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CYTOTOXICITY EFFECT OF DOCETAXEL-LOADED-MICROEMULSION IN A549 NON-SMALL CELL LUNG CANCER AND HCT116 COLON CANCER CELLS

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ABSTRACT

Docetaxel, an anti-mitotic chemotherapeutic agent, is mainly used for the treatment of various cancers. The objective of this study was to evaluate the antitumor activity of docetaxel-loaded-microemulsion in A549 non-small cell lung cancer and HCT 116 colon cancer cells. The safe effect of the formula was evaluated in HFS human foreskin cells. It has been found that the storage of the drug in the microemulsion formulation for around two weeks (O-ME) did not affect the toxicity of the drug formulations against the erythrocytes as the hemolysis activities of 1mg/ml of both of freshly prepared docetaxel-loaded-microemulsion (F-ME) and O-ME were 36 ± 7.61 and 32.15 ± 6.32, respectively. The cytotoxicity screening revealed that 5µM of F-ME and O-ME were more cytotoxic than 5 µM of Taxotere by two folds when subjected unto A549 and HCT 116 cells and were less than 5 µM of Taxotere by approximately one and half folds when administered into HFS cells. Formulating the docetaxel in a microemulsion formula improved its efficiency besides reducing its side effect.

KEYWORDS: Antitumor activity; ApopNexin FITC apoptosis detection kit; Hemolysis activity; sulphorhodamine B assay.

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INTRODUCTION

Docetaxel is a semi-synthetic compound belonging to the taxane family of anticancer drugs. The taxanes are a unique class of cytotoxic drugs that promote the polymerization of microtubules, which leads to the disruption of mitosis, cell cycle arrest at G2/M, and cell death. Docetaxel has a significant antitumor activity against various human malignancies and is approved by the Food Drug Administration for the treatment of breast cancer, ovarian cancer, non-small cell lung cancer, and prostate cancer. In spite of its high efficiency, side effects limit the clinical use of docetaxel, due to its high lipophilicity and practically insoluble in water (3µg/ml) [1]. The current formulation of docetaxel contains 40 mg/ml docetaxel and 1040 mg/ml polyoxyethylated surfactant, polysorbate 80 (Tween 80), which requires further dilution with 13% ethanol before addition to intravenous infusion solution. Adverse reactions due to either the drug itself [2] or the solvent system [3] have been reported. The presence of Tween 80 in docetaxel formula resulted in severe side effects. Therefore, many alternative delivery formulations with free Tween-80 or with addition of a low concentration of Tween 80 were invented. Docetaxel was encapsulated in several delivery systems that improved its efficiency [4-13].

Furthermore, in order to improve patient compliance and for efficient combination therapy with other antineoplastic agents, an oral formulation of docetaxel would be useful since oral chemotherapy could ease the use of more chronic regiments [14, 15]. Studies have shown that cyclosporine A or interferon-alpha could increase the oral bioavailability of docetaxel by inhibiting P-gp [16, 17]. However, pretreatment with a P-gp inhibitor before the oral administration of docetaxel is far from improving patient compliance and may lead to more side effects. Amongst the various drug delivery systems, the microemulsion system is considered an ideal alternative for the oral delivery of poorly water-soluble compounds [18, 19]. Microemulsion (MEs) is a lipid based delivery system demonstrating absorption enhancement. The major advantages include high solubilization potential, thermodynamic stability, improved dissolution of lipophilic drugs and surfactant-induced permeability enhancement [20, 21]. Additionally, several excipients commonly used in these systems including Cremophor, Tween 80, Labrasol and Transcutol could inhibit the function of P-gp making the microemulsion system an attractive choice for docetaxel oral delivery [22-24]. In this study, the microemulsion formula, consisted of weight percentages of 37 of cremophor/transcutol mixture (2:1, w/w), 29 of capryol 90 and 34 of water, was produced according to a method described by Yin *et al.* [9]. The anticancer activity of docetaxel-loaded-microemulsion was evaluated in A549 and HCT 116 cell while the side effect was examined in HFS cells.

MATERIALS AND METHODS

Cremophor EL, transcutol, capryol 90 and docetaxel were purchased from Jassomah Establishment (Jeddah, Saudi Arabia). Taxotere was generously gifted from King Abdulaziz University Hospital. Modified eagle medium (MEM), vitamin solution, fetal calf serum (FCS), non-essential amino acid, penicillin ,streptomycin, phenol red, phosphate buffered saline, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer solution (HEPES), trypsin, sulforhodamine B (SRB) and trichloroacetic acid (TCA) were purchased from Sigma-Aldrich Chemical Co, St Louis, MO, USA. The distilled water was purified using a water purification system from Bibby sterilin ltd, UK. ApopNexin FITC Apoptosis Detection Kit was purchased from Millipore, MA, USA. All other reagents were of analytical grades. The human cell lines of A549 non-small cell lung cancer, HCT116 colon cancer and HFS human foreskin were obtained from the Tissue Culture Bank at King Fahd Medical Research Center, Jeddah, KSA.

