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ANTICANCER EFFECT OF GEMCITABINE COMBINED WITH 5-FLUROURACIL IN MICROEMULSION ON THE HCT-116 COLON CANCER CELLS

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ABSTRACT

Combination therapies of chemotherapeutic agents in nanoparticelsare promising strategy in releasing the drugs selectively into the targeted tissues and cells. 5-Flurouracil (5-FU) and Gemcitabine (GEM) are antimetabolites used as anticancer drugs. In this study, the antitumor activity of the combination of GEM and 5- FU encapsulated in a micro emulsion (ME) was *in vitro* evaluated in HCT- 116 colon cancer cells. The inhibition of cell growth was examined by the 3(4,5-dimethyl) ethiazole (MTT) assay. The mechanism of cell death was determined by observing the morphological changes of treated cells under light microscope, identifying apoptosis by using 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 combining 25µM of 5-FU with 5µM of GEM in a ME formula (5GEM/25FU-ME) has enhanced the cytotoxicity of 5GEM-ME and preserved the strong cytotoxicity of 25FU-ME against HCT-116 cells. The current study proved that formulating GEM with 5-FU in ME has improved the therapeutic potential of GEM and 5-FU as anticancer drugs.

INTRODUCTION

Colon cancer, a malfunctioning of the large intestine cells, stems from cecumin the abdomen and keep spreading until it reach the rectum in the deep pelvis (Yeatman, 2001). The most common treatments involved in cancers include surgery, radiotherapy, and chemotherapy. In cases where the tumor is not surgically resected, radiation and chemotherapy are typically delivered (Liang *et al.,* 2005). While proven to be effective in reducing the size of tumors, these treatments also have a high potential to damage healthy cells and may not necrotize the entire tumor. The ideal strategy to treat cancer is to formulate drugs that are selective and destructive to the cancer cells (Bayazitoglu *et al.,* 2013). There are several restrictions of the chemotherapeutics that are limiting their use for cancer treatment such as limited bioavailability, poor specificity and short circulating half-lives (Sachan and Singh, 2014). Thus, most of the pharmaceutical industries are attempting to develop delivery systems that would overcome the complications associated with the cancer treatment drugs (Kayser *et al.,* 2005). One of the most promising approaches is to produce nanocarriers that would encapsulate minimum amount of drugs and hence reduce their side effects without affecting their efficacy (Parveen *et al.,* 2012). Microemulsion (ME) system isa heterogeneous system that consists of surfactant, cosurfactant, oil and water. The suspended droplets of the ME, which have diameters in the range of 10-200 nm, are thermodynamically stable. Because of the composition and structure of ME, loading drugs on the ME droplets would enhance the drug absorption in the intestine (Charman and Stella, 1991; Mandal *et al.,* 2010). 5-Fluorouracil (5-FU), an antimetabolite of the pyrimidine analogue type, is an antiproliferating agent used to treat various types of cancers (Wilson *et al.,* 2012).Gemcitabine (GEM), a purine analogue, is an antimetabolite used to treat a broad spectrum of cancers (Mini *et al.,* 2006; Joerger *et al.,* 2002). The major objective of the current study was to evaluate the anticancer activity of the combination of GEM and 5-FU, loaded onto ME, in HCT-116 cells.

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MATERIALS AND METHODS

Subjects

GEM hydrochloride was purchased from United States pharmacopeia convention. 5-FU, Sorbitan monolaurate (Span 20), Polyoxyethylene sorbitan monooleate (Tween 80),

Isopropyl Myristate (IPM), sodium phosphate dibasic anhydrous, were obtained from Sigma (Missouri, US). The 3-(4,5-dimethylthiazole-2-yl)-2,5-diphyneltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO) and Coomassie brilliant blue were obtained from biomatik (Ontario, Canada). Ethanol and formaldehyde were purchased from Fisher Chemical (UK). The heat inactivated fetal bovine serum (FBS) and phosphate buffered saline (PBS, pH7, 10mM) were obtained from Lonza Walkersville (USA). Dulbeccos modified eagle medium (DMEM), trypsin and penicillin streptomycin antibiotic were obtained from Gibco life technologies (New York, US). The 4′,6-diamidino-2-phenylindole (DAPI) dihydrochloride was purchased from Invitrogen life technologies (New York, US). The ApopNexin™ FITC Apoptosis Detection Kit (Lot. No. 2053919) was purchased from Millipore (Massachusetts, US). The human colon cancer cell line (HCT116) was procured from American Type Tissue Culture Collection (Manassas, VA, USA).

