

## Cytotoxic Effect of the Combination of Gemcitabine and Atorvastatin Loaded in Microemulsion on the HCT116 Colon Cancer Cells

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### ABSTRACT

The combination of anticancer drugs in nanoparticles has great potential as a promising strategy to maximize efficacies by eradicating resistant, reduce the dosage of the drug and minimize toxicities on the normal cells. Gemcitabine (GEM), a nucleoside analogue, and atorvastatin (ATV), a cholesterol lowering agent, have shown anticancer effect with some limitations. The objective of this *in vitro* study was to evaluate the antitumor activity of the combination therapy of GEM and ATV encapsulated in a microemulsion (ME) formulation in the HCT116 colon cancer cells. The cytotoxicity and efficacy of the formulation were assessed by the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The mechanism of cell death was examined by observing the morphological changes of treated cells under light microscope, identifying apoptosis by using the ApopNexin apoptosis detection kit, and viewing the morphological changes in the chromatin structure stained with 4',6-diamidino-2-phenylindole (DAPI) under the inverted fluorescence microscope. It has been found that reducing the concentration of GEM loaded on ME (GEM-ME) from 5  $\mu$ M to 1.67  $\mu$ M by combining it with 3.33  $\mu$ M of ATV in a ME formulation (GEM/2ATV-ME) has preserved the strong cytotoxicity of GEM-ME against HCT116 cells. The current study proved that formulating GEM with ATV in ME has improved the therapeutic potential of GEM and ATV as anticancer drugs.

**Keywords:** Apoptosis, Nanoparticles, MTT assay, ApopNexin apoptosis detection kit, DAPI assay.

### INTRODUCTION

Colon cancer is the abnormal growth of the large intestine cells, which can be originated from both inherited or somatic gene mutations that continue over the course of a lifetime<sup>1</sup>, while colorectal cancer (CRC) results from an accumulation of genetic and epigenetic alterations in colon epithelial cells, which transmute them into adenocarcinomas<sup>2</sup>. CRC has been ranked the first among the male population and the third among the female population. It has affected 52.4% of males and 47.6% of females with the median age of 60 and 55 years, respectively, in Saudi Arabia<sup>3</sup>.

Gemcitabine (GEM), commonly used in the clinic as an anticancer drug, is an antimetabolite that induces apoptosis and inhibits DNA synthesis. GEM, the first line chemotherapeutic drug for pancreatic cancer, has been validated to exhibit anticancer activity against a wide variety of cancers, including colon, lung, breast, bladder and ovarian cancer<sup>4,5</sup>. However, GEM has severe side effects when injected intravenously because it metabolizes rapidly in the blood and thus, has a very short plasma half-life. Following intracellular transport, GEM is phosphorylated by deoxycytidine kinase to GEM diphosphate and GEM triphosphate. Both active forms inhibit processes required for DNA synthesis<sup>6</sup>.

A combination of agents for cancer chemoprevention has great potential as a promising strategy to improve efficacies by eradicating resistant variants or to reduce the

dosage of the drug and minimize toxicities<sup>7</sup>. Combinations of low doses of antitumor agents that have different synergistic effects may have considerably ameliorated efficacy for impeding tumor growth when compared with the single treatments of the anticancer drugs.

Statins, cholesterol-lowering drugs, are one of the most commonly prescribed drugs worldwide to treat hypercholesterolemia. They have a potent inhibitor of 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase, which catalyzes the conversion of HMG-CoA to mevalonate, and thus reduce the levels of mevalonate and its downstream products<sup>8</sup>. These products have been shown to deter cancer cell growth and stimulate apoptosis. Atorvastatin (ATV) calcium, commercially called Lipitor, the best-selling pharmaceutical in history, is a lipid-lowering agent that belongs to the statin group with a high molecular weight (1209.4 g/mol). It displayed an antitumor effect on various human cancer cell lines, T24 and J82 bladder, Huh7 hepatoma and HCT116 colon<sup>9,10,11</sup>. The design and development of a new drug delivery system, which are the methods or processes of administering a pharmaceutical compound to accomplish a therapeutic effect, intended to treat a disease<sup>12</sup>. They are promising approaches to overcome the complications of the traditional therapy and major obstacles associated with drug development like poor water solubility, low bioavailability and drug toxicity. Microemulsions (MEs)

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are excellent candidates as a promising drug delivery system with practical applications for pharmaceuticals<sup>13</sup>. ME, a system of water, oil and surfactant, often in combination with cosurfactant, is a single optically isotropic and thermodynamic stable liquid solution with a droplet size range 1-100 nm. ME improves the cytotoxic effect with respect to the free drug, implying a better effective drug uptake inside the cells. In other words, the anticancer drug accumulation in the cancerous cells would get enhanced besides reducing its side effect on the other cells<sup>14,15,16</sup>. The objective of this *in vitro* study was to examine the antitumor activity of the combination therapy of GEM and ATV, encapsulated in a ME formulation designed by Tsai *et al.*<sup>17</sup>, in HCT116 human colon cancer cells.

## MATERIALS AND METHODS

### Materials and subjects

ATV calcium was obtained from Jamjoom Pharma (Jeddah, KSA). GEM hydrochloride, Span 20, Tween 80, Isopropyl myristate (IPM), sodium phosphate dibasic anhydrous, an antibiotic solution of 10,000 (U/ml) penicillin/streptomycin (10 mg/ml) were obtained from Sigma (Missouri, US). Coomassie brilliant blue was obtained from biomatik (Ontario, Canada). Ethanol and formaldehyde were purchased from Fisher Chemical (UK). Dulbeccos modified eagle medium (DMEM), heat inactivated fetal bovine serum (FBS), and phosphate buffered saline (PBS, pH7, 10mM), trypan blue (0.4%), 4',6-diamidino-2-phenylindole (DAPI) dihydrochloride were purchased from Invitrogen life technologies (New York, US). Trypsin was obtained from HyClone (Utah, US). Dialysis membrane (molecular weight cut-off size 12,000-14,000 Da) was obtained from spectra lab (California, US). The 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was obtained from Cayman's chemical (Michigan, US). ApopNexin™ FITC Apoptosis Detection Kit (Lot. No. 2053919) was procured from Millipore (Massachusetts, US). The human cell line, HCT116, was obtained from America Type Tissue Culture Collection (Manassas, VA, USA).