(i) Preparation of the docetaxel -loaded – microemulsion

Docetaxel-loaded-microemulsion formula was prepared using the method described by Yin *et al*.[9]. The microemulsion formula was produced by mixing the components in subsequent steps. First, the surfactant, cremophore EL, was mixed with the cosurfactant, transcutol, at a fixed weight ratio of 2:1. Then, the oil, capryol 90, was added slowly to the desired weight fraction of the surfactant and cosurfactant until the phase was formed. After that, the fixed fraction of the aqueous phase was added drop wise. Finally, the mixture was vortexed and shaken at room temperature to be clear and transparent. The microemulsion formula was stored at 25°C. To prepare docetaxel-loaded-microemulsion, sufficient amount of 1mg/ml of docetaxel was dissolved directly and vortexed well in the liquid microemulsion formula. The examined microemulsion formulations involved in this study were blank microemulsion (B-ME), freshly prepared docetaxel-loaded-microemulsion (F-ME) and stored docetaxel-loaded-microemulsion for around two weeks (O-ME).

(ii) Size and morphology characterization of docetaxel -loaded -microemulsion using scanning electron microscope

The particle size analysis was carried out using SEM at Faculty of Science, King Abdulaziz University. To prepare the specimens of B-ME and F-ME for the SEM, they were first fixed with Karnovsky's glutaraldehyde fixative (2g of Paraformaldehyde, 2 - 4 drops of 1M Sodium hydroxide, 5ml of 50% glutaraldehyde, and 20ml of 0.2M cacodylate buffer (pH 7.4)) and then taken through a graded alcohol dehydration series. Once dehydrated, the specimens were placed in a critical point dryer, mounted and placed in a gold coater. Once gold coating is complete, specimens were ready to be viewed on the SEM. Images were scanned on a digital imaging system by computer enhancement.

(iii) Hemolysis assay of docetaxel –loaded-microemulsion

The hemolysis assay was performed as described by Bulmus *et al.* [25]. In brief, freshly collected human blood in 5 ml Ethylenediamine tetraacetic acid (EDTA) containing vacutainers were transferred into a clean test tube, marked with the total volume of the added blood, and then centrifuged at 4000 rpm for 5 min in order to separate the red blood cells (RBCs). The serum was removed and the RBCs were washed three times with 150 mM of NaCl solution. After removing the saline solution from the last wash, the volume was raised to the total volume of blood with 100 mM sodium phosphate buffered saline (PBS, pH 7.4) and gently mixed by inversion. In order to examine the effect of the drug microemulsion formulations (B-ME, 1mg/ml of F-ME and O-ME), a 200 µl of the tested sample was mixed with 200 µl of RBCs and 600 µl of PBS. The negative control was prepared by mixing 200 µl of RBCs with 800 µl of PBS, whereas the positive control was produced by adding 200 µl of RBCs into 800 µl of de-ionized water. All of the samples were mixed by inversion and placed in a water bath at 37ºC for one hour and then, centrifuged for 5 min. Finally, the supernatant was collected and its absorbance was measured at 541nm.The hemolysis percentage was calculated by the following equation:

$$\text{Hemolytic activity (\%)} = \frac{(\text{Absorbance of sample} - \text{Absorbance of negative control})}{\text{Absorbance of positive control}} \times 100$$

(iv) Cell culture

A549, HCT-116 and HFS cell lines were cultivated in a tissue culture flask (75 cm^2) containing 10 ml of MEM media supplemented with 10% (v/v) heat inactivated fetal calf serum at 37°C in a 95% air and 5% humidified CO_2 incubator. Medium was discarded from the tissue culture flask and changed at 48 h intervals. Whenever the cells get confluent after continuous feeding, they were collected by trypsinization, washed and passaged every 3 days. Cells, used for experiments, were

between passages 7 and 11. They were dissociated with 2 ml of trypsin (0.15M) added to the tissue culture flask, left for few seconds and then discarded two times with expanding the second time to three min. The experimental cells were incubated in a MEM media (10% FBS) for 24 h in a 95% air and 5% humidified CO_2 incubator at 37°C.