Preparation of the ME formulas

ME formulas were produced as described by Tsai et al. (2010). 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. The drug-loaded- ME were 1µM of GEM (1GEM-ME), 5µM of GEM (5GEM-ME), 25µM of 5-FU (25FU-ME), 10µM of 5-FU (10FU-ME), several combinations consisted of 1µM of GEM and 25µM of 5-FU (1GEM:25FU-ME), 5µM of GEM and 25µM of 5-FU (5GEM:25FU-ME) and 5µM of GEM and 10µM of 5-FU (5GEM:10FU-ME). Similarly, another group of solution formulas was produced by dissolving the drugs in water instead of ME and each formula was designated as 1GEM-D.W, 5GEM-D.W, 25FU-D.W, 1GEM:25FU-D.W and 5GEM:25FU-D.W.

***In Vitro* evaluation of antitumor activity**

Cell culture

HCT-116 were cultivated in a 25cm^2 cell culture flask, containing DMEM which was supplemented with 10% (v/v) FBS and 1% (v/v) penicillin-streptomycin, were incubated in a 5% CO_2/95% humidified atmosphere at 37°C. In the meantime, the medium was discarded from the cell culture flask and changed at 48h intervals. Cells were fed until confluence and confluent cells were washed with 2ml of PBS, detached by adding 2ml of trypsin, and incubated at 37°C.

Cytotoxicity screening using MTT assay

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_2 incubator for cell attachment. Then, cells were treated with 100µl of different ME and solution formulas, followed by incubation at 37°C in a CO_2 incubator at 24 and 48h for HCT-116. After that, a 5 µl of MTT reagent was added to each well, mixed gently for one minute and incubated for 3 to 4h at 37°C in a CO_2 incubator. Then, the culture medium containing MTT reagent was removed, followed by adding a 100 µl of DMSO and incubation for 2h. 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:

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

Characterization of cell morphology using inverted microscope

In order to assess the morphological changes of the treated cells, the phase contrast inverted microscope (Olympus 1X51, Japan) was utilized as described by Alkhatib and Alkhayyal (2014). Cells were counted and plated at a density of 5,000 cells per well into 24-well plate containing 500µl of growth media in each well. Then, they were incubated with 500µl of the desired drug formula for 24h. Cells were washed twice with 300µl of PBS and fixed by the addition of 200µl of 4% formaldehyde for 5 min. After that, the fixation solution was discarded and the cells were washed with 300µl of PBS and stained with 200µl of 10% Coomassie blue for 10 min. Then, the stain solution was discarded, washed with 300µl of 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 dual 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 binds to the DNA of the lysed cells. Cells, plated at a density of 5x10^4 cells per well into 24-well plate containing 500µl of culture media, were treated with 500µl of the ME and solution formulas and were incubated for 24h. Then, cells were washed with 300µl of PBS, dissociated by 200µl of trypsine and spin down at 1000 rpm for 5min. After that, the supernatants were removed and cells were re-suspended in 3ml ice cold PBS and spin down twice at 1000 rpm for 5min. Detached cells were re-suspended in 200 µl of 1X binding buffer (10 mM of 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid buffer solution /NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). After that, the mixture was incubated with 3µl of FITC and 2µl of PI for 15min at room temperature in the dark. All cells were evaluated by a BD FACSAriaTM III Flow Cytometer (BD Biosciences, US). Data, analyzed using FACS Diva software version 6.1.3. The positive FITC indicates the 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. 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. Cells were counted and plated at a density of 5× 10^4 cells per 500µl of growth media in each well of 24 well-plate.