### Preparation of the ME formulations

ME formulas were produced as described by Tsai *et al.*<sup>17</sup>. In brief, a 40% (v/v) of surfactant mixture, consisted of 3:2 ratio of Tween 80 to Span 20, respectively, was mixed with 50% (v/v) IPM. The remaining 10% of the aqueous phase containing 40% ethanol was added to the mixture dropwise. The resulted ME formula (Blank-ME) was vortexed until it becomes clear and transparent. A 1 mg/ml of drug formulations loaded in ME were GEM-loaded-ME (GEM-ME), ATV-loaded-ME (ATV-ME), the combination of GEM and ATV loaded-MEs mixed at different ratios of 1:1 (GEM/ATV-ME), 1:2 (GEM/2ATV-ME) and 2:1 (2GEM/ATV-ME). Similarly, 1mg/ml of the entire drug formulations were produced by dissolving the drug in water instead of ME and were designated as GEM, ATV, GEM/ATV, GEM/2ATV and 2GEM/ATV. The micromolar concentrations of the drug formulas, loaded in either water or ME, were prepared as illustrated in Table 1.

### Cell culture

The HCT116 cell line, cultivated in a 25cm<sup>2</sup> cell culture flask containing DMEM supplemented with 10% (v/v) FBS and 1% (v/v) penicillin-streptomycin, was incubated in a 5%CO<sub>2</sub>/95% humidified atmosphere at 37°C. In the meantime, the medium was discarded from the cell culture flask and changed at 48 h intervals. Cells were fed until confluence and confluent cells were washed with PBS, detached by adding 2ml of trypsin, and incubated at 37 °C.

### MTT assay for cytotoxicity screening

The toxicity of the chemotherapeutic agents against the cancerous cells is evaluated by the MTT assay. A 100 µl of culture media containing 5,000 cells, counted using a countess automated cell counter (Invitrogen, US), was seeded in each well of a 96-well plate and was incubated overnight at 37°C in a CO<sub>2</sub> incubator for cell attachment. Then, cells were treated with 100µl of 1&5µM of different ME formulations and solutions and were incubated for 48 h at 37°C in a CO<sub>2</sub> incubator. After that, a 5 µl of MTT reagent was added to each well, mixed gently for one minute and incubated for 4h at 37°C in a CO<sub>2</sub> incubator. Then, the culture media containing MTT reagent were removed followed by adding a 100 µl of crystal dissolving solution. The absorbance (Abs) was read at 540 nm using a microplate reader (BioTek, US). Wells, included culture media, were considered negative control while culture media containing cells served as a positive control. The percentages of cell viabilities were determined by the following equation:

Cell viability (%)

$$= \frac{(\text{Abs of treated cells} - \text{Abs of negative control})}{\text{Abs of positive control}} \times 100$$

### Characterization of cell morphology using inverted microscope

In order to assess the morphological changes of the treated HCT116 cells, the phase contrast inverted microscope (Olympus 1X51, Japan) was utilized as described by Alkhatib and Alkhayyal<sup>14</sup>. Cells were counted and plated at a density of 5,000 cells per well into 24-well containing 500 µl of growth media in each well. Then, they were incubated with 500 µl of 5 µM of the desired drug formula for 24 and 48h. Cells were washed twice with 300 µl of PBS and fixed by the addition of 200 µl of 4 % formaldehyde for 5 min. After that, the fixation solution was discarded and the cells were washed with PBS and stained with 200 µl of 10 % Coomassie blue for 10 min. Then, the stain solution was discarded, washed with distilled water twice and left to dry at room temperature, 25°C.

### ApopNexin™ FITC apoptosis detection assay

Apoptosis was analyzed by flow cytometry using ApopNexin™ FITC Apoptosis Detection Kit. This kit uses a double staining protocol in which the apoptotic cells are stained with annexin V conjugated with fluorescein isothiocyanate (FITC, green fluorescence) which stains phosphatidylserine (PS) that is normally located in the inner surface of the cell membrane of viable cells but it would get translocated into the outer membrane surface when the cell undergo apoptosis. The necrotic cells are stained with propidium iodide (PI, red fluorescence) which

Table 1: The micromolar concentration of the drug formulations, loaded in either water or microemulsion (ME) (gemcitabine (GEM), atorvastatin (ATV) and combination of GEM and ATV (GEM/ATV, GEM/2ATV and 2GEM/ATV)).

Concentration of the drug formula ( $\mu\text{M}$ )	Micromolar concentration of the components of the drug formulation				
	GEM	ATV	GEM/ATV	GEM/2ATV	2GEM/ATV
1	1	1	0.5: 0.5	0.33:0.66	0.66:0.33
5	5	5	2.5:2.5	1.6:3.4	3.4:1.6

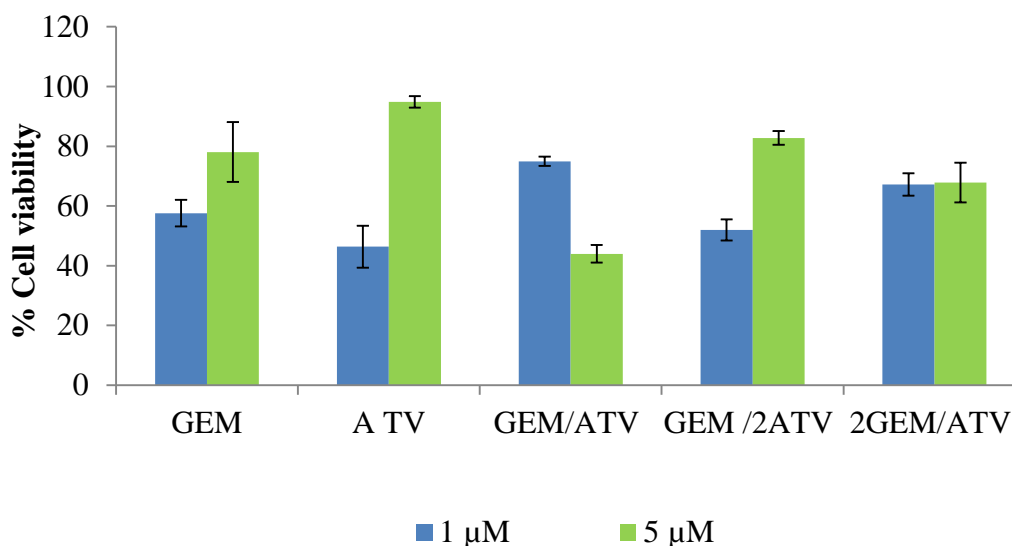


Figure 1: Percentages of cell viability of 1 & 5  $\mu\text{M}$  of the solution formulation subjected into HCT 116 cells for 48h. Error bars represent the standard deviation.