(v) Screening of the antiproliferative effect using SRB assay

SRB assay was performed according to the method of Skehan *et al.* [26]. Cultured cells, A549, HCT116 and HFS, were counted using hemocytometer and seeded at a density of 1 x10^5 cells per well containing 0.1 ml of growth medium. After that, cells were incubated with 0.1 ml of media containing (1, 5 and 10) µM of B-ME, F-ME and O-ME and Taxotere solubilized in the media (triplicate wells were prepared for each individual concentration) and re-incubated for additional 48 h at 37°C in a humidified 5% CO_2. Untreated cells were used as control. After the time of incubation, cells were fixed by gentle layering with 50 µl of cold 50% TCA on the top of growth media in each well. The cultures were incubated at 4ºC for one hour and then washed five times with tap water to remove TCA and left for drying at room temperature. TCA-fixed cells were stained for 30 min with 0.4% (wt/vol) of SRB dissolved in 1% acetic acid. At the end of the staining period, SRB was removed and cultures were quickly rinsed four times with 1% acetic acid to remove the unbound dye. Bound dye was solubilized with 10 mM unbuffered Tris-EDTA (100 µl/well) for 5 min on a gyratory shaker. The numbers of living cells were assayed by measuring the color intensity using enzyme-linked immunosorbent assay, ELISA, reader at a wave length of 490 nm. The ratios of vital cell to dead cells were determined to evaluate the cytotoxicity of B-ME, D-ME, O-ME and Taxotere against A549, HCT116 and HFS cells. The cytotoxicity effect was determined by measuring the percentages of cell viability using the following equation:

$$\text{Cell viability (\%)} = \frac{\text{Absorbance af the sample}}{\text{Absorbance of the control}} \times 100 ,$$

where the absorbance of the sample and the absorbance of the control were defined as the absorbance of the treated and untreated cells, respectively.

(vi) Characterization of cell morphology using light microscope

Cultured cells of A549, HCT-116 and HFS were counted and plated at a density of 1 x10^5 cells per well into 96-well, flat-bottomed tissue culture plates containing 0.1 ml of growth medium per well. Cells were incubated with 200 µl of media containing (1, 5, 10) µM of B-ME, F-ME and O-ME. Then, they were washed with 100 µl of PBS for 5 min. After that, 4% of formaldehyde was added for 5 min and then, discarded and stained with a 100 µl of 10% Coomassie blue dye for 10 min. Finally, the stain was discarded and the cells were washed with tap water five times, and left to dry overnight at room temperature. Morphological changes were observed by phase contrast inverted microscope (1X17 Olympus, Japan).

(vii) Apoptosis detection using ApopNexin FITC assay

The signs of apoptosis induced by 5 µM of B-ME, F-ME, O-ME and Taxotere were inspected by ApopNexin FITC Apoptosis Detection kit (Millipore, Lot. No. 2053919, Billerica, MA, USA). This kit uses a staining protocol in which the apoptotic cells are stained with annexin V conjugated with fluorescein isothiocyanate (FITC) (*green fluorescence*) which stains phosphatidylserine (PS). All cultured cells of A549, HCT-116 and HFS were plated in 24-well plates (2×10^4 cells per well) and incubated for 24 h. The formulations of 5 µM of B-ME, F-ME, O-ME and Taxotere were introduced to the cells and incubated for another 48 h. The supernatant containing detached cells was removed and put into a tube, centrifuged at 400 x g for 5 min. Then, the supernatant was taken

and the adherent cells were washed twice with cold buffer solution. After that, the detached cells were re-suspended in ApopNexin FITC diluted in 1X binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) and transferred back into the container of adherent cells and incubated for 15 min at room temperature. The supernatant containing detached cells was removed and spun down at 400 x g for 5 min; the cells were re-suspended in 1X binding buffer. Adherent cells were harvested by scraping with a rubber policeman in 200 µl of 1X binding buffer, centrifuged at 400 x g for 5 min and re-suspended in 1X binding buffer (1×10^4 cells/mL). After transferring 100µl of adherent and detached cells to a tube, 2 µl of propidium iodied (PI) were added followed by incubation for 15 min in ice-cold and dark. All cells were viewed using fluorescence microscope (BX61, DP72 Olympus, USA). The positive of Annexin V-FITC indicates the out-releasing of phospholipid phosphatidylserine (PS), which happens in the early stage of apoptosis. Therefore, the apoptotic cells were identified as Annexin V-FITC$^+$ and PI$^-$. The nonviable cells were identified as Annexin V-FITC$^+$ and PI$^+$ and viable cells as Annexin V-FITC$^-$ and PI$^-$.

(viii) Statistical analysis

All values were expressed as mean ± standard deviation $(\bar{X} \pm SD)$ of the obtained data from the experiments (each experiment was performed in triplicate). Statistical analyses were performed using one- way analysis of variance (ANOVA) test, two- way ANOVA test and independent sample *t*-test using the MegaStat. The statistical significance difference was considered when p-value ≤ 0.05.

