preparation

The synthesis of 5-FU and TQ-loaded $CaCO_3$ nanoparticles was carried out as previously described by Hamidu et al. [14]. Stock solutions of all drug formulations, including free 5-FU, TQ, PM, and the combination treatment (FTP), as well as nanoparticle formulations 5FU-$CaCO_3$ and TQ-$CaCO_3$ were prepared in phosphate-buffered saline (PBS) containing Tween 80.

2.5. MTT assay for cell viability

The cytotoxic effects of free drug formulations or $CaCO_3$-encapsulated formulations on SW620 and SW480 cells were evaluated using the standard MTT assay. Cells were seeded into 96-well plates at a density of 5×10^3 cells per well and allowed to adhere for 24 h. Subsequently, cells were treated with varying concentrations (3.13, 6.25, 12.5, 25, 50, and 100 μg/mL) of the free drug formulations or $CaCO_3$-encapsulated formulations dissolved in DMSO, ensuring the final DMSO concentration remained below 0.1 %. After 24, 48, and 72 h of incubation, 20 μL of MTT stock solution (5 mg/mL) was added to each well and incubated for 4 h at 37°C in a humidified incubator. Following incubation, the medium was then carefully removed, and 150 μL of DMSO was added to dissolve the formazan crystals. Absorbance was measured at 570 nm with a reference wavelength of 620 nm using a Bio-Rad microplate reader (Infinite F50, TECAN). The IC_{50} values were determined using nonlinear regression by plotting the percentage of cell viability against drug concentrations.

2.6. Combination Index (CI)

The combination index (CI) for both free drugs and $CaCO_3$-encapsulated formulations on the SW620 and SW480 cells was determined using Compusyn software (Version 1.0, Compusyn, Inc., and Paramus,

NJ, USA) based on the Chou-Talalay method. A fixed-ratio of 1:2:4 (5-FU:TQ:PM) was selected according to the IC_{50} values obtained from prior MTT cytotoxicity screening. Stock solutions were prepared at 5 μg/mL (5-FU or 5-FU-$CaCO_3$), 10 μg/mL (TQ or TQ-$CaCO_3$), and 20 μg/mL (PM), combined to yield a 1000 μg/mL master mixture, and serially diluted to a final working range of 3.125 – 100 μg/mL. For combination treatments, two sets were prepared: (i) FTP (free 5-FU, TQ, and PM at 5, 10, and 40 μg/mL, respectively) and (ii) FT-NP/PM (5-FU-$CaCO_3$ and TQ-$CaCO_3$ combined with non-encapsulated PM). These concentrations were applied consistently across all assays to evaluate the dose-dependent effects at 24, 48, and 72 h. Cells were treated with either individual agents or the fixed-ratio combinations, followed by MTT assay to determine cell viability and CI values. CI interpretations were as follows: CI> 1.3 antagonism; CI 1.1–1.3 moderate antagonism; CI 0.9–1.1 additive effect; CI 0.8–0.9 slight synergism; CI 0.4–0.8 synergism; CI 0.2–0.4 strong synergism [8].

2.7. Flow cytometry analysis of apoptosis

Cells in the presence or absence of treatment were trypsinized, washed twice with ice-cold PBS, and resuspended in 1X Binding Buffer at a concentration of 1×10^6 cells/mL. A 100 μL aliquot (1×10^5 cells) was transferred into a 5 mL FACS tube, followed by the addition of 5 μL Annexin V-FITC and/or 5 μL of PI to the respective samples. The cells were gently vortexed and incubated at room temperature (25°C) in the dark for 15 min. After incubation, 400 μL of 1X Binding Buffer was added to each tube, and the samples were analyzed within 1 h by BD FACS flow cytometry (BD Bioscience, USA).

2.8. Statistical analysis

Data were presented as mean±SD from at least three independent experiments, each conducted in triplicate. Statistical analysis was performed using GraphPad Prism (version 10) with a one-way ANOVA, followed by Tukey's post-hoc test to determine significant differences between groups. A p-value of less than 0.05 was considered statistically significant.

3. Results

3.1. Cytotoxicity and combination effects of free drug and $CaCO_3$-encapsulated formulations on SW620 and SW480 cells

The results for the free drug formulations, as shown in Fig. 1A, demonstrated a clear time- and dose-dependent cytotoxic effect across all tested compounds in SW620 cells. Notably, PM exhibited a cytotoxic effect with a mean IC_{50} of 59.51 μg/mL at 24 h, 47.97 μg/mL at 48 h, and 22.11 μg/mL at 72 h, which indicates a pronounced time-dependent reduction in viability (Fig. 1B). Meanwhile, TQ exhibited a cytotoxic profile with a mean IC_{50} values of 30.00 μg/mL, 28.20 μg/mL, and 28.10 μg/mL at 24, 48, and 72 h, respectively. In contrast, 5-FU displayed a time-dependent effect, with IC_{50} decreasing from 42.70 μg/mL at 24 h to 31.65 μg/mL at 48 h and further declining to 18.20 μg/mL at 72 h. For 5-FU-$CaCO_3$, the average IC_{50} values were 44.55 μg/mL at 24 h, 29.85 μg/mL at 48 h, and 18.00 μg/mL at 72 h. The TQ-$CaCO_3$ formulation exhibited more or less similar cytotoxicity to its free counterpart, with a mean IC_{50} value of 35.30 μg/mL, 28.90 μg/mL, and 24.45 μg/mL at 24, 48, and 72 h, respectively. The encapsulation of these single agents in $CaCO_3$ nanoparticles resulted in inhibitory effects