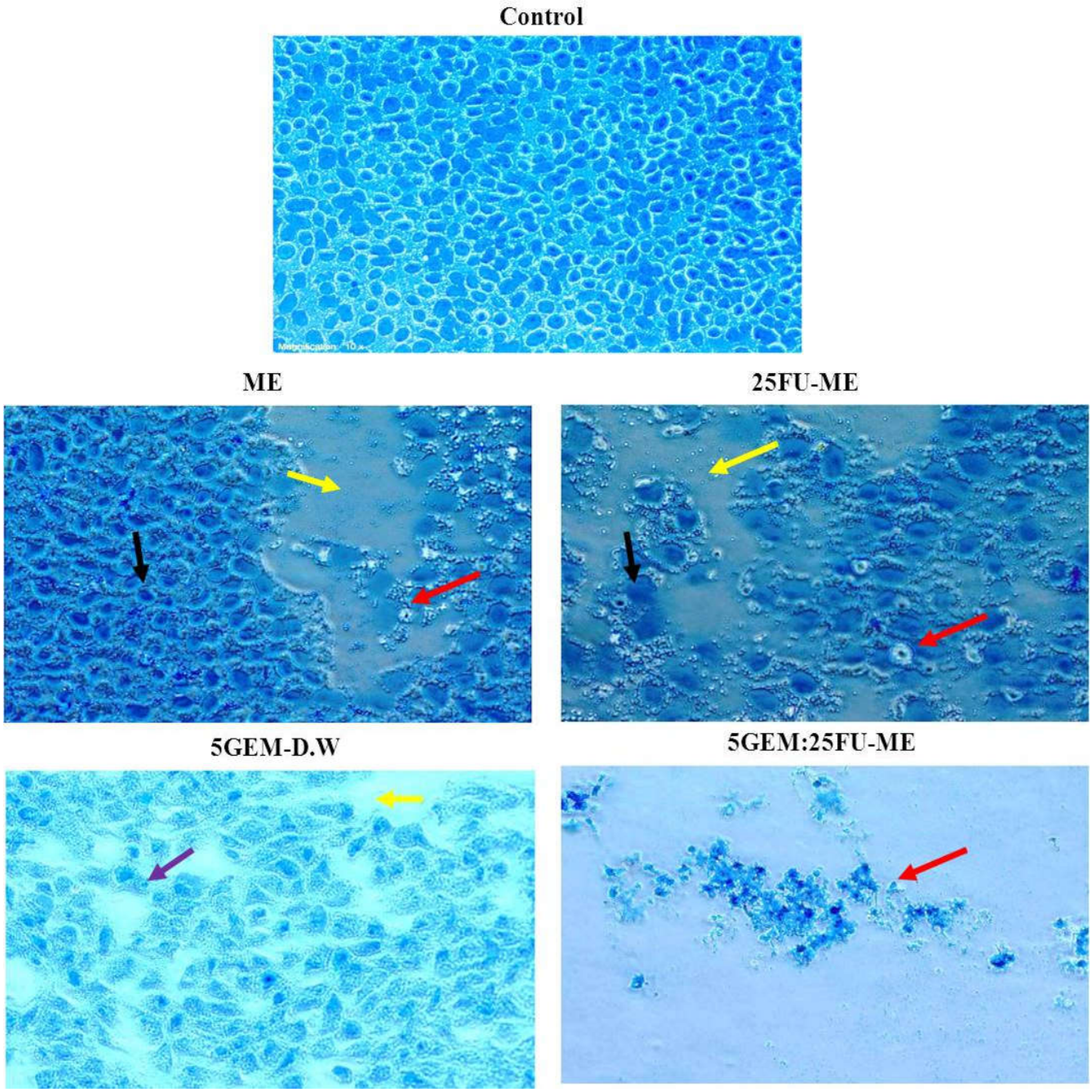

Figure 1. Light microscopy images of HCT -116 cells treated with the solution and ME formulas for 24h. Signs of apoptosis are represented by the purple arrows (membrane blebbing), black arrows (chromatin condensation), red arrows (apoptotic bodies) and yellow arrows (intercellular space)

Cells, treated with 500µl of the desired formula, were incubated for 24h. Then, cells were equilibrated with 300µl of PBS, fixed by 200µl of formaldehyde and stained with 300 µl 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 (Leica CRT6000, Germany). The percentages of fluorescent intensity were estimated by ImageJ 1.48v.