RESULTS

1 Characterization of docetaxel -loaded - microemulsion using SEM

The morphologies and sizes of the microemulsion droplets were determined for both of B-ME and F-ME. As exhibited in Figure 1, it has been found that the mean diameter of microemulsion droplets sizes of B-ME was 89.32 ± 25.49 nm with a percentage of variation coefficient (%CV) of 28.54, measured by dividing the standard deviation by the average multiplied by 100. On the other hand, the droplet sizes of F-ME have increased to 205 ± 13.10 nm with a %CV of 6.38. Both of the droplets of B-ME and F-ME were spherical.

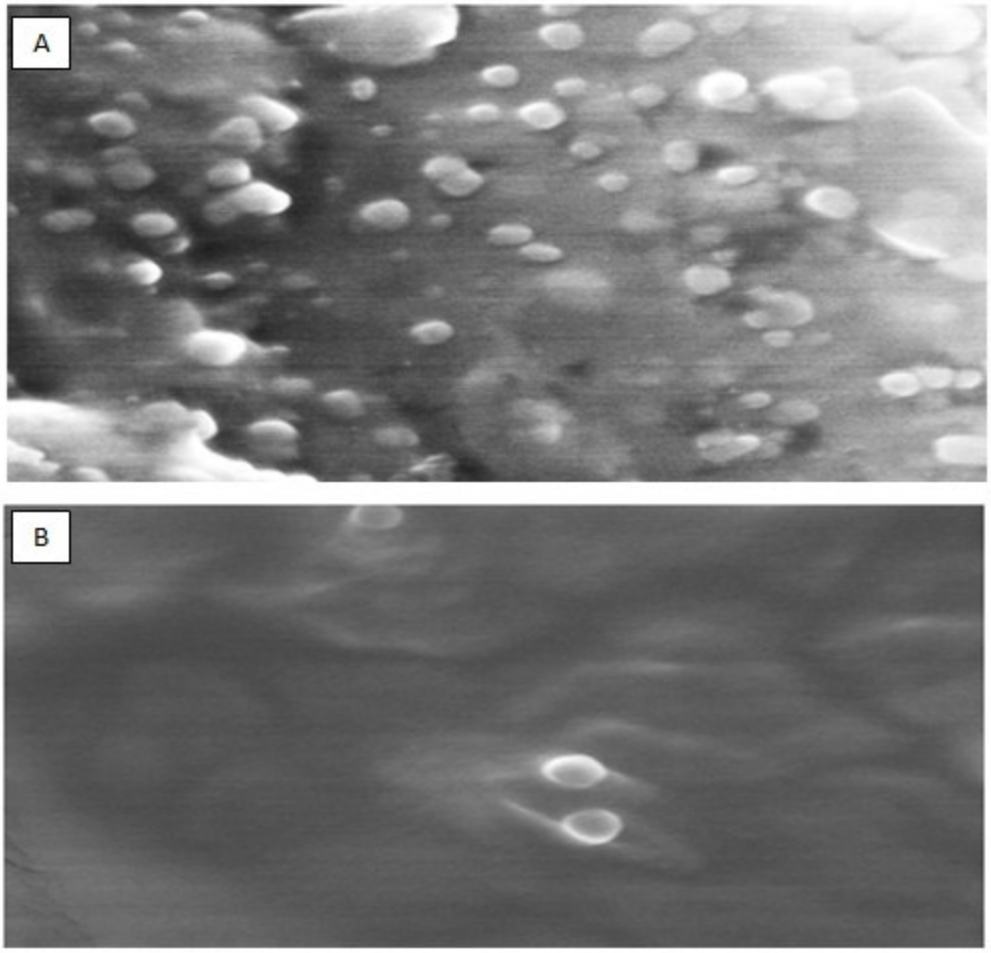

Figure 1
Scanning electron micrographs of (A) B-ME and (B) F-ME.

2 Hemolysis Activity of docetaxel -loaded –microemulsion

The hemolytic activity assay was performed in order to detect the percentages of the RBCs that lysed when get administered with the microemulsion formulations (B-ME, 1mg/ml of F-ME and O-ME). According to Figure 2, it has been found that both of F-ME and O-ME have more hemolytic activity than B-ME. Regarding the docetaxel-loaded microemulsion formulations, the time of the drug storage did not affect the hemolytic activity as there was no significant difference between the F-ME and O-ME ($p > 0.05$).