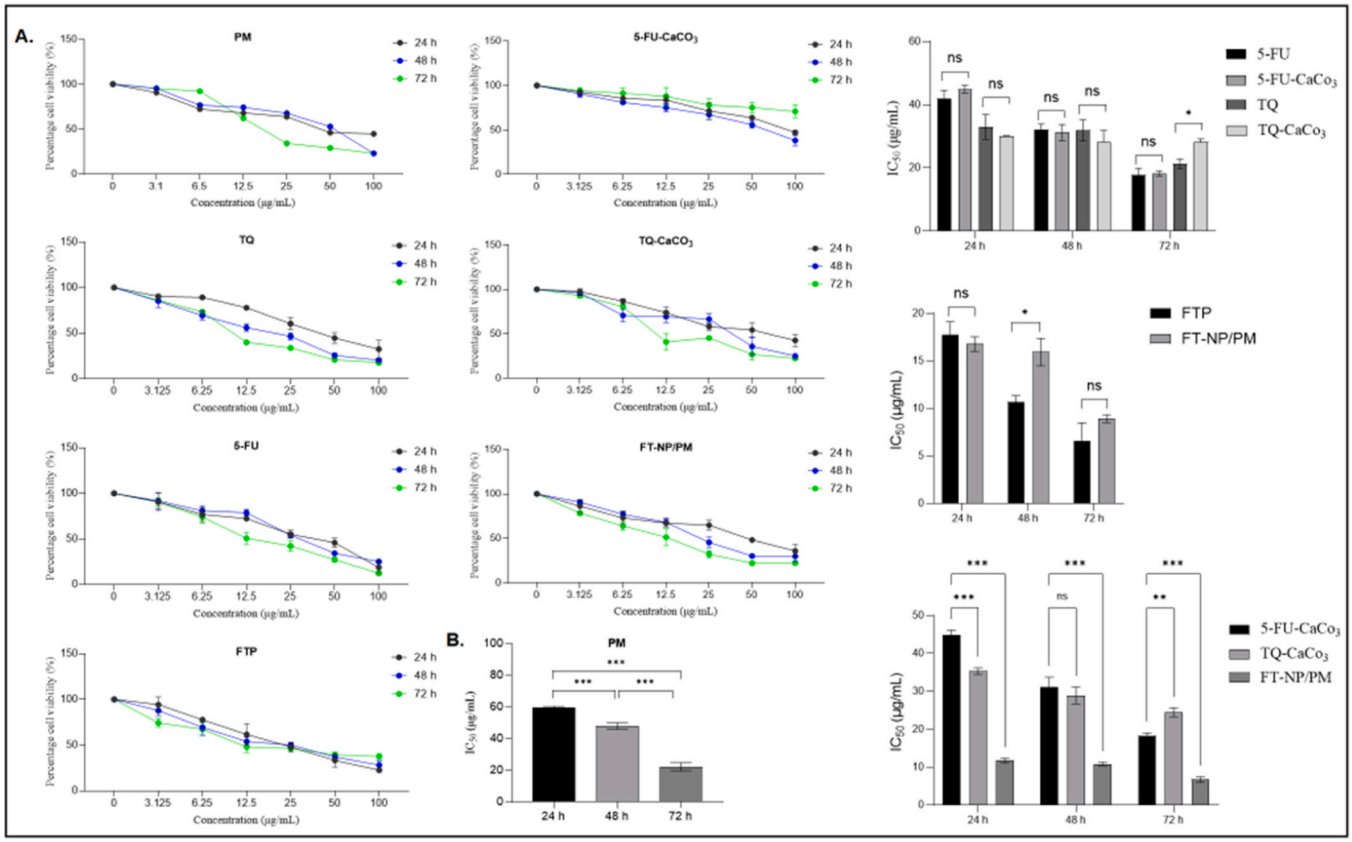

Fig. 1. Cytotoxicity and combination effects of free drug and $CaCO_3$-encapsulated formulations on SW620 colorectal cancer cells. (A) shows a time- and dose-dependent cytotoxic effect of PM, 5-FU, TQ, 5-FU-$CaCO_3$, TQ-$CaCO_3$, FTP, and FT-NP/PM; (B) compares the IC_{50} values of PM, 5-FU, TQ, 5-FU-$CaCO_3$, TQ-$CaCO_3$, FTP, and FT-NP/PM in SW620 cells at 24, 48, and 72 h. Data are presented as Mean±SD; ns: non-significant ($p > 0.05$); *$p < 0.05$; ***$p < 0.001$ compared to 5-FU. 5-FU: 5-Fluorouracil; TQ: Thymoquinone; PM: *Polygonum minus*; FTP: 5-FU+TQ+PM; FT-NP/PM: 5-FU-$CaCO_3$+TQ-$CaCO_3$+PM.

on cell proliferation comparable to their single free-drug counterparts. Moreover, the combination therapy of free-drug (FTP: 5-FU+TQ+PM) exhibited cytotoxic potency, with IC_{50} values of 17.75 μg/mL, 10.66 μg/mL, and 6.54 μg/mL at 24, 48, and 72 h, respectively. Combination of $CaCO_3$-encapsulated formulations (FT-NP/PM: 5-FU-$CaCO_3$+TQ-$CaCO_3$+PM) also exhibited slightly better cytotoxicity than its free counterpart, with a mean IC_{50} values of 11.75 μg/mL, 10.84 μg/mL, and 6.70 μg/mL at 24, 48, and 72 h, respectively.

In SW480 colorectal cancer cells, treatment with free drug formulations resulted in pronounced, time- and dose-dependent reduction in cell viability across all tested agents (Fig. 2A). The IC_{50} values obtained from cytotoxicity assays (Fig. 2B) illustrate the varying degrees of potency among the agents. The IC_{50} values of 5-FU declined from 36.70 μg/mL at 24 h to 29.15 μg/mL at 48 h and 22.50 μg/mL at 72 h. TQ followed a similar trend, with a mean IC_{50} values of 34.38 μg/mL, 33.28 μg/mL, and 21.66 μg/mL at 24, 48, and 72 h, respectively. In contrast, PM was relatively more potent, with a consistent time-dependent decline in IC_{50} from 23.48 μg/mL at 24 h to 16.39 μg/mL by 72 h. When these drugs were encapsulated in $CaCO_3$ nanoparticles, their cytotoxic profiles remained broadly similar to their free forms. The IC_{50} values for 5-FU-$CaCO_3$ were 33.10 μg/mL at 24 h, 32.40 μg/mL at 48 h, and 25.45 μg/mL at 72 h, slightly aligning with those of the free drug. Likewise, TQ-$CaCO_3$ displayed IC_{50} values of 36.37 μg/mL, 32.50 μg/mL, and 22.87 μg/mL at 24, 48, and 72 h, respectively. On the other hand, the triple-drug combination (FTP) produced enhanced cytotoxic effects, with IC_{50} values of 15.38 μg/mL, 13.28 μg/mL, and 7.68 μg/mL at 24, 48, and 72 h, respectively. The FT-NP/PM showed a modestly higher IC_{50} values of 16.98 μg/mL at 24 h, 16.78 μg/mL at 48 h, and 8.81 μg/mL by 72 h.

3.2. Combination index (CI) of free drug and $CaCO_3$-encapsulated formulations on SW620 and SW480 cells

The combination index of 5-FU, TQ, and PM, either as free drugs (FTP) or $CaCO_3$-encapsulated formulations (FT-NP/PM), was evaluated in SW620 and SW480 cells using CompuSyn software based on the Chou-Talalay method. A fixed ratio of 1:2:4 (5-FU:TQ:PM), established from prior IC_{50} screening, was applied across all assays. Both free and encapsulated formulations produced dose-dependent reductions in cell viability, with CI values confirming synergistic interactions in both cell lines.

In SW620 cells, a stronger synergistic interaction was observed after 72 h of treatment, particularly with the FT-NP/PM combination (Table 1). At concentrations below 50 μg/mL, CI values ranged from 0.38 to 0.25, indicative of strong synergism. At the highest tested concentration (100 μg/mL, 72 h), FT-NP/PM still demonstrated synergism, with a CI value of 0.78. In contrast, the free-drug combination (FTP) exhibited strong to moderate synergism at lower concentrations (CI: 0.39–0.58 at 3.125 – 12.5 μg/mL, 72 h). However, at higher concentrations, the interaction shifted towards additivity (CI: 1.08) or even antagonism (CI: 1.08–3.23).

In SW480 cells, both FTP and FT-NP/PM combinations demonstrated strong synergism at the lowest tested concentration (3.125 μg/mL, 72 h), with CI values of 0.29 and 0.33, respectively (Table 2). For the FTP combination, moderate synergism was observed across intermediate concentrations (CI: 0.48–0.89 at 6.25–25 μg/mL, 72 h), whereas antagonistic interactions emerged at higher concentrations (CI: > 1.3 at 50–100 μg/mL, 72 h). In contrast, the FT-NP/PM combination maintained moderate to slight synergism over a broader range of