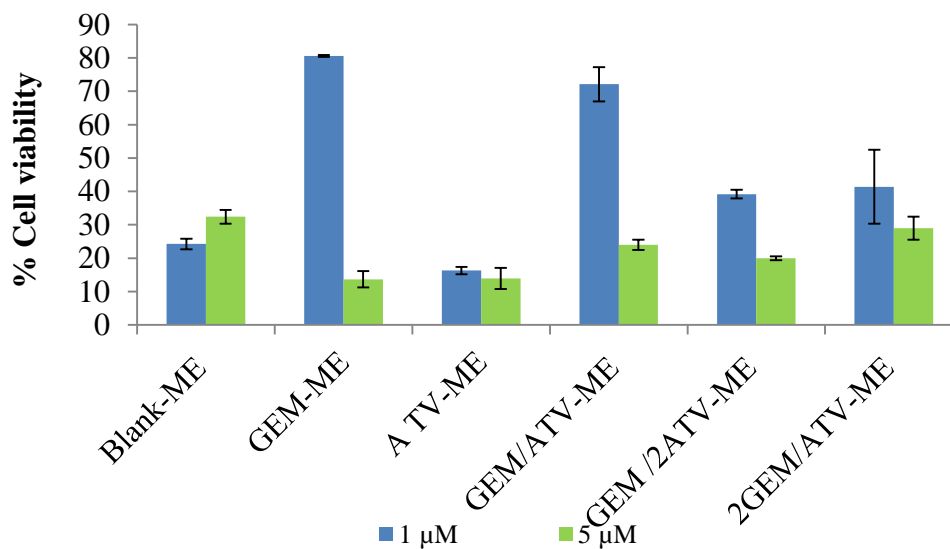


Figure 2: Percentages of cell viability of 1 & 5  $\mu\text{M}$  of ME formulations subjected into HCT 116 cells for 48h. Error bars represent the standard deviation.

binds to the DNA of the lysed cells.

Cells, plated at a density of  $5 \times 10^4$  cells per well into 24-well plate containing 500  $\mu\text{l}$  of culture media, were treated with 500  $\mu\text{l}$  of 5  $\mu\text{M}$  of the drug ME and solution formulas and were incubated for 24 and 48 h. They were detached, harvested by centrifugation at 400X g for 5 min and washed twice with ice cold PBS. Detached cells were re-suspended in 200  $\mu\text{l}$  of 1X binding buffer (10 mM 4-(2-

hydroxyethyl)-1-piperazine ethane sulfonic acid buffer solution /NaOH, pH 7.4, 140 mM NaCl, 2.5 mM  $\text{CaCl}_2$ ) at a concentration of  $1 \times 10^6$  cells/ml. After that, the mixture was incubated with 3  $\mu\text{l}$  of FITC and 2  $\mu\text{l}$  of PI for 15 min at room temperature in the dark. All cells were evaluated by a BD FACS Aria<sup>TM</sup> III Flow Cytometer (BD Biosciences, US). Data, analyzed using FACSDiva software version 6.1.3. The positive FITC indicates the

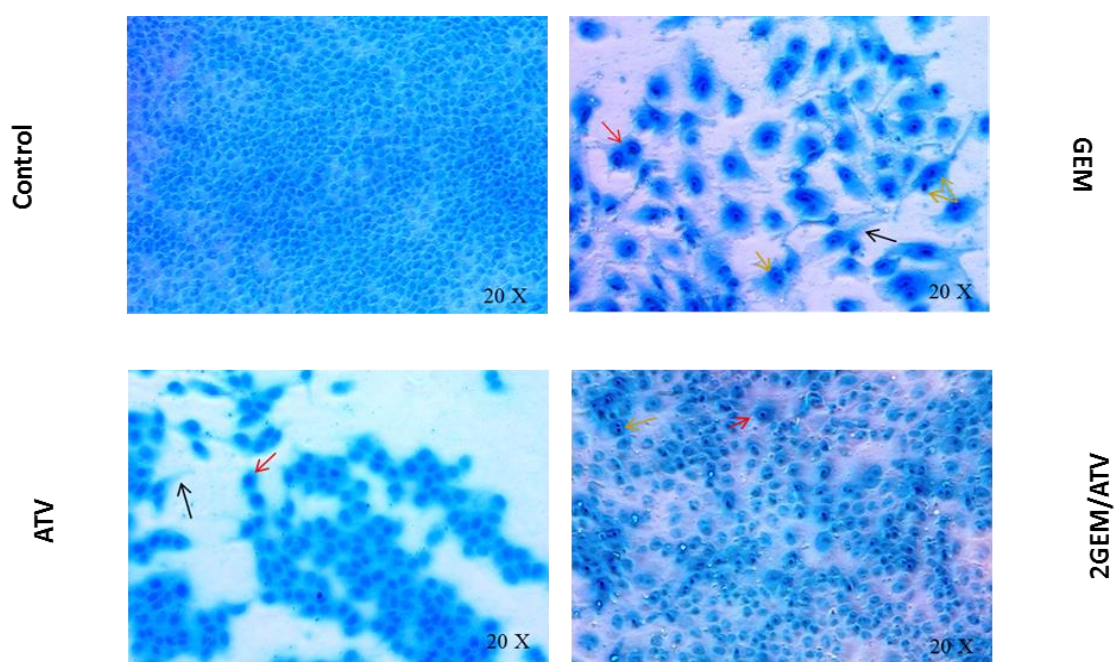


Figure 3: Light microscopy images of HCT 116 colon cancer cells treated with 5  $\mu$ M of the solution formulation for 48h. Black arrows represent membrane blebbing and gold arrows represent nuclear fragmentation while red arrows represent chromatin condensation.