Statistical analysis

Data were expressed as mean ± standard deviation ($\bar{X}$±SD) as eachexperiment was performed in triplicate. Statistical analyses were performed with one-factor analysis of variance (ANOVA) using MegaStat excel (version 10.3, Butler University). The statistical significance differences were considered *p*-value <0.05.

RESULTS

MTT assay for cytotoxicity screening: The effect of the ME and solution formulas on the proliferation of the HCT-116 cells is displayed in Table 1.At 24h, the ME formulas that have considerable cytotoxicity with % cell viabilities less than 60% were ME, 25FU-ME and 5GEM:25FU-ME. By increasing the period of time to 48h, 5-GEM-ME have showed significant toxicity on the cells as well as ME, 25FU-ME and 5GEM:25FU-ME have shown improved cytotoxicity. It is noteworthy to mention that the combination formula, 5GEM:25FU-ME, when subjected for 24h, has a cytotoxicity in the middle between the single treatments of 5GEM-ME and 25FU-ME since the % of cell viabilities treated with 5GEM:25FU-ME was less than %cell viabilities treated with 5GEM-ME and more than the %cell viabilities treated with 25FU-ME.

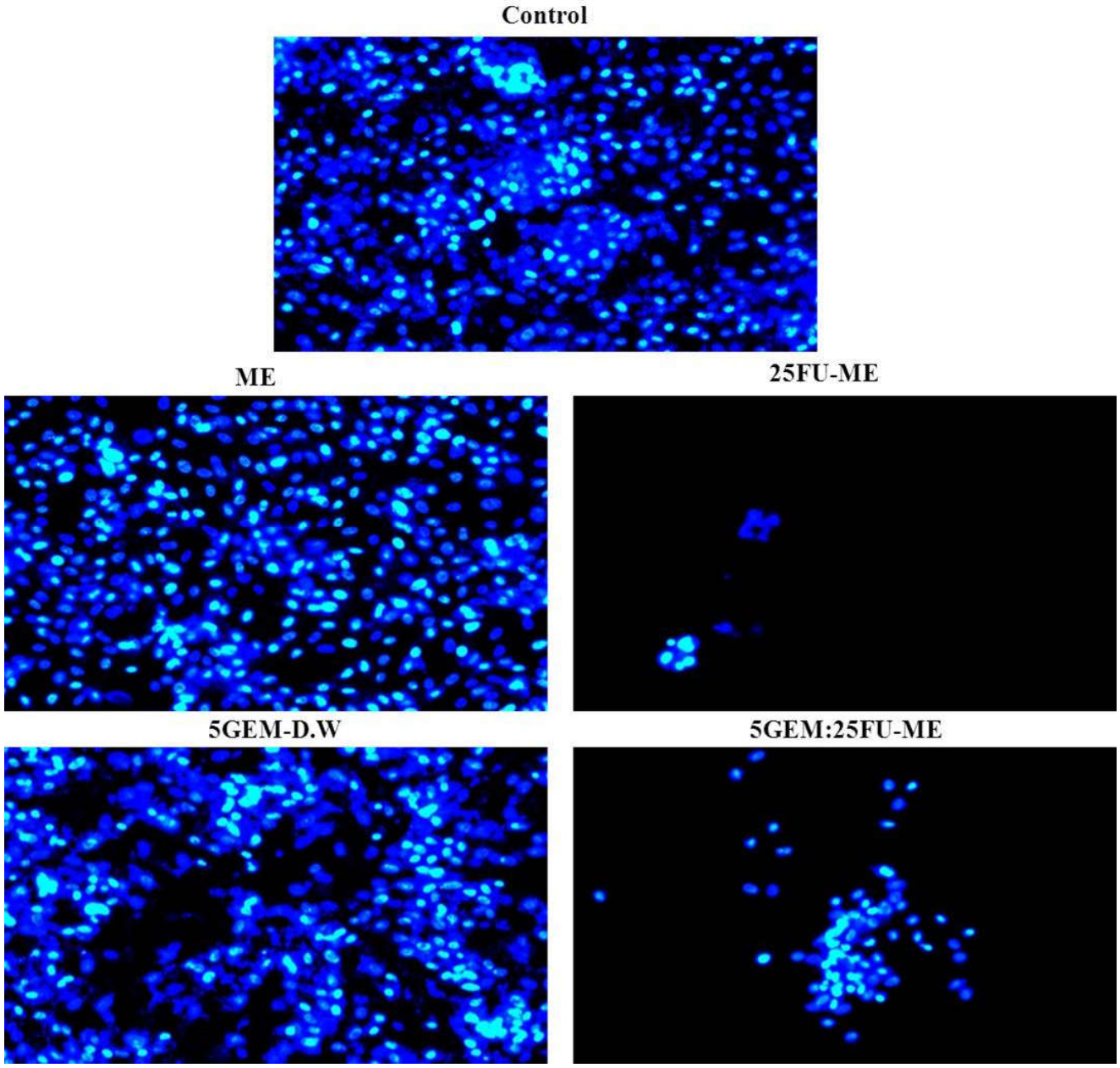

Figure 2. Fluorescence microscopy images of HCT -116 cells stained with DAPI when treated with the solution and ME formulas for 24h. Images were magnified at 20X

In other words, combining 5GEM with 25FU in ME has improved the cytotoxicity of GEM and reduced the cytotoxicity of FU. Among the solution formulas, it was only the 5GEM-D.W that has considerable cytotoxicity.

Characterization of cell morphology using inverted microscope