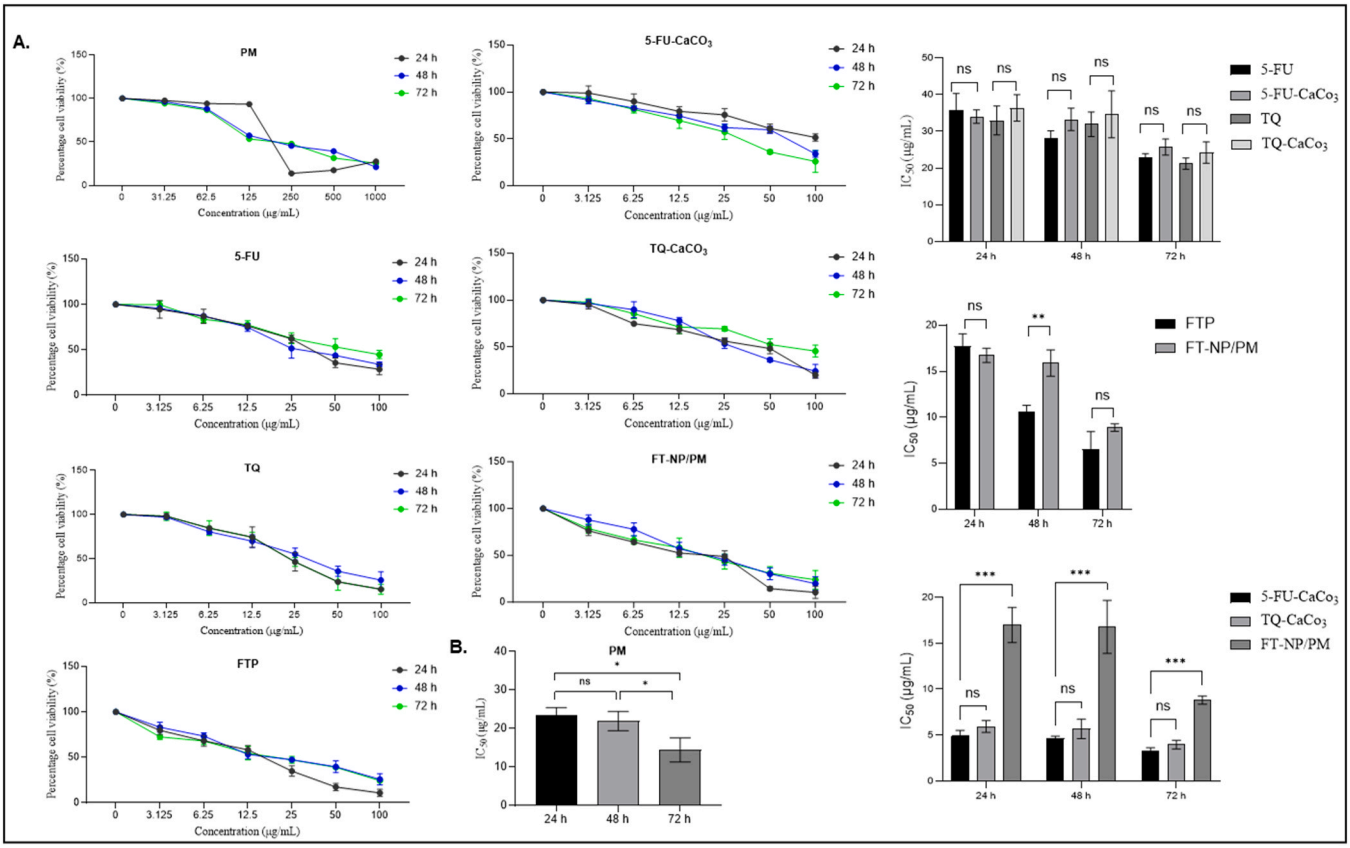

Fig. 2. Cytotoxicity and combination effects of free drug and $CaCO_3$-encapsulated formulations on SW480 colorectal cancer cells. (A) shows a concentration- and time-dependent decrease in cell viability following treatment with PM, 5-FU, TQ, 5-FU-$CaCO_3$, TQ-$CaCO_3$, FTP, and FT-NP/PM; (B) a comparative analysis of the IC_{50} values for PM, 5-FU, TQ, 5-FU-$CaCO_3$, TQ-$CaCO_3$, FTP, and FT-NP/PM over 24, 48, and 72 h. Data are presented as Mean±SD; ns: non-significant ($p > 0.05$); **$p < 0.01$; ***$p < 0.001$ compared to 5-FU. 5-FU: 5-Fluorouracil; TQ: Thymoquinone; PM: *Polygonum minus*; FTP: 5-FU+TQ+PM; FT-NP/PM: 5-FU-$CaCO_3$+TQ-$CaCO_3$+PM.

Table 1
Combination index (CI) values for fixed-ratio combinations of 5-FU, TQ, and PM free drug (FTP) and $CaCO_3$-encapsulated formulations (FT-NP/PM) in SW620 colorectal cancer cells.

Cells	Combine Concentrations (μg/mL)		3.125 μg/mL	6.25 μg/mL	12.5 μg/mL	25 μg/mL	50 μg/mL	100 μg/mL
	Incubation time (h)	Treatment						
SW620	24 h	CI (Free drug, FTP)	0.95793	0.75260	0.53311	0.50836	0.45878	0.49450
	48 h		0.87240	0.50092	0.51218	0.86610	0.99334	1.29270
	72 h		0.38673	0.57766	0.57725	1.07621	1.67596	3.23102
	24 h	CI (Encapsulated, FT-NP/PM)	0.6583	0.2968	0.54452	0.93626	0.72769	0.72405
	48 h		0.5685	0.46822	0.55381	0.39076	0.38407	0.75477
	72 h		0.25469	0.25349	0.31283	0.30404	0.38733	0.78423

Table 2
Combination index (CI) values for fixed-ratio combinations of 5-FU, TQ, and PM free drug (FTP) and $CaCO_3$-encapsulated formulations (FT-NP/PM) in SW480 colorectal cancer cells.

Cells	Combine Concentrations (μg/mL)		3.125 μg/mL	6.25 μg/mL	12.5 μg/mL	25 μg/mL	50 μg/mL	100 μg/mL
	Incubation time (h)	Treatment						
SW480	24 h	CI (Free drug, FTP)	0.55949	0.62462	0.69595	0.78821	0.91865	1.35686
	48 h		0.46993	0.96468	0.74451	0.86552	1.33513	1.61363
	72 h		0.29088	0.48405	0.57373	0.87925	1.29623	1.39873
	24 h	CI (Encapsulated, FT-NP/PM)	0.22843	0.28863	0.40037	0.71306	0.39284	0.60472
	48 h		0.51702	0.5499	0.48793	0.66493	0.78762	1.01486
	72 h		0.32638	0.32638	0.47613	0.54703	0.66936	0.97004

concentrations (CI: 0.33–0.67 at 6.25–50 μg/mL, 72 h), but shifted to additivity at the highest concentration (CI: 0.97 at 100 μg/mL, 72 h).

Combination index (CI) values were determined using CompuSyn software (Version 1.0) based on the Chou-Talalay method. Cells were treated with fixed-ratio combinations of free drug, 5-FU:TQ:PM (1:2:4) (FTP) or (FT-NP/PM) at the concentrations (3.125 – 100 μg/mL) for 24, 48, and 72 h. CI values were interpreted as follows: CI > 1.3, antagonism; CI 1.1–1.3, moderate antagonism; CI 0.9–1.1, additive effect; CI 0.8–0.9, slight synergism; CI 0.4–0.8, synergism; CI 0.2–0.4, strong synergism.

3.3. Analysis of free drug-induced cell death in SW620 and SW480 cells

To evaluate the effects of different drug formulations on SW620 and SW480 cells, cells were stained with Annexin V-FITC and PI and analyzed using flow cytometry. The analysis revealed that the treatments induced varying levels of apoptosis and necrosis over time, depending on the drug type, combination, and encapsulation within $CaCO_3$ nanoparticles. In SW620 cells, free 5-FU induced a moderate pro-apoptotic effect. At 24 h, the percentage of apoptotic cells was relatively low, but a significant increase was detected at 48 h, followed by only a minimal change at 72 h. In contrast, free TQ elicited a more pronounced

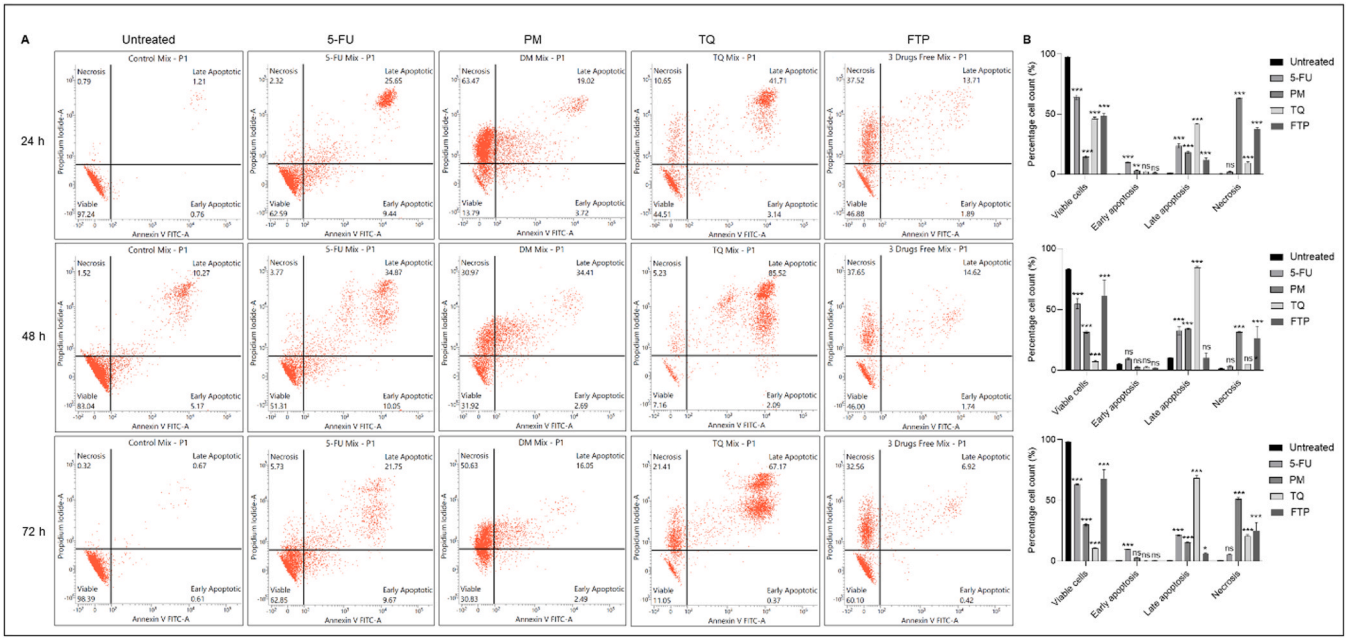

Fig. 3. Flow cytometry analysis of free drug-induced cell death in SW620 cells treated with single agents over time. (A) SW620 cells were treated with 5-FU, PM, TQ, and FTP for 24, 48, and 72 h. The dot plots represent the distribution of viable (Annexin V^-/PI^-), early apoptotic (Annexin V^+/PI^-), late apoptotic (Annexin V^+/PI^+), and necrotic (Annexin V^-/PI^+) cells over time for each treatment; (B) Quantification of percentage cell count at different cell populations. Data are presented as Mean ±SD; ns: non-significant ($p > 0.05$); *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ compared to untreated control. 5-FU: 5-Fluorouracil; TQ: Thymoquinone; PM: *Polygonum minus*; FTP: 5-FU+TQ+PM.