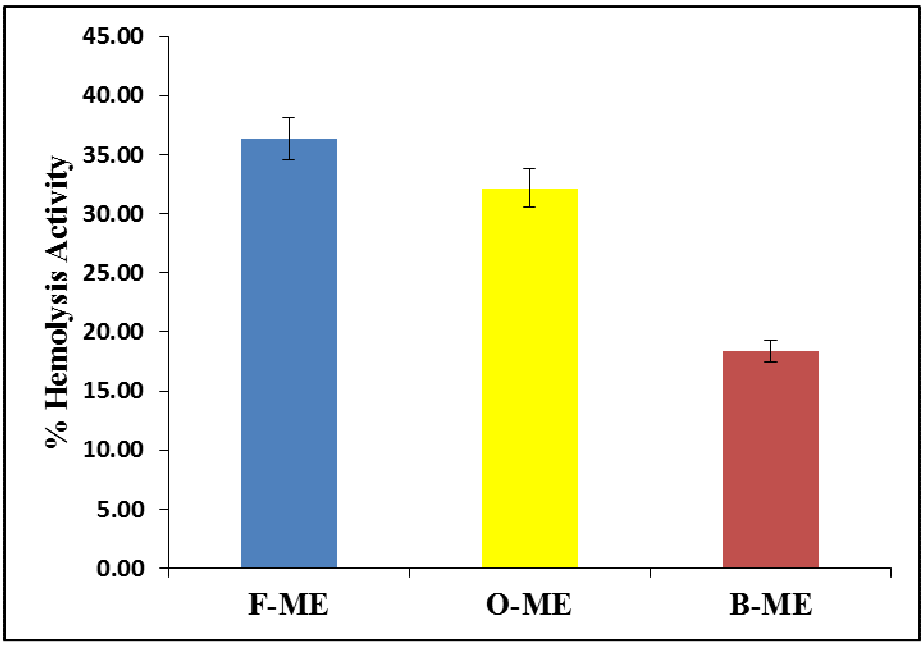

Figure 2
Hemolysis activity of microemulsion formulations. Error bars indicate the standard deviations of triplicate determinations (n = 3).

3 Screening of the antiproliferative effect using SRB assay

As illustrated in Figure 3a, when different micromolar concentrations (1, 5 and 10) of the microemulsion formulations (F-ME, O-ME and B-ME) and Taxotere were subjected into A549 cells, it has been found that all of the microemulsion formulations have the least percentages of cell viability in the range of 14.87- 22.99 at a concentration of 5µM, while the formulas at concentrations of 1 and 10 µM were having similar cytotoxicity in the range of 42.38 – 58.32. In contrast, the percentages of cell viability have decreased as the concentration of Taxotere increased from 1 to 10 µM. It should be noted that the cytotoxicity of the entire microemulsion formulations and Toaxotere were similar at 1 µM. However, the percentages of cell mortalities, when treated with 5 µM of F-ME, O-ME and B-ME, were significantly more than 5 µM of Taxotere by 2.38, 2.6 and 2 fold, respectively. On the contrary, all of the microemulsion formulations were significantly less cytotoxic than Taxotere at 10 µM. In contrast, microemulsion formulations subjected unto HCT116 cells have had similar antiproliferative effect at different concentrations, which were significantly more cytotoxic than Taxotere (Figure 3b). On average, the cell mortalities, when subjected into the microemulsion formulations at different concentrations, were more than Taxoere by around 2 folds. In order to examine the safe effect of the microemulsion formulation on the healthy human cells, F-ME, O-ME, B-ME and Taxotere were applied into HFS human foreskin (Figure 3c). At 1 and 10 µM, it has been found that O-ME was having the least cytotoxic effect, while Taxotere was having the most cytotoxic effect against HFS cells. On the other hand, F-ME was having the most cytotoxic effect, while O-ME was having the least cytotoxic effect at 5 µM.