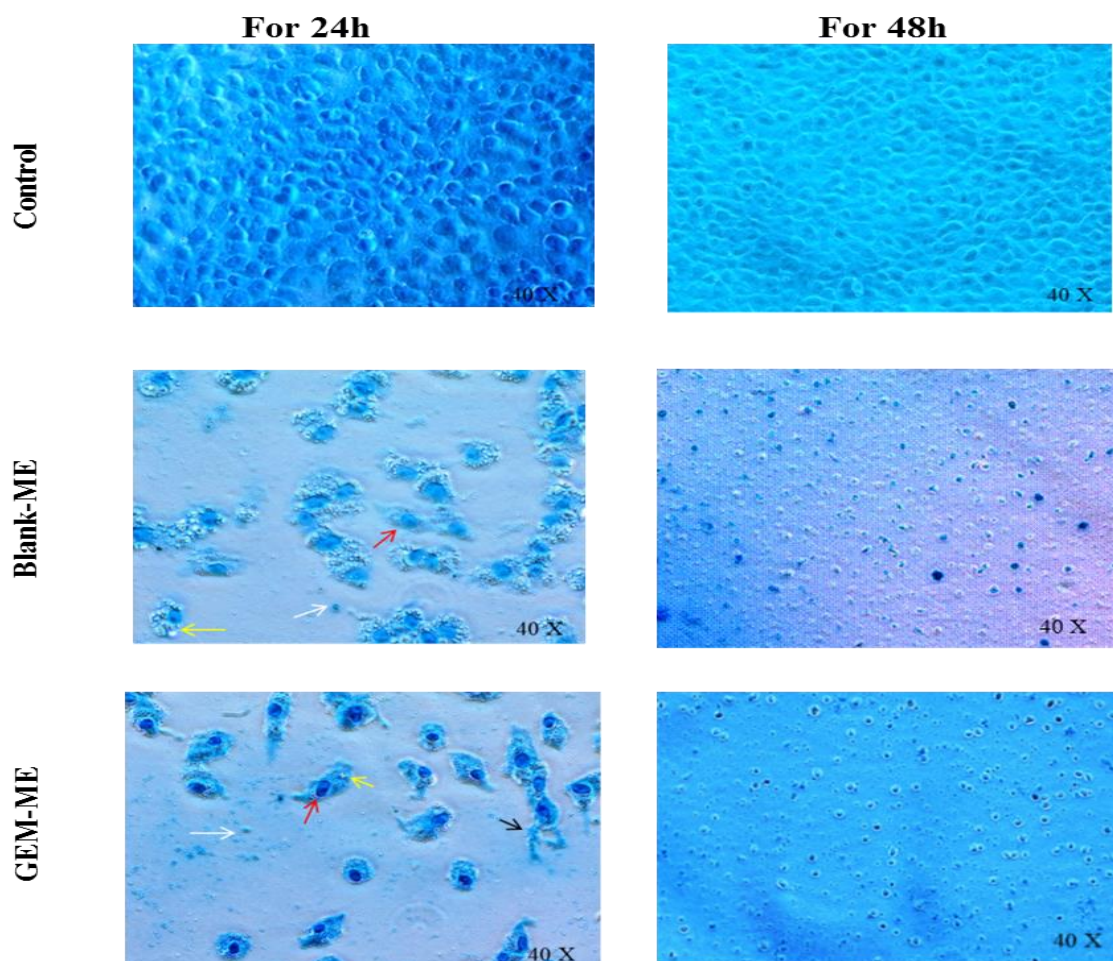


Figure 4: Light microscopy images of HCT 116 colon cancer cells treated with 5  $\mu$ M of ME formulations for 24 and 48h. Signs of apoptosis are represented by the black arrows (membrane blebbing), red arrows (chromatin condensation), white arrows (apoptotic bodies) and yellow arrows (vacuoles).



release of PS, which happens in the early stage of apoptosis and the positive of PI indicates lysed cells.

#### *Nuclear DNA staining for apoptosis detection*

The nuclear condensation of the treated cells undergoing apoptosis was detected using the DAPI stain. Cells were counted and plated at a density of  $5 \times 10^4$  cells per 500  $\mu$ l of growth media into each well of 24 well-plate. Cells, treated with 500  $\mu$ l of 5  $\mu$ M of the ME and solution formulas, were incubated for 24 and 48h. Then, cells were equilibrated with 300  $\mu$ l of PBS and fixed by the addition of 200  $\mu$ l of 4 % formaldehyde for 5min. After that the cells were stained with 300  $\mu$ l of 300 nM of DAPI solution and incubated for 1-5 min at 25°C. The stain solution was discarded and cells were observed by the inverted

fluorescent microscope with appropriated filter at 461nm (Leica CRT6000, Germany).

#### *Statistical analysis*

Data were expressed as mean  $\pm$  standard deviation ( $\bar{X} \pm SD$ ) as each experiment was performed in triplicate. Statistical analyses were performed with one-factor analysis of variance (ANOVA) and two-factor ANOVA tests using MegaStat (version 10.3, Butler University). The statistical significance difference was considered when  $p$ -value  $< 0.05$ .