pro-apoptotic response, with statistically significant increases in apoptotic populations at 24, 48 and 72 h compared to untreated controls (Fig. 3). A slight but significant increase in necrotic cells was observed in TQ-treated groups across all time points. PM treatment induced both apoptosis and necrosis, with necrotic cell death being more pronounced in the other single-drug treatments. A significant rise in necrotic cells was observed at 24 h compared with the controls, which became progressively greater at 48 and 72 h. Similarly, PM treatment also induced a significant increase in apoptotic cell population across all time points compared to the control group. The triple combination (FTP: 5-FU+TQ+PM) produced a significant necrotic response in SW620 cells across all time points. At 24 h, necrosis was significantly increase compared to the untreated control, which became more effective at 48 and 72 h. In parallel, apoptosis was also significantly increased across all time points in the FTP-treated group compared to the untreated control.

In SW480 cells, free 5-FU treatment induced a time-dependent increase in apoptosis. At 24 h, the pro-apoptotic cells population were significantly elevated compared to untreated controls, and this effect became more pronounced at 48 and 72 h. Necrotic cell death in the 5-FU-treated group, however, remained low and did not show significant variation across the three time points. In contrast, TQ demonstrated a more potent pro-apoptotic effect. Elevated levels of both early and late apoptosis were evident as early as 24 h, with the response further intensified at 48 and 72 h. A mild but consistent increase in necrotic cell death was also detected in TQ-treated groups, particularly at later stages. Compared to 5-FU, TQ consistently demonstrated stronger apoptotic activity in SW480 cells.

PM treatment in SW480 cells induced a pronounced increase in both apoptosis and necrosis. At 24 h, a moderate but statistically significant increase in apoptotic cells was observed compared with untreated controls, which continued to increase at 48 and 72 h. Necrosis was more prominent in PM-treated cells than in other single-agent groups, with a significant increase detected as early as 24 h, which became more substantial after 72 h (Fig. 5). In contrast, the combination therapy (FTP) produced the strongest cytotoxic effect on SW480 cells. At all-time points, FTP significantly increased the apoptotic cell population, with a sharp increase between 48 and 72 h. Compared with individual treatments, FTP induced the highest levels of apoptosis compared to untreated controls (Fig. 6). A modes but significant increase in necrosis was also detected, particularly at 72 h, although apoptosis remained the dominant mode of cell death.

3.4. Analysis of $CaCO_3$-encapsulated drug-induced cell death in SW620 and SW480 cells

The encapsulation of 5-FU in $CaCO_3$ nanoparticles did not enhance apoptotic activity in SW620 cells compared to free 5-FU. Apoptotic cell populations in the 5-FU-$CaCO_3$ group showed no significant increase over time, but statistical comparison with the untreated control group revealed a significant increase in late apoptotic cell populations across all time points (Fig. 7). In contrast, TQ-$CaCO_3$ induced a significantly higher level of apoptosis compared with free TQ, especially at 72 h, where apoptosis was significantly elevated. A modest rise in necrotic cell death was also detected in the TQ-$CaCO_3$ group, particularly at 72 h. Similarly, the FT-NP/PM treatment induced substantial apoptosis, with significantly higher levels at 48 and 72 h compared to the controls. Necrosis in the FT-NP/PM group showed a slight but consistent increase at 48 and 72 h when compared to the free FTP group.

In SW480 cells, the 5-FU-$CaCO_3$ did not significantly enhanced apoptotic activity compared to its free form. Apoptotic populations in this group remained relatively stable across time points, with no major upward trend. However, when compared to the untreated control, the treatment revealed a statistically significant increase in late apoptotic cells after 48 h, but did not outperform free 5-FU (Fig. 8). Necrotic cell populations in the 5-FU-$CaCO_3$ group were also significantly increased when compared to the untreated control, but remained minimal after 72 h. In contrast, TQ-$CaCO_3$ demonstrated enhanced pro-apoptotic activity in SW480 cells relative to free TQ, with a stronger induction of both early and late apoptosis (Fig. 9). The increase was significant when compared to both the untreated control and free TQ. A slight rise in necrotic cell populations was also detected at later stages, especially at 24 and 72 h. Treatment with the combination formulation FT-NP/PM induced substantial levels of apoptosis with minimal necrosis. Apoptosis was significantly increase at all time points compared to the untreated control, while necrotic cell populations in the FT-NP/PM group were also significantly higher, particularly at 24 and 72 h.

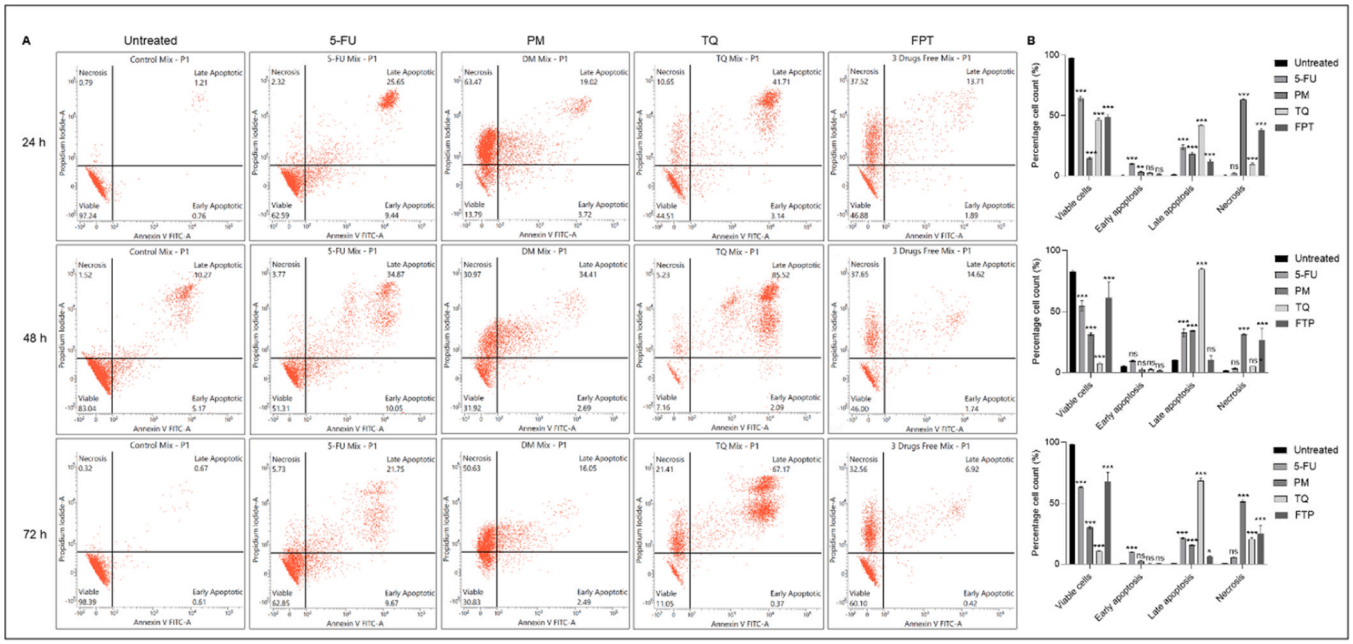

Fig. 4. Flow cytometry analysis of free drug-induced cell death in SW480 cells. (A) Apoptotic and necrotic responses of SW480 cells treated with 5-FU and TQ for 24, 48, and 72 h. (B) Quantification of the percentage cell count at different cell populations. Data are presented as Mean±SD; ns: non-significant ($p > 0.05$); *$p < 0.05$; ***$p < 0.001$ compared to untreated control. 5-FU: 5-Fluorouracil; TQ: Thymoquinone.