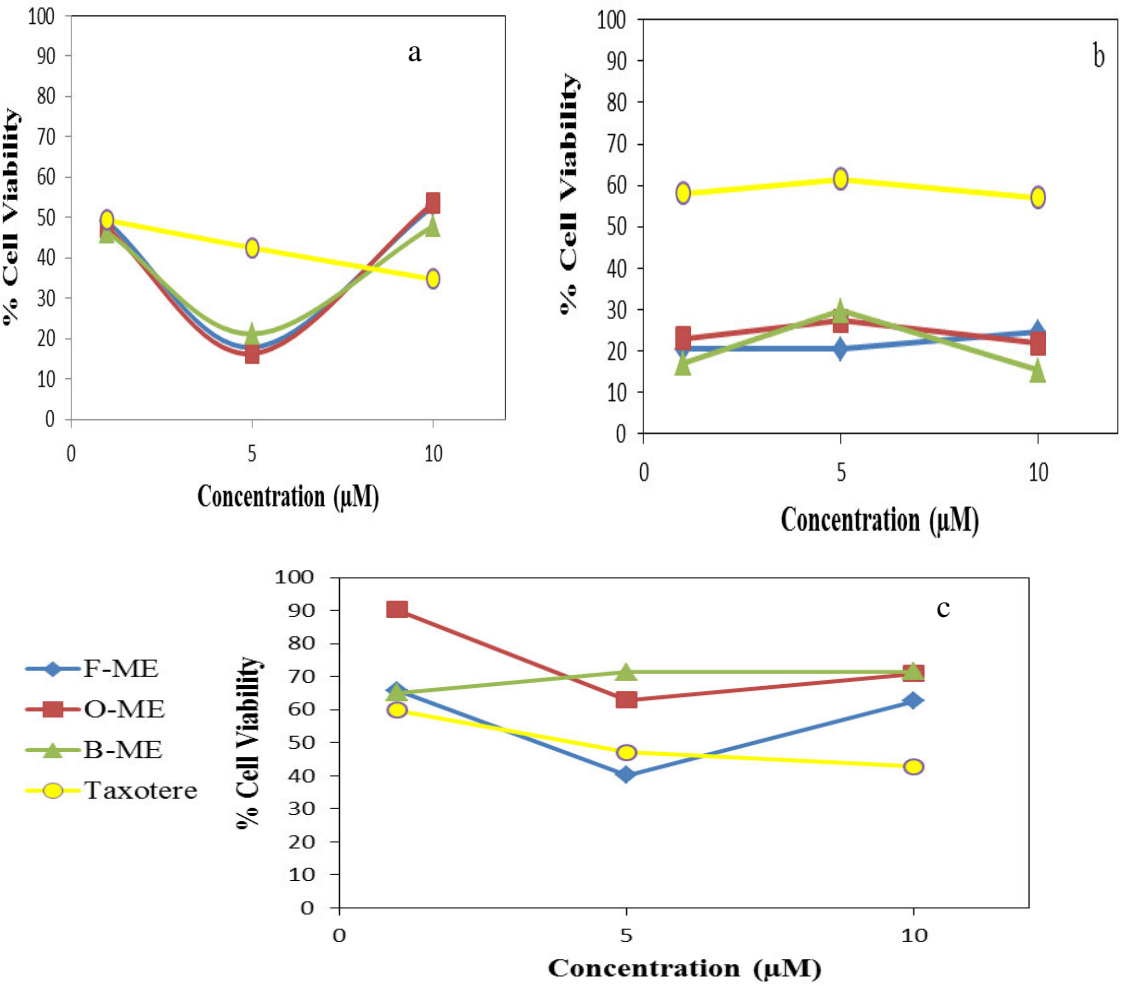

Figure 3

The percentages of cell Viability. (a) A549 (b) HCT116 and (c) HFS cells were subjected for 48 h into different micromolar concentrations (1, 5 and 10) of B-ME, F-ME, O-ME and Taxotere.

4 Characterization of cell morphology using light microscope

As exhibited in Figure 4, it was so obvious that the chromatids of A549 cells were fragmented and condensed, when treated with B-ME, O-ME and F-ME. Additionally, membrane blebbing and more intracellular spaces took place between the treated cells. Interestingly, the treated A549 cells were reduced, got accumulated and became more condensed, when treated with 5 µM of the entire microemulsion formulations (F-ME, O-ME and B-ME). The effect of the microemulsion formulations on the HCT 116 cells was similar to their effect on A549 cells, especially at 5 µM, as shown in Figure 5. However, at concentrations of 1 and 10 µM of all of the microemulsion formulations, the number of the cells get reduced as well as their shape have got changed. In order to study the safe effect of the microemulsion formulations, they were subjected in to the HFS cells. As displayed in Figure 6, it was so clear that they did not cause any change in the structure of the cells except the 1µM of O-ME that reduced the number of the cells besides changing their shape and forming apoptotic bodies.