## RESULTS

#### *MTT assay for cytotoxicity screening*

The MTT cell proliferation assay was used to determine the viability of the HCT116 cells when treated with various

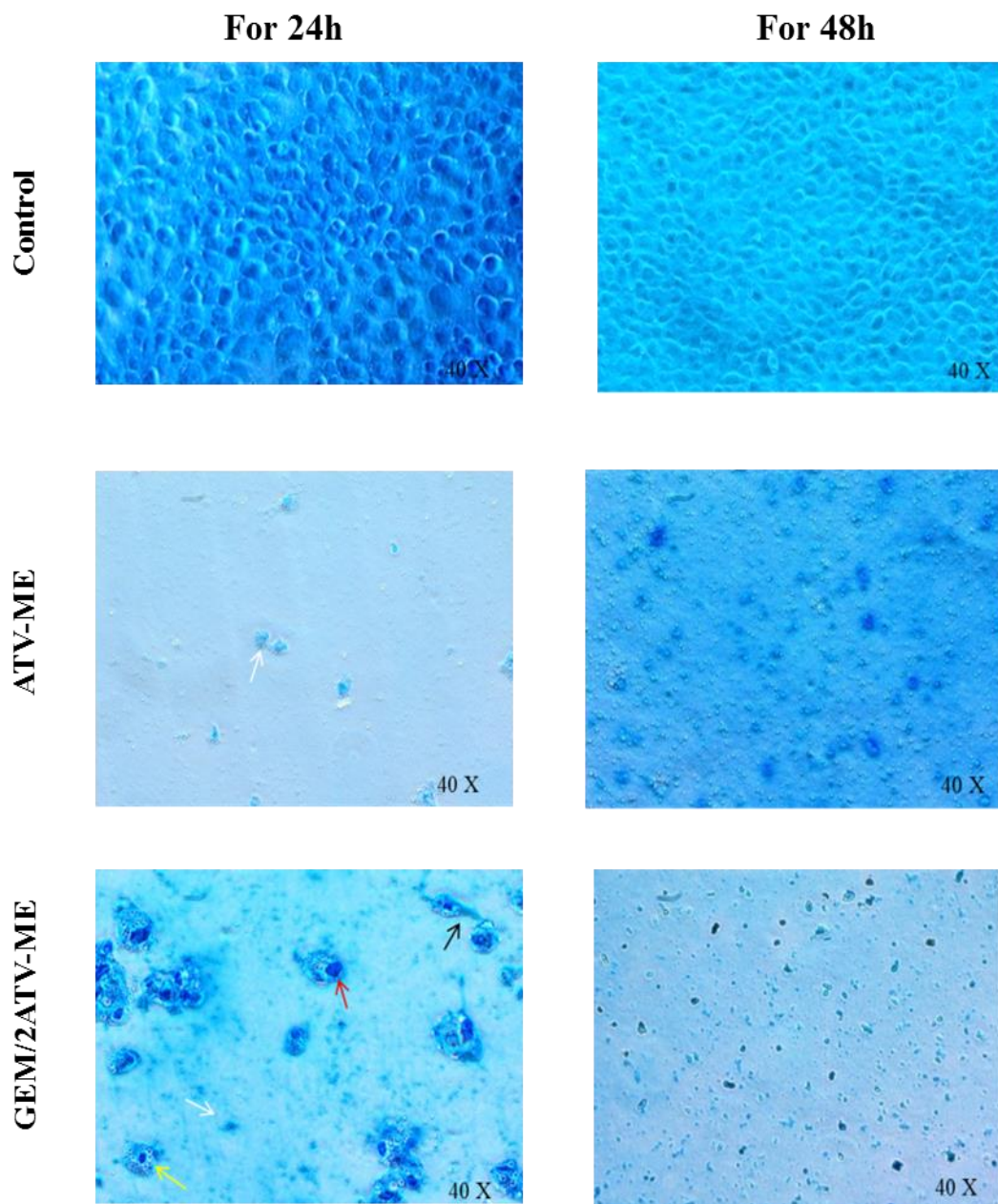


Figure 5: Light microscopy images of HCT 116 colon cancer cells treated with 5 $\mu$ M of ME formulations for 24 and 48h. Signs of apoptosis are represented by the black arrows (membrane blebbing), red arrows (chromatin condensation), white arrows (apoptotic bodies) and yellow arrows (vacuoles).

solutions and ME formulations at different concentrations as demonstrated in Figure 1. When HCT116 cells were treated with solution formulations, the greatest inhibition was observed for the 1  $\mu\text{M}$  of GEM, ATV and GEM/2ATV. However, when their concentrations have increased to 5  $\mu\text{M}$ , they become more carcinogenic as their toxicity have decreased significantly. The other combination formulas have different behavior as the toxicity of GEM/ATV has increased when the concentration increased while the effect of 2GEM/ATV was not sensitive to concentration.

The effect of the ME formulations on the proliferation of the cancerous cells, HCT116, is shown in Figure 2. At 1  $\mu\text{M}$ , the most effective inhibition of the ME formulas on the HCT116 was recorded for ATV-ME followed by Blank-ME while both of GEM-ME and GEM/ATV-ME were having the least cytotoxic effect. In contrast, all of

the ME formulations at 5  $\mu\text{M}$  were having a similar inhibition effect. As a consequence, the selected formulas to make further studies was 5  $\mu\text{M}$  of GEM/2ATV-ME since it has the highest anti-proliferative effect on HCT116 cells.

#### *Characterization of cell morphology using inverted microscope*

The signs of apoptosis that would be observed under the inverted microscope include shrinkage of the cells, membrane blebbing, chromatid condensation and formation of apoptotic bodies. HCT116 cells subjected into the water formulations were mostly affected by GEM as shown in Figure 3. Cells have got enlarged, the chromatins have got condensed and fragmented, and the membrane of the cell has got bulged. Also, the number of cells has got decreased. Incubating the cells with ATV resulted in the enhancement of the nucleus size and decreasing the number of the cells. In contrast, cells treated