In order to understand the mechanism of cell death, the HCT-116 cells morphologies were analyzed using light microscopy as shown in Figure 1. In general, slight changes in the cells shape, chromatid condensation, decreased total number of cells, formation of apoptotic bodies and increased intercellular spaces between the cells treated with ME and 25FU-ME formulas were observed compared with the untreated cells. Cells treated with 5GEM:25FU-ME have shown dramatic changes in shape, the massive formation of apoptotic bodies, extremely increased intercellular spaces and clearance of the cells.

On the other hand, slight changes were observed in the cells treated with 5GEM-D.W such as membrane blebbing and limited intercellular spaces.

ApopNexin™ FITC apoptosis detection assay

The effect of the solution and ME formulas on the HCT-116 is summarizedas the percentages of the cells undergoing necrosis and different stages of apoptosis is (Table 2). Cells, undergone necrosis, were more abundant when treated with ME formulas compared to the solution formula. The percentages of the viable cells as well as the apoptotic cells have significantly increased when they were subjected into the solution formula.

Nuclear DNA staining for apoptosis detection

Figure 2 exhibits the effect of the solution and ME formulas on the morphology of the nuclei of HCT-116 cells. There were discrepancies between the formulas in affecting the nuclei of HCT-116 cells.

Table1. Cytotoxicity screening of ME and solution formulas at different concentrations subjected into HCT- 116 cells. The percentages of cell viabilities were expressed as $\overline{X}$ ± SD

Formulas	% Cell viability	
	24h	48h
ME	56.20±1.83	49.49±2.6
1GEM-ME	98.24±2.34	90.23±1.01
5GEM-ME	85.91±0.17	9.54±1.58
25FU-ME	9.49±1.01	1.85±1.38
1GEM:25FU-ME	90.98±1.21	72.51±1.49
5GEM:25FU-ME	58.10±2.56	0.63±0.31
10FU-ME	74.82±1.25	70.21±2.34
5GEM:10FU-ME	98.69±0.72	91.31±2.11
1GEM-D.W	90.56±5.31	82.95±1.12
5GEM-D.W	64.44±24.34	54.99±1.11
25FU-D.W	95.76±0.07	91.45±2.11
1GEM:25FU-D.W	97.64±7.47	96.25±3.98
5GEM:25FU-D.W	84.31±2.77	83.27±3.17