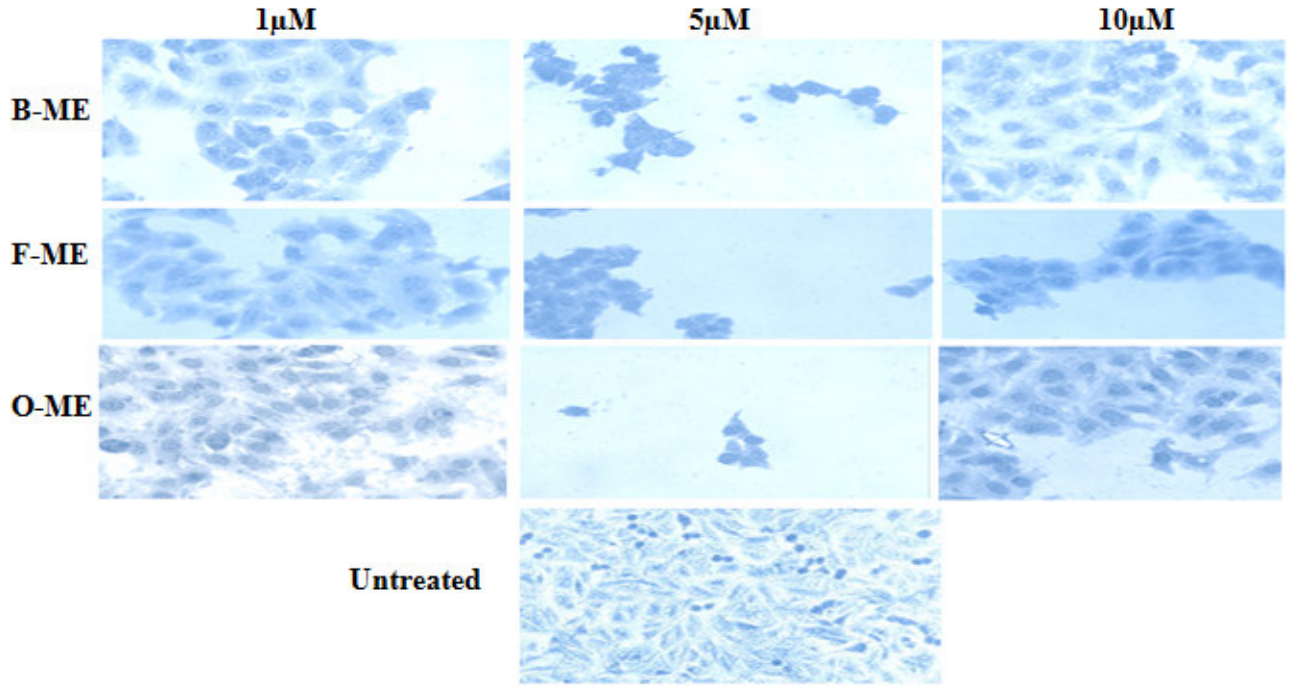

Figure 4

Light microscopic images of A549 cells. Cells were treated with B-ME, F-ME and B-ME at different concentrations of (a) 1 µM, (b) 5 µM, (c)10 µM. Images were magnified at 400 µm.

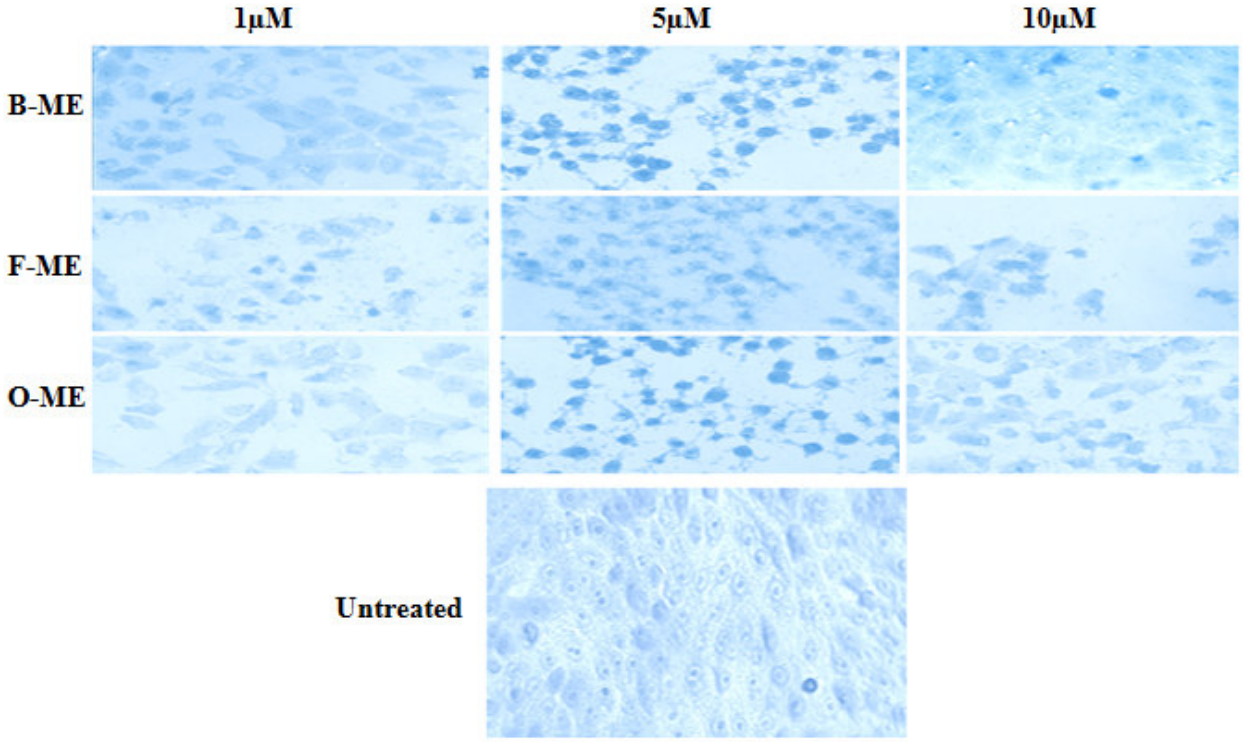

Figure 5

Light microscopic images of HCT116 cells. Cells were treated with B-ME, F-ME and B-ME at different concentrations of (a) 1 µM, (b) 5 µM, (c)10 µM. Images were magnified at 400 µm.