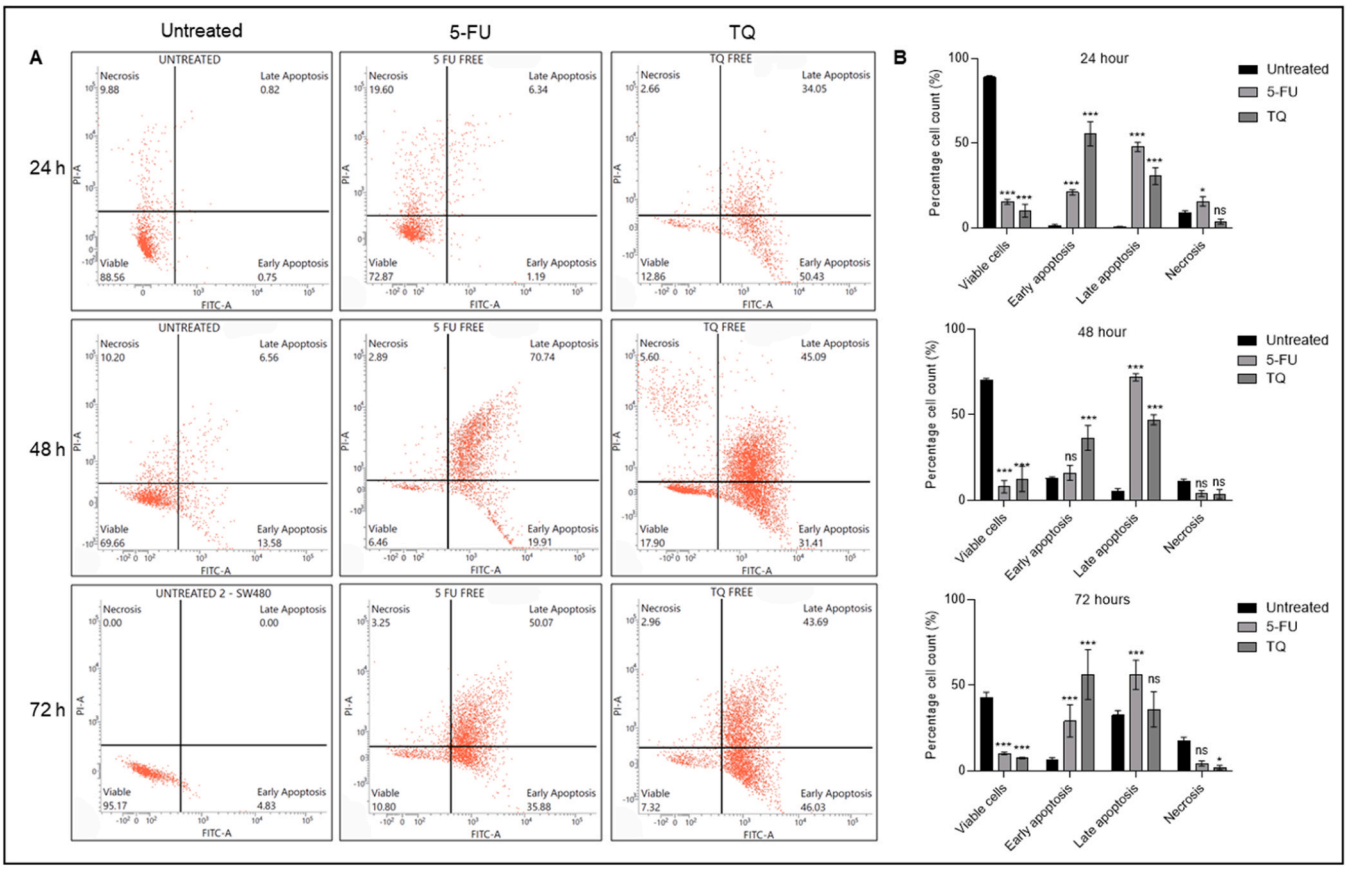

Fig. 5. Flow cytometry analysis of free drug-induced cell death in SW480 cells. (A) Apoptotic and necrotic responses of SW480 cells treated with PM for 24, 48, and 72 h. The dot plots represent the distribution of viable (Annexin V^-/PI^-), early apoptotic (Annexin V^+/PI^-), late apoptotic (Annexin V^+/PI^+), and necrotic (Annexin V^-/PI^+) cells over time for each treatment; (B) Quantification of the percentage cell count at different cell populations. Data are presented as Mean±SD; ns: non-significant ($p > 0.05$); *$p < 0.05$; ***$p < 0.001$ compared to untreated control. PM: *Polygonum minus*.

4. Discussion

Colorectal cancer remains a devastating disease with drug resistance posing a significant challenge to treatment efficacy. While the SW620 cell line, derived from metastatic colorectal cancer, is known for its resistance to chemotherapeutic agents, including 5-FU [26], the SW480 cell line, derived from primary tumor tissue, is relatively more sensitive to treatment. This resistance is largely attributed to enhanced DNA repair mechanisms, overexpression of drug efflux transporters, and alterations in apoptotic signaling pathways [13,23,24]. Previous findings have shown that colorectal cancer cells treated with 5-FU at different time points exhibit resistance to the drug. However, combining 5-FU with other agents enhances its anticancer activity against colon cancer [12,16,19,21,29]. In this study, the cytotoxic effects of 5-FU, TQ, and PM indicate a time-dependent inhibition of SW620 cell proliferation, with the combination therapy (FTP) producing enhanced cytotoxic effects. The significant reduction in IC_{50} values over time suggests that the combinatorial approach may help overcome the intrinsic resistance of SW620 cells, aligning with previous reports that combination therapies can enhance chemosensitivity in resistant colorectal cancer models [2,33]. 5-FU, a widely used chemotherapeutic for colorectal cancer, showed moderate cytotoxicity in SW620 cells, with a minimal decrease in IC_{50} over time.

Interestingly, our findings indicate that $CaCO_3$ encapsulation did not enhance the efficacy of single-drug treatments, except for the FTP combination therapy. Previous studies suggest that $CaCO_3$ encapsulation of 5-FU with other agents, such as TQ, can enhance its anti-colon cancer effects [10,11]. The FT-NP/PM formulation in this study exhibited significantly lower IC_{50} values (11.75 μg/mL) after 24 h compared to the free FTP formulation (17.75 μg/mL). However, the IC_{50} remained the same over time compared to the free FTP formulation. This indicates that while $CaCO_3$ encapsulation may not enhance drug activity across individual compounds, it has a significant impact on the combined formulation, potentially due to synergistic effects or improved intracellular delivery mechanisms. In contrast, SW480 cells demonstrated a much greater sensitivity to 5-FU, with lower IC_{50} values across all time points, which reflect a more favorable therapeutic profile. Similarly, TQ and PM exhibited more pronounced cytotoxic effects in SW480 than in SW620, indicating that the efficacy of these agents is influenced by the intrinsic drug responsiveness of the cell line. While the FTP combination also enhanced cytotoxicity, the degree of improvement was less dramatic than in SW620, likely because the baseline sensitivity to single agents was already high. This indicates that combination therapy is particularly valuable in overcoming resistance rather than simply amplifying already effective responses. Furthermore, $CaCO_3$ encapsulation did not provide substantial additional benefit for single-agent treatments either, due to the already high sensitivity of the cell line.