Table 2. The percentages of the HCT -116 cells, detected by FITC/PI flow cytometry plots subjected into the solution and ME formulas for 24h. Cells are classified as necrotic (Q1), late apoptotic (Q2) viable (Q3) and early apoptotic (Q4) cells

Quadrants	Control	ME	5GEM-D.W	25FU-ME	5GEM:25FU-ME
Q1	5.1	54	3.2	49.1	57.4
Q2	0.1	16.9	13.3	22.6	16.2
Q3	95.5	26.4	54.4	20	23.5
Q4	2.4	2.7	29.2	8.4	2.8
Q2+Q4	2.5	19.6	42.5	31	19

ME and 5GEM-D.W have the least cytotoxic effect as the nuclei have intensely fluoresced and their shapes have slightly got changed, indicating that the cells have endured early stages of apoptosis. In contrast, 25FU-ME and 5GEM:25FU-ME formulas have had a massive effect on the nuclei of the cells as less intense blue spots were observed, indicating that the cells have endured late stages of apoptosis.

DISCUSSION

In this study, MTT assay was employed to evaluate the cytotoxicity of GEM, 5-FUand their combination, when loaded in ME and distilled water, in HCT-116, at different periods of time. It has been found that all ME formulas have antitumor activity against HCT-116 cancer cells better than the solutions formulas in agreement with many previous studies. Yuan et al. (2015) have validated that encapsulating 5-FU in nanoparticle consisting of methoxy poly(ethylene glycol)–poly(lactide) was having enhanced cytotoxic effect and a more effective uptake of 5-FU inside the cells compared to free 5-FU. Furthermore, this study revealed that the cell viability was significantly reduced in the combination drug-loaded ME. This may be due to the better uptake of the carrier into the cells. While 25 µM of 5-FU in combination with 5 µM of GEM resulted in a decrease of 97% in the cell viability. Guo et al. (2015) have reported that there is a synergistic anticancer effect of paclitaxel and GEM-loaded N-succinyl chitosan nanoparticles toward colon cancer cells (HT-29). Surprisingly, HCT-116 cells were sensitive to the change in concentration of GEM-ME from 1 to 5 µM in the combination formula (1GEM:25FU-ME to 5GEM:25FU-ME). Similarly, Alkhatib and Alkhayyal (2014) exhibited that increasing the concentrations of GEM-ME from 0.03 to 0.3 %v/v did result in a significant change in the cytotoxicity of GEM-ME against HCT-116.

In order to understand the mechanism of cell death, the morphologies of the cells were characterized using light microscopy, DAPI assay and Apop Nexin™ FITC apoptosis detection assay.In this study, ME formulas have a necrotic effect on HCT-116 cells. This is possibly due to the cellular uptake of GEM-loaded ME which is a critical step in ensuring the cytotoxic efficacy of the drug as it acts by incorporation of its active triphosphate form (5'-triphosphate GEM) into DNA strand, halting its elongation and causing cell death (Garg *et al.,* 2012). Furthermore, based on morphological changes seen under a light microscope, among all of the treated cells, solutions formulas slightly affected the cells and changed their shapes. In contrast, ME formulas showed the most effect on all of the cells. Their efficiency has got enhanced as they enormously killed the cells and late signs of apoptosis were seen, such as chromatid condensations and apoptotic bodies. On the other hand, at solutions formulas, the number of cells decreased, their shape has changed and revealed early signs of apoptosis. This can probably be explained by better uptake by the cancer cells due to the small droplet size of ME formulas. It has been reported that the cut off size in tumor vessels ranged between 200 nm and 2 µm (Hobbs *et al.,* 1998; Hashizume *et al.,* 2000).

Conclusion

It has been found that combining 5-FU with GEM in ME has improved the efficacy of GEM considerably. It is recommended to apply the combination formulas in ME on other cancer cells.

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REFERENCES

Alkhatib, M. and Alkhayyal, N. 2014. Cytotoxicity of gemcitabine-loaded-microemulsions in breast and colon cancer cells. *Trop J Pharm Res.,* 13(2): 217.