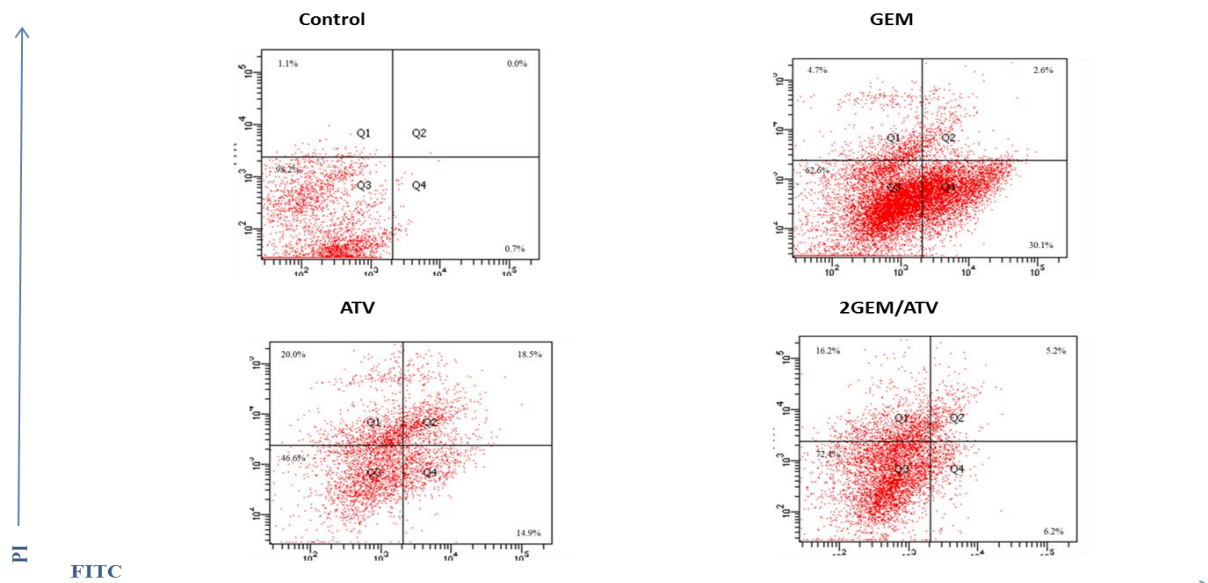


Figure 6: The FITC/PI flow cytometry plots of 5  $\mu\text{M}$  of solution formulations subjected into HCT 116 colon cancer cells for 48h.

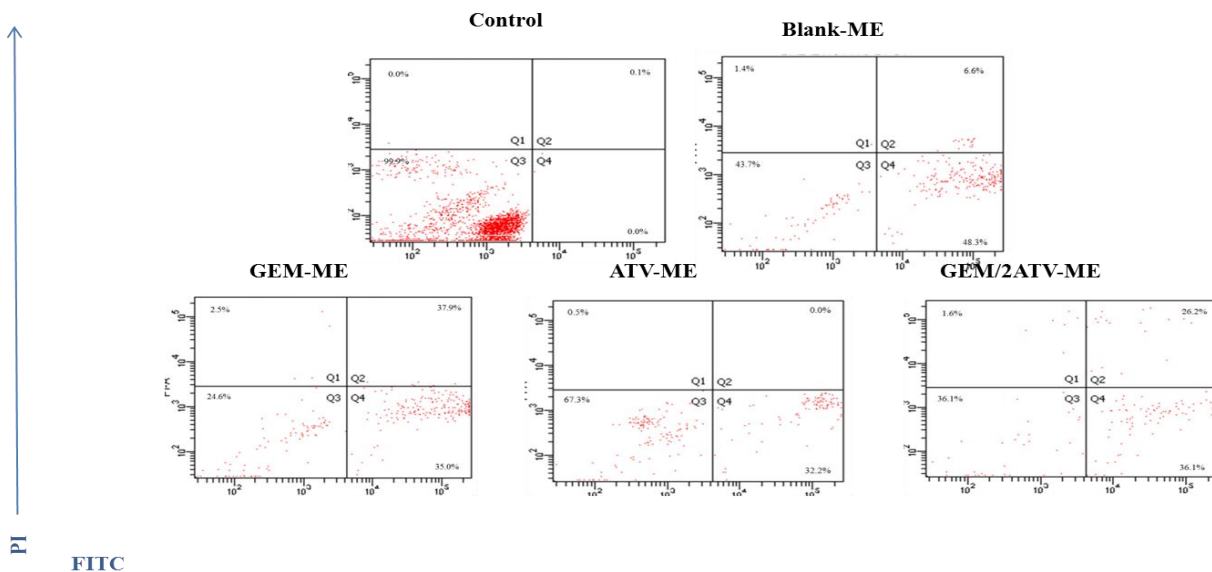


Figure 7: The FITC/PI flow cytometry plots of 5  $\mu\text{M}$  of ME formulations subjected into HCT 116 colon cancer cells for 24h.

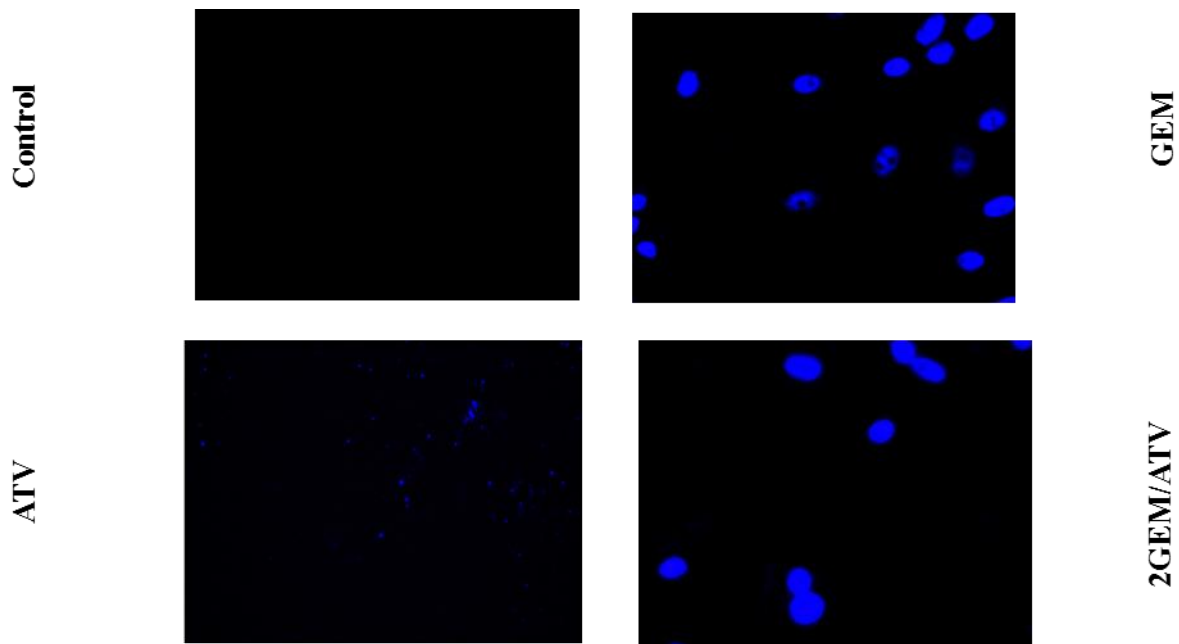


Figure 8: Fluorescence microscopy images of HCT 116 cells stained with DAPI after treated with 5  $\mu$ M of the solution formulation for 48h. Images were magnified at 20X.