The combination index (CI) analysis provided insights into the differential responses of SW480 and SW620 cells to the triple-drug formulations. In SW480 cells, both the free-drug (FTP) and encapsulated (FT-NP/PM) combinations exhibited strong synergism at low concentrations (3.125 μg/mL, 72 h), with CI values of 0.29 and 0.33, respectively. These findings suggest that early-stage colorectal carcinoma cells (SW480, derived from the primary tumor) are highly susceptible to combinatorial treatment at sub-cytotoxic doses, a strategy that may allow dose reduction while preserving therapeutic efficacy. Previous studies have shown that drug combinations are particularly effective in

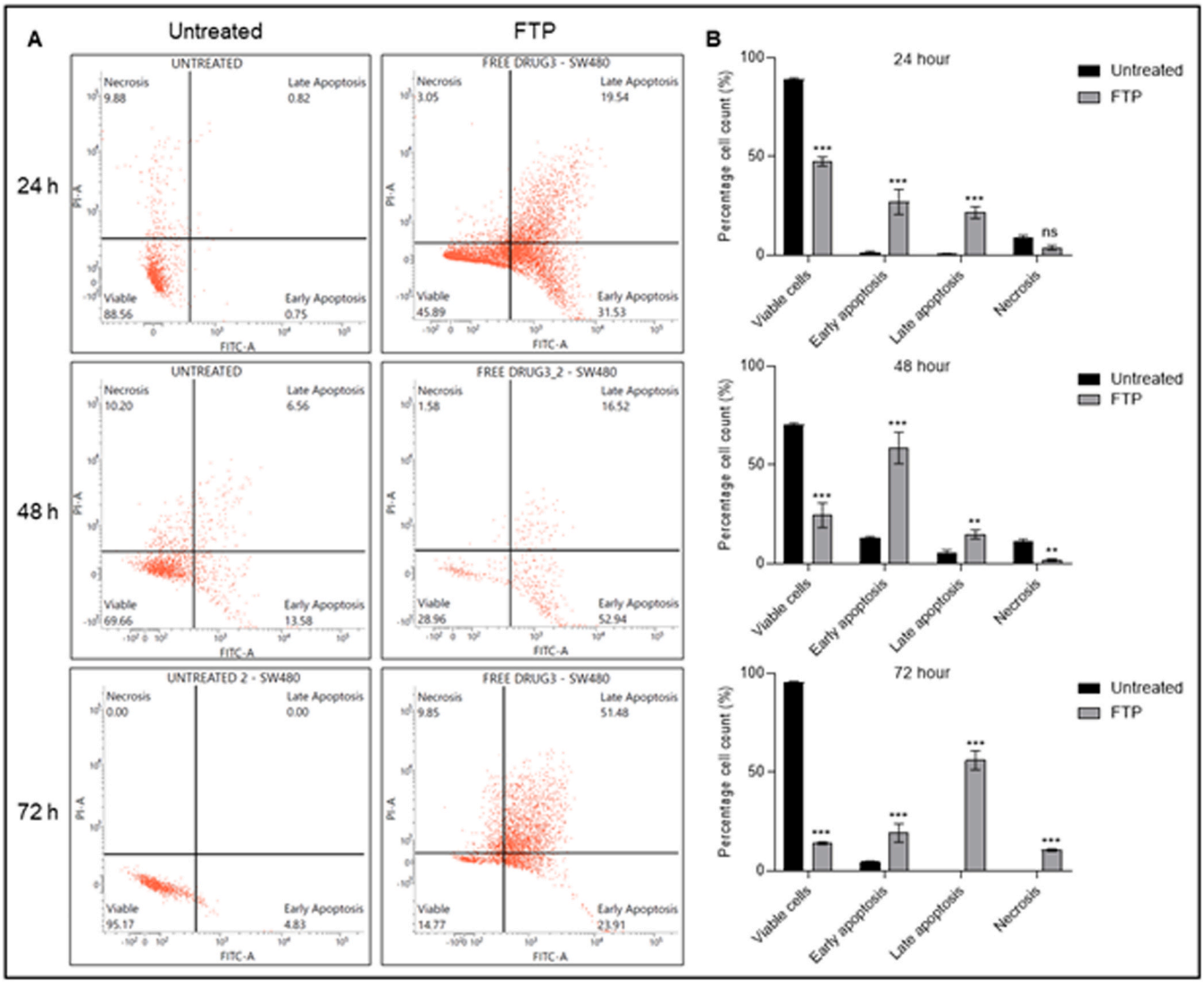

Fig. 6. Flow cytometry analysis of free drug-induced cell death in SW480 cells. (A) Effects of the FTP combination on SW480 cell death treated for 24, 48, and 72 h. The dot plots represent the distribution of viable (Annexin V^-/PI^-), early apoptotic (Annexin V^+/PI^-), late apoptotic (Annexin V^+/PI^+), and necrotic (Annexin V^-/PI^+) cells over time for each treatment; (B) Quantification of the percentage cell count at different cell populations. Data are presented as Mean±SD; ns: non-significant ($p > 0.05$); **$p < 0.01$; ***$p < 0.001$ compared to untreated control. FPT: 5-FU+PM+TQ.

less resistant phenotypes, where synergism at lower doses can overcome single-agent limitations and reduce toxicity profiles ([8]; Bayat [3]).

In contrast, SW620 cells (derived from a metastatic site of the same patient) demonstrated a more resistant profile. Strong synergism was observed only at 72 h with the FT-NP/PM formulation at concentrations below 50 μg/mL (CI: 0.25–0.38), while free-drug combinations shifted toward additive or antagonistic interactions at higher concentrations. This aligns with the intrinsic chemoresistant nature of metastatic colorectal cancer cells, which often exhibit altered drug uptake, enhanced efflux, and adaptive survival pathways [15,22]. The improved synergism seen with the nanoparticle-encapsulated formulations in SW620 cells indicates that $CaCO_3$-based delivery enhanced intracellular accumulation and sustained release of the active compounds, thereby partially overcoming drug resistance mechanisms.

The observed antagonism or additivity at higher concentrations (≥50 μg/mL, 72 h) in both cell lines may reflect saturation of cellular targets, induction of compensatory survival mechanisms, or non-specific cytotoxicity. Such biphasic responses are common in combination chemotherapy, where optimal synergism is dose- and time-dependent [8]. Importantly, the stronger synergism in SW480 compared to SW620 underscores the importance of disease stage and cell phenotype in determining therapeutic outcomes. These findings highlight that rationally designed, low-dose fixed-ratio combinations, particularly when delivered via nanocarriers, can enhance treatment efficacy while minimizing the risk of toxicity, offering translational potential for colorectal cancer therapy.

Flow cytometry analysis of SW620 cells treated with individual and combined formulations of 5-FU, PM, and TQ provides important understanding into their respective cytotoxic effects, primarily through apoptosis and necrosis. SW620 cells are known for their chemoresistant profile, particularly to 5-FU, which is a first-line chemotherapeutic agent for colorectal cancer [20]. In this study, flow cytometry results suggest that while 5-FU alone induces a modest increase in apoptosis, the response is relatively delayed and limited, consistent with previous reports [11]. Necrotic cell death in the 5-FU group remained minimal throughout the treatment period, indicating that 5-FU primarily triggers programmed cell death rather than direct cytotoxicity. TQ, on the other hand, exhibited more pronounced pro-apoptotic activity compared to 5-FU. TQ is known to induce apoptosis via mitochondrial disruption and oxidative stress mechanisms, which may explain its enhanced efficacy