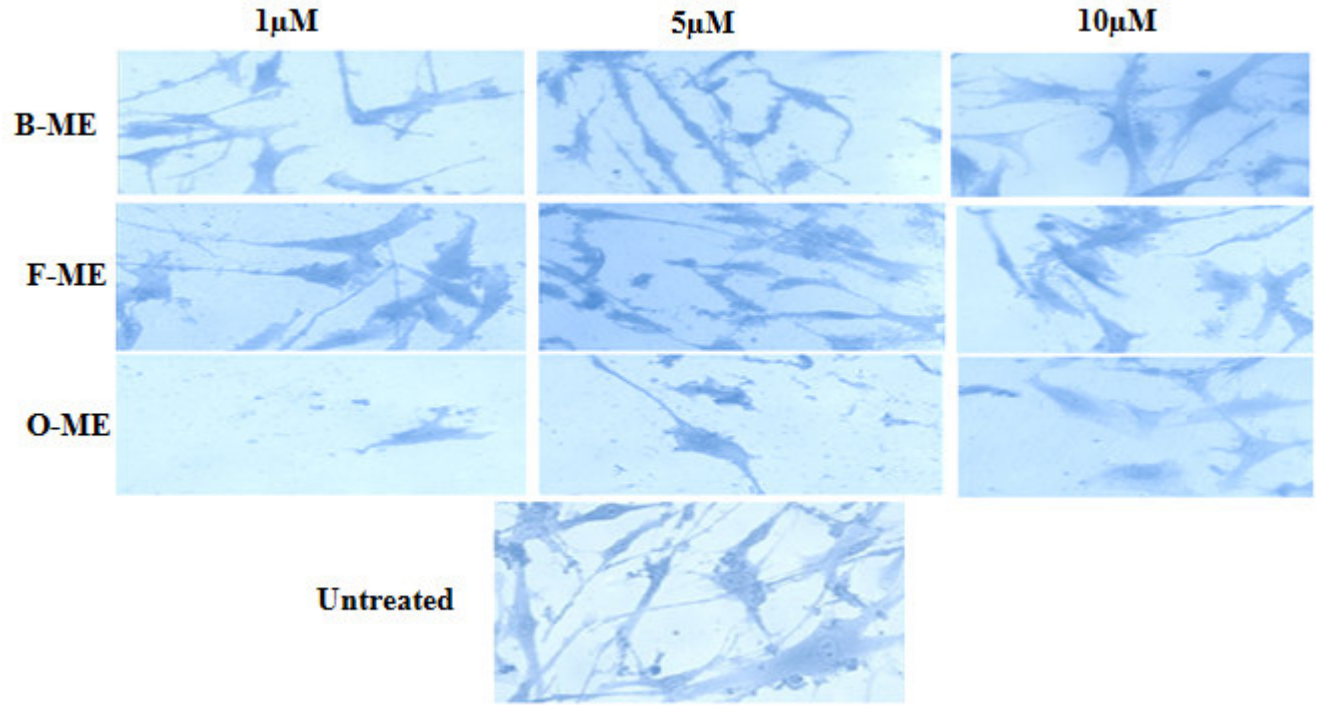

Figure 6

Light microscopic images of HFS cells. Cells were treated with B-ME, F-ME and B-ME at different concentrations of (a) 1 µM, (b) 5 µM, (c)10 µM. Images were magnified at 400 µm.

3.5 Apoptosis detection using ApopNexin FITC assay

In order to clarify the mechanism of cell death whether there were signs of apoptosis or necrosis, ApopNexin FITC apoptosis detection kit was employed. As shown in Figure 7, the untreated cells didn't stain positively with neither dyes which indicates the viability of cells, while all the treated cells (A549, HCT116, HFS) with F-ME, B-ME and Taxotere displayed green fluorescent dye with different levels of intensity which indicates that the percentages of the cells undergone apoptosis varied by the applied formula. On the other hand, some cells exhibited red fluorescent dye which implies that the cells were lysed and have passed secondary necrosis due to the presence of late stage apoptotic bodies since they do not get deleted by the phagocytosis in suspension cultures. It has been found that A549 cells were more affected by both of B-ME and F-ME than Taxotere, unlike HCT 116 cells that were affected by all of the subjected formulas. In contrast, HFS cells were slightly affected by all of the formulas as slight fluorescent dyes were observed.