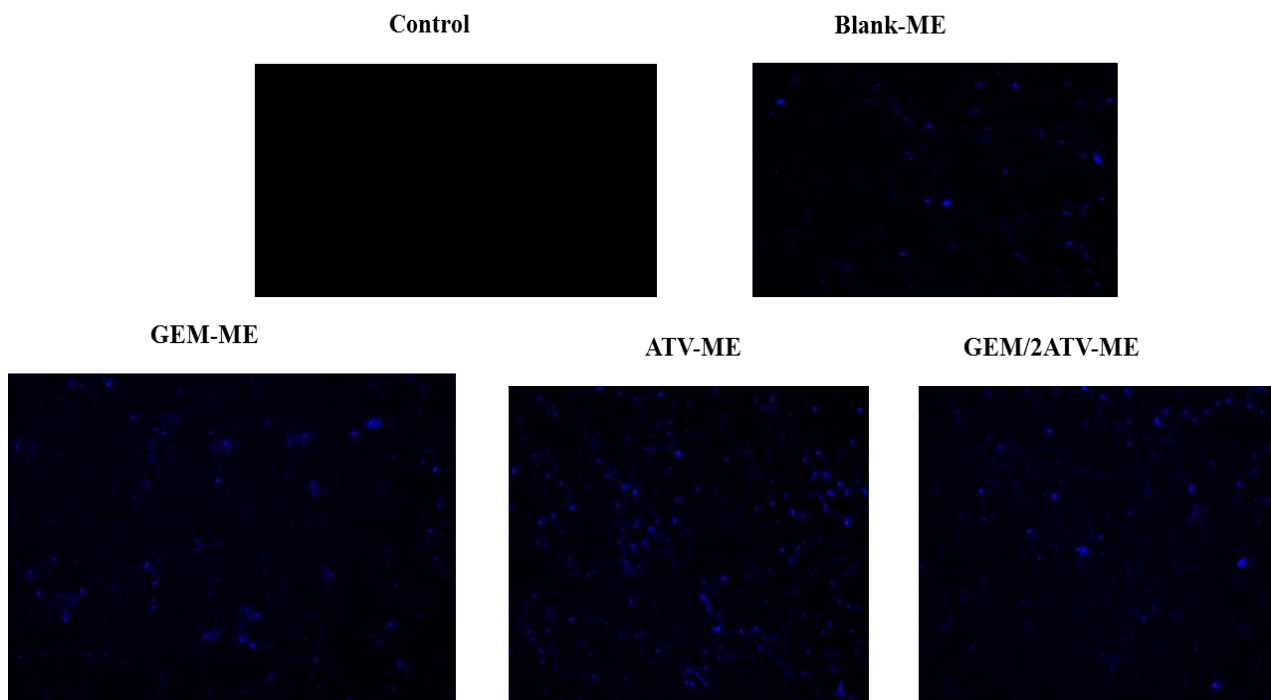


Figure 9: Fluorescence microscopy images of HCT 116 cells stained with DAPI after treated with 5  $\mu$ M of ME formulations for 24h. Images were magnified at 20X.

with the combination formula 2GEM/ATV have slightly got affected as the number of the cells did not change relative to the control. However, early signs of apoptosis started to appear as the chromatin has got condensed and intracellular spaces between the cells was displayed suggesting that if the incubation time have got increased, cells might undergo apoptosis.

On the other hand, the effect of all of the ME formulations on the HCT116 cells, exhibited in Figures 4 and 5, has damaged most of the cells within 48h. In fact, the

chromatin condensation and the formation of vacuoles were observed in the cells incubated with the Blank-ME and GEM-ME for 24h, meanwhile the accumulation of the apoptotic bodies was clearly seen in the cells treated with GEM/2ATV-ME. Interestingly, subjecting ATV into HCT116 cells has killed all of the cells within 24h.

*ApopNexin™ FITC apoptosis detection assay*

Double staining with FITC/PI can distinguish between necrotic (Q1), viable (Q3), early apoptotic (Q4) and late apoptotic (Q2) cells. It should be noted that all of the

Table 2: The percentages of the HCT 116 colon cancer cells, detected FITC/PI flow cytometry plots, undergoing necrosis and different stages of apoptosis when subjected into solution formulations and microemulsion (ME) formulations for 48h and 24h, respectively. Cells are classified as necrotic (Q1), late apoptotic (Q2) viable (Q3) and early apoptotic (Q4) cells. Data were expressed as  $\bar{X} \pm SD$ .