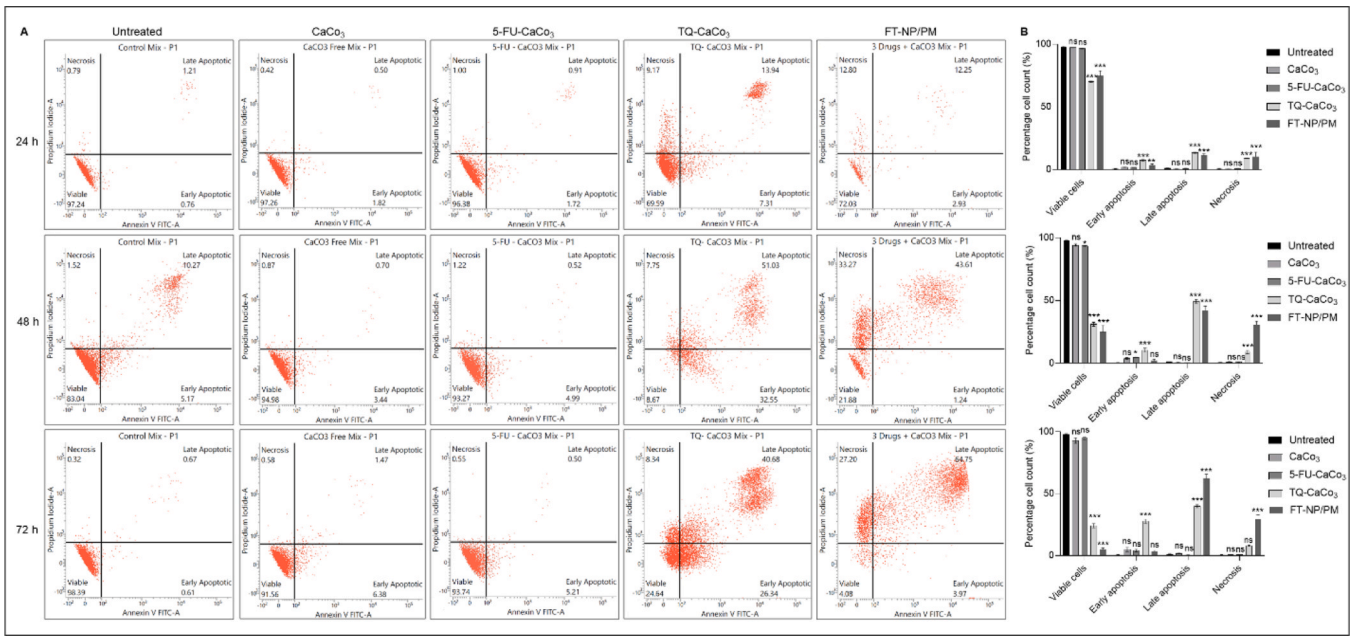

Fig. 7. Flow cytometry analysis of $CaCO_3$-encapsulated drug-induced cell death in SW620 cells. (A) SW620 cells treated with $CaCO_3$, 5-FU-$CaCO_3$, TQ-$CaCO_3$, and FT-NP/PM for 24, 48, and 72 h. The dot plots represent the distribution of viable (Annexin V^-/PI^-), early apoptotic (Annexin V^+/PI^-), late apoptotic (Annexin V^+/PI^+), and necrotic (Annexin V^-/PI^+) cells over time for each treatment; (B) Quantification of percentage cell count at different cell populations. Data are presented as Mean ±SD; ns: non-significant ($p > 0.05$); *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ compared to untreated control. 5-FU: 5-Fluorouracil; TQ: Thymoquinone; FT-NP/PM: 5-FU-$CaCO_3$+TQ-$CaCO_3$+PM.

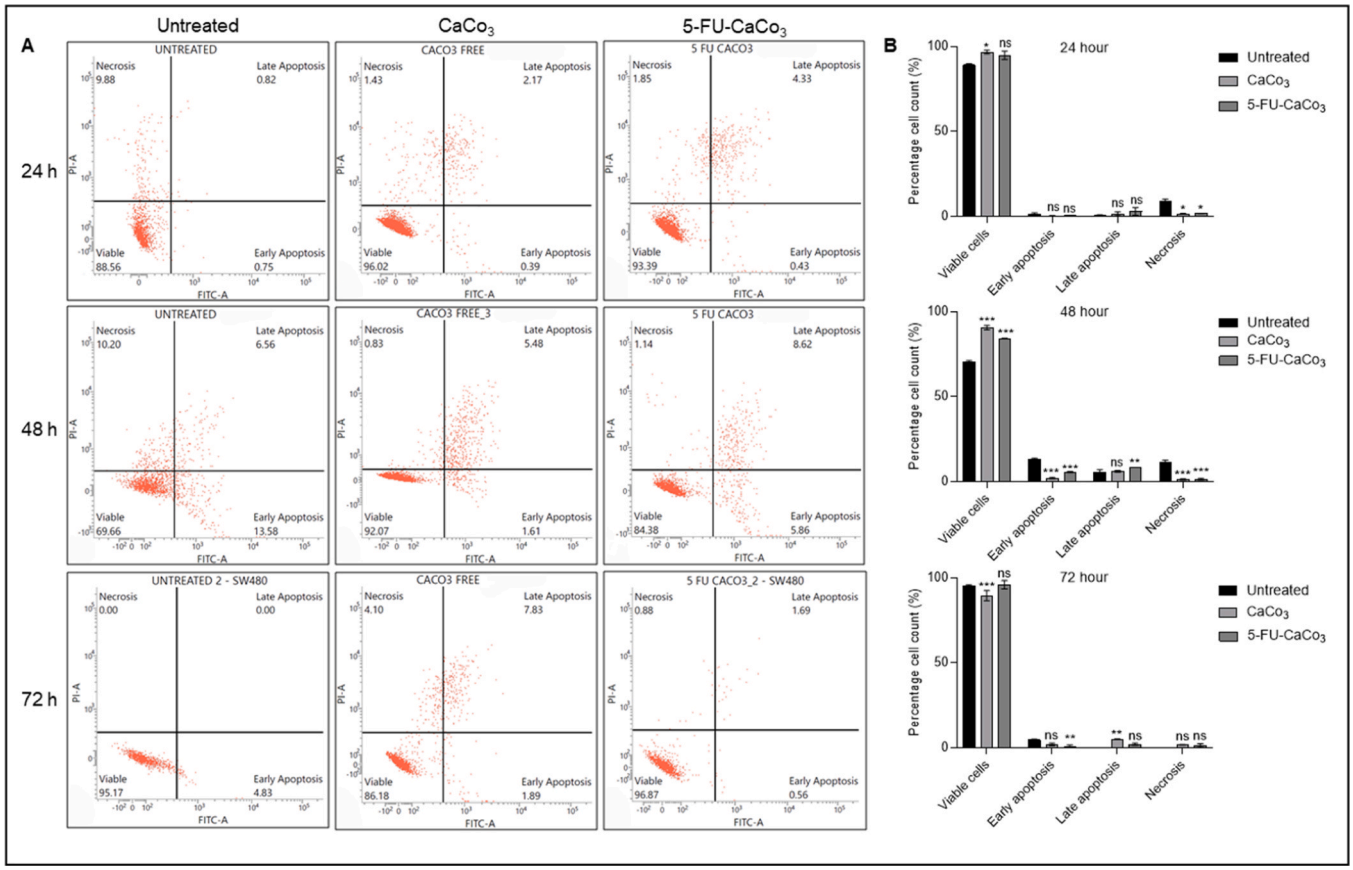

Fig. 8. Flow cytometry analysis of $CaCO_3$-encapsulated 5-FU-induced cell death in SW480 cells. (A) Apoptotic and necrotic responses of SW480 cells treated with $CaCO_3$ and 5-FU-$CaCO_3$ for 24, 48, and 72 h. The dot plots represent the distribution of viable (Annexin V^-/PI^-), early apoptotic (Annexin V^+/PI^-), late apoptotic (Annexin V^+/PI^+), and necrotic (Annexin V^-/PI^+) cells over time for each treatment; (B) Quantification of the percentage cell count at different cell populations. Data are presented as Mean±SD; ns: non-significant ($p > 0.05$); *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ compared to untreated control. 5-FU: 5-Fluorouracil.