Bayazitoglu, Y., Kheradmand, S. and Tullius, T. 2013. An overview of nanoparticle assisted laser therapy. *Int J Heat Mass Transf.,* 67: 469-486.

Charman, W. and Stella, V. 1991. Transport of lipophilic molecules by the intestinal lymphatic system. *Adv. Drug Deliv. Rev.,* 7(1): 1-14.

Garg, N., Dwivedi, P., Campbell, C. and Tyagi, R. 2012. Site specific/targeted delivery of gemcitabine through anisamide anchored chitosan/poly ethylene glycol nanoparticles: an improved understanding of lung cancer therapeutic intervention. *Eur. J. Pharm. Sci.*, 47(5): 1006-1014.

Guo, X., Wang, P., Du, Q., Han, S., Zhu, S., Lv, Y., Liu, G. and Hao, Z. 2015. Paclitaxel and gemcitabine combinational drug-loaded mucoadhesive delivery system in the treatment of colon cancers. *Drug Res.*, 65(04): 199-204.

Hashizume, H., Baluk, P., Morikawa, S., McLean, J., Thurston, G., Roberge, S., Jain, R. and McDonald, D. 2000. Openings between defective endothelial cells explain tumor vessel leakiness. *Am. J. Pathol.,* 156(4): 1363-1380.

Hobbs, S., Monsky, W., Yuan, F., Roberts, W., Griffith, L., Torchilin, V. and Jain, R. 1998. Regulation of transport pathways in tumor vessels: Role of tumor type and microenvironment. *Proc. Natl. Acad. Sci.,* U.S.A. 95(8): 4607-4612.

Joerger, M., Gunz, A., Speich, R. and Pestalozzi, B. 2002. Gemcitabine-related pulmonary toxicity. Swiss Med. Wkly. 132(1): 17-20.

Kayser, O., Lemke, A. and Hernandez-Trejo, N. 2005. The impact of nanobiotechnology on the development of new drug delivery systems. Curr Pharm Biotechnol. 6(1): 3-5.

Liang, H., Wan, L., Bai, C. and Jiang, L. 2005. Gold hollow nanospheres: Tunable surface plasmon resonance controlled by interior-cavity sizes. *J. Phys. Chem. B.*, 109(16): 7795-7800.

Mandal, S., Mandal, S. and Sawant, K. 2010. Design and development of microemulsion drug delivery system of atorvastatin and study its intestinal permeability in rats. *Int. J. of Drug Del.*, 2(1): 69-75.

Mini, E., Nobili, S., Caciagli, B., Landini, I. and Mazzei, T. 2006. Cellular pharmacology of gemcitabine. *Ann. Oncol.,* 17(5): 7-12.

Parveen, S., Misra, R. and Sahoo, S. 2012. Nanoparticles: A boon to drug delivery, therapeutics, diagnostics and imaging. Nanomed.: *Nanotechnol., Biol. Med.,* 8(2): 147-166.

Sachan, A. and Singh, S. 2014. Nanoparticles: Nasal delivery of drugs. *IJPRS*, 3(3): 2277 - 7873.

Tsai, Y., Hsieh, Y., Huang, Y., Chang, J., Huang, C. and Wu, P. 2010. Microemulsions for intravesical delivery of gemcitabine. *Chem. Pharm. Bull.,* 58(11): 1461-1465.

Wilson, B., Ambika, T., Dharmesh Kumar Patel, R., Jenita, J. and Priyadarshini, S. 2012. Nanoparticles based on albumin: Preparation, characterization and the use for 5-flurouracil delivery. *Int. J. Biol. Macromolec.,* 51(5): 874-878.

Yeatman, T. 2001. Colon cancer.Encyclopedia of Life Sciences.

Yuan, Z., Qu, X., Wang, Y., Zhang, D., Luo, J., Jia, N. and Zhang, Z. 2015. Enhanced antitumor efficacy of 5-fluorouracil loaded methoxy poly(ethylene glycol)-poly(lactide) nanoparticles for efficient therapy against breast cancer. *Colloids Surf B Biointerfaces,* 128: 489-497.

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