% of cells					
Quadrants	Control	GEM	ATV	2GEM/ATV	
Q1	0.60 ± 0.56	11.64 ± 0.35	33.63 ± 3.20	22.47 ± 0.85	
Q2	0.00 ± 0.00	1.57 ± 1.91	9.33 ± 2.28	2.13 ± 2.70	
Q3	99.10 ± 0.85	69.10 ± 1.65	49.20 ± 0.55	72.67 ± 3.45	
Q4	0.30 ± 0.35	17.70 ± 2.58	7.84 ± 3.15	2.63 ± 0.09	
% of cells					
Quadrants	Control	Blank-ME	GEM-ME	ATV-ME	GEM/2AT-ME
Q1	2.07 ± 0.78	3.03 ± 0.15	6.17 ± 0.00	5.17 ± 1.94	5.87 ± 3.99
Q2	0.03 ± 0.06	22.26 ± 0.30	22.27 ± 0.30	21.47 ± 0.20	29.20 ± 0.30
Q3	97.33 ± 0.75	29.00 ± 0.15	27.50 ± 0.20	30.46 ± 0.60	25.27 ± 3.33
Q4	0.57 ± 0.05	45.70 ± 0.35	44.07 ± 0.50	42.90 ± 0.75	39.67 ± 1.00

cancer cells were incubated with solution formulations for 48h while the ME formulations were subjected into the cells for 24h. The effect of the solution and ME formulations on the HCT116 is demonstrated in the flow cytometry plots displayed in Figures 6 and 7, while a summary of the percentages of the cells undergoing necrosis and different stages of apoptosis is illustrated in Table 2. Cells, undergone necrosis, were more abundant when treated with the solution formulation compared to the ME formulations. On the other hand, most the cells, treated with the ME formulations, were early apoptotic. Interestingly, the percentages of late apoptotic cells treated with the ME formulations, were significantly more than the ones treated with solution formulations. The percentages of the viable cells have significantly increased when they were subjected into the solution formulations.

#### Nuclear DNA staining for apoptosis detection

DAPI dye is able to permeate the cell undergoing apoptosis and get attached to A-T rich regions in DNA to give a strong blue fluorescent dye. As revealed in Figure 8, the water formulations have clearly affected the nuclei of the HCT116 cells when applied for 48h. When cells were subjected into GEM and 2GEM/ATV, the nuclei have got enlarged and their morphology has got altered indicating that the cells are undergoing late stages of apoptosis. Meanwhile, cells treated with ATV have immensely got affected as small blue stains were observed, suggesting that the nuclei were fragmented, a sign of late stage of apoptosis. Similarly, Figure 9 displayed that all of the ME-formulations subjected into the HCT116 cells for 24h have massively affected the nuclei and resulted in the clearance of the cells as very small blue spots have shown up.

## DISCUSSION

In this study, MTT assay was applied to evaluate the cytotoxicity of GEM-ME, ATV-ME and their combination loaded-ME on HCT116 cells at 1 and 5 $\mu$ M. It has been found that all ME formulations have antitumor activity against HCT116 cells in agreement with many previous studies. Celano *et al.*<sup>18</sup> have validated that encapsulating GEM in liposomes was having enhanced cytotoxic effect and a more effective uptake of GEM inside the cells compared to free GEM. Li *et al.*<sup>19</sup> have shown that GEM-

loaded-albumin nanospheres improved the antitumor activity of GEM against BxPC-3 human pancreatic cancer cells. Hosseinzadeh *et al.*<sup>20</sup> demonstrated that encapsulating GEM in Chitosan-Pluronic nanoparticles could be considered as an efficient oral formulation for colon cancer treatment. Moreover, Mekhail *et al.*<sup>21</sup> found out that ATV loaded in micelles has more antiproliferative activity against MCF-7 and HCT116 cells than ATV alone. In order to understand the mechanism of cell death, the morphologies of the cells were characterized using light microscopy, DAPI assay and ApopNexin™ FITC apoptosis detection assay. Within 24h, different levels of apoptosis were observed in the cancer cells subjected into all of the ME formulations. There are several possible explanations for this result. The presence of Tween 80 in the ME formula would improve the permeability of the drugs into the cell membrane while inhibiting the P-glycoprotein efflux system<sup>22,23</sup>.

The current study displayed that the ME formulation caused apoptosis in the cancer cells that was integrated with autophagocytosis. This finding is in agreement with Alkhatib and AL-Qaidi<sup>24</sup> findings, which showed that docetaxel-loaded-ME have stimulated the formation of endosomes and vacuoles, containing cell debris and organelles, in the HepG2 cells. Furthermore, Gupta *et al.*<sup>25</sup> have exhibited that gelatin nanoparticles, prepared by W/O ME systems, were uptaken by the human fibroblasts through endocytosis, thereby forming vacuoles in the cell body.

Regarding the solution formulations, the cytotoxicity screening using MTT assay has revealed that a 5 $\mu$ M of 2GEM/ATV (3.4  $\mu$ M GEM/1.6  $\mu$ M ATV) enhanced the inhibition effect of GEM on HCT 116 cells. Mistafa and Stenius<sup>26</sup> indicated that in combination treatment, 4 $\mu$ M of ATV has increased the antiproliferative of 0.1 $\mu$ M of GEM in pancreatic cancer cells (Panc-1). There was a strong synergy in the growth inhibition of HCT116 and HT29 cell when administered into the combination of 4 $\mu$ M of ATV and 25 $\mu$ M of celecoxib<sup>9</sup>. Chen *et al.*<sup>27</sup> demonstrated that the combination of 2.5 $\mu$ M of ATV and 5 $\mu$ M of carboplatin had strongly inhibited the cell proliferation of A549 NSCLC cells.



Surprisingly, ATV solution has significantly inhibited the growth of HCT116 cells at lower concentration (1  $\mu$ M). A previous study found that ATV has a stronger antiproliferative effect on the HCT116 than HT29<sup>9</sup>. In fact, ATV inhibited the growth of different types of cancer cells, MCC-2 myeloma cells and CEM leukemic cells<sup>28</sup>; PBMCs peripheral blood mononuclear cells and (A20 and EL4 cells) lymphoma cells<sup>29</sup> and T24 and J82 cells<sup>11</sup>, in a dose and time-dependent manner.

## CONCLUSION

It has been found that combining GEM with ATV at a ratio of 1:2, respectively, in a ME has significantly improved the inhibition effect of GEM on the HCT116 cells. Interestingly, increasing the concentration of GEM/2ATV –ME from 1 –to- 5  $\mu$ M has improved the cytotoxic effect of the combination formula, whereas increasing the same combination formula in water (GEM/2ATV) has decreased the cytotoxic effect of the mixed formulation. Based on this study, it is recommended to establish further *in vivo* researches in order to give extensive study of the drug combination-ME formula effect on the body tissues and organs. Also, further studies have to be performed to discover the mechanisms responsible for the antitumor activity of the drug-loaded-ME.

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