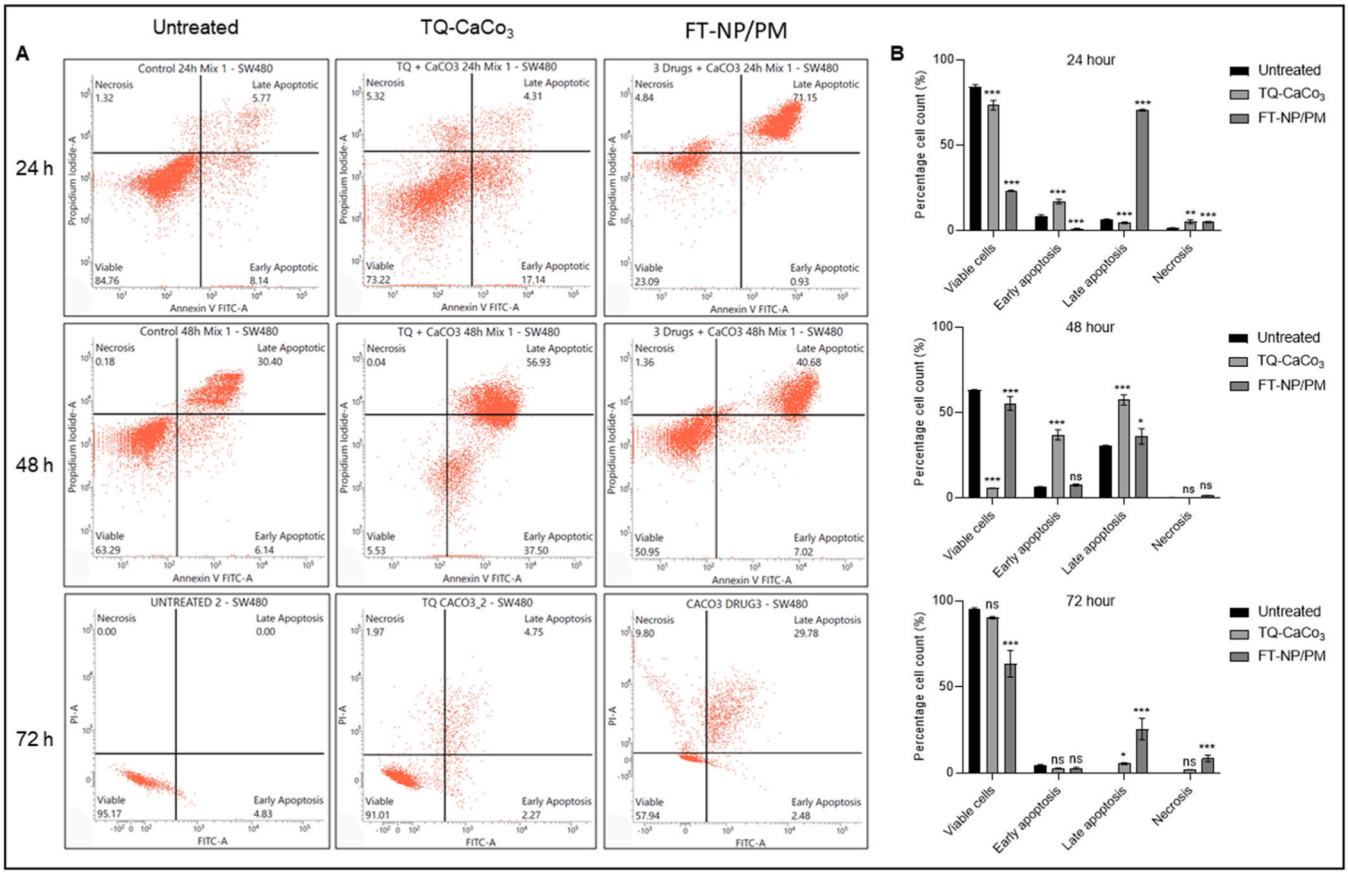

Fig. 9. Effects of the TQ-$CaCO_3$ and FT-NP/PM on SW480 cells. (A) Apoptotic and necrotic responses of SW480 cells treated with TQ-$CaCO_3$ and FT-NP/PM for 24, 48, and 72 h. The dot plots represent the distribution of viable (Annexin V^-/PI^-), early apoptotic (Annexin V^+/PI^-), late apoptotic (Annexin V^+/PI^+), and necrotic (Annexin V^-/PI^+) cells over time for each treatment; (B) Quantification of the percentage cell count at different cell populations. Data are presented as Mean±SD; ns: non-significant ($p > 0.05$); *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ compared to untreated control. TQ: Thymoquinone; FT-NP/PM: 5-FU-$CaCO_3$+TQ-$CaCO_3$+PM.

[2]. A slight increase in necrosis observed in TQ-treated cells at later time points may be attributed to excessive ROS production leading to secondary necrosis. In SW620 cells treated with PM, a higher level of necrotic cell death was observed, in contrast to the predominantly apoptotic responses elicited by 5-FU and TQ. The necrotic effect of PM appeared to be sustained over time, suggesting that PM may exert its cytotoxic effects through membrane-disruptive or pro-inflammatory mechanisms rather than classical apoptotic pathways. Interestingly, encapsulation of 5-FU and TQ in $CaCO_3$ nanoparticles did not enhance apoptotic effects when used as individual drugs, corroborating previous findings [11]. The underlying reason for this observation remains unclear, although it could indicate limited improvements in drug stability or intracellular delivery in these specific formulations. However, the FT-NP/PM group demonstrated higher levels of necrosis compared to the free FTP combination, along with a concomitant increase in apoptosis over time. The synergistic interaction among 5-FU, PM, and TQ, combined with $CaCO_3$-mediated delivery, may contribute to enhanced apoptosis and the inhibition of multiple survival pathways, resulting in greater cytotoxicity against SW620 cells. This triple therapy approach aligns with previous studies indicating improved anticancer effects on colon cancer cells when 5-FU, TQ, and coenzyme Q10 are combined [2]. In contrast, SW480 cells responded more robustly across all treatments. 5-FU, TQ, and PM all induced significant levels of apoptosis, with TQ being the most potent single agent. PM also caused higher levels of necrosis compared to other agents. FTP treatment led to the most extensive apoptotic response, and a combination treatment consisting of 5-FU-$CaCO_3$ and TQ-$CaCO_3$, administered together with PM (FT-NP/PM), helped sustain and slightly enhance this effect, particularly after 24 h. Unlike in SW620, where $CaCO_3$ delivery seemed critical for optimizing treatment efficacy, in SW480, the benefit was more in maintaining cytotoxicity over time rather than overcoming resistance.

In this study, only 5-FU and TQ were encapsulated within $CaCO_3$ nanoparticles, while PM was administered in free form. This approach reflects the technical challenges of encapsulating crude extracts with multiple phytoconstituents, yet it still enabled us to demonstrate clear synergistic interactions between the agents. Similar findings have been reported in other drug-phytochemical combinations, where herbal extracts potentiated the efficacy of chemotherapeutics despite differences in formulation [4,7]. Nevertheless, future studies should investigate co-encapsulation or controlled delivery strategies for all three agents to improve co-localization, bioavailability, and stability, thereby maximizing therapeutic outcomes.

While the present study demonstrates clear synergistic effects of 5-FU, TQ, and PM in both free and $CaCO_3$ nanoparticles-encapsulated formulations, the findings are limited to in vitro models. The absence of mechanistic assays such as caspase activation, Bcl-2 family regulation, or ROS generation, as well as the lack of in vivo validation, restricts the translational scope. Future investigations will therefore focus on elucidating the molecular basis of the observed synergy, including apoptosis-related pathways (caspase-3/9 activation, Bax/Bcl-2 modulation), oxidative stress responses (ROS accumulation, Nrf2 signaling), and survival signaling cascades (PI3K/Akt, NF-KB, and p53). Validation of these findings in animal models will further clarify how the combined treatment overcomes resistance and enhances therapeutic efficacy in colorectal cancer.

5. Conclusion

In summary, this study demonstrates that while the cytotoxicity profiles of single free agents were comparable to their $CaCO_3$-encapsulated counterparts, the combination therapies, particularly FTP and FT-NP/PM, yielded significantly greater cytotoxicity effects than single agents alone. Importantly, FT-NP/PM displayed superior combination index values compared to FTP in both SW480 and SW620 cells, indicating a stronger synergistic interaction. Mechanistically, FT-NP/PM treatment induced higher levels of necrosis alongside a time-dependent increase in apoptosis, suggesting that the synergistic interplay among 5-FU, TQ, and *Polygonum minus*, facilitated by $CaCO_3$-mediated delivery, enhances apoptotic signaling and disrupts survival pathways, especially in the more resistant SW620 line. While FTP treatment elicited the most extensive apoptosis response, FT-NP/PM sustained and slightly improved this effect, particularly at early time points (24 h). Notably, the $CaCO_3$ delivery system was more critical in overcoming resistance in SW620 cells, whereas in SW480 cells, its role was primarily to maintain cytotoxic efficacy over time. Collectively, these findings highlight the therapeutic potential of $CaCO_3$-based co-delivery of phytochemicals and chemotherapeutics as a promising strategy for colorectal cancer treatment.

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CRediT authorship contribution statement

Ahmad Faizal Abdull Razis: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition. **Hamidu Ahmed:** Writing – review & editing, Formal analysis, Data curation, Conceptualization. **Md Zuki Abu Bakar:** Writing – review & editing, Visualization, Validation, Supervision, Project administration, Formal analysis. **Kim Wei Chan:** Writing – review & editing, Visualization, Supervision, Data curation, Conceptualization. **Mohammed Alsubbi:** Resources, Data curation, Conceptualization. **Noorjahan Banu Mohammed Alitheen:** Writing – review & editing, Supervision, Data curation, Conceptualization. **Ibrahim Malami:** Writing – review & editing, Data curation, Conceptualization. **Kshidan Marim Abdullah Salem:** Writing – review & editing, Writing – original draft, Validation, Formal analysis, Data curation, Conceptualization. **Norsharina Ismail:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships, which may be considered as potential competing interests: Norsharina Ismail reports that financial support was provided by Universiti Putra Malaysia. Reports a relationship with that includes:. Has a patent pending to. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

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Data availability

Data will be made available on request.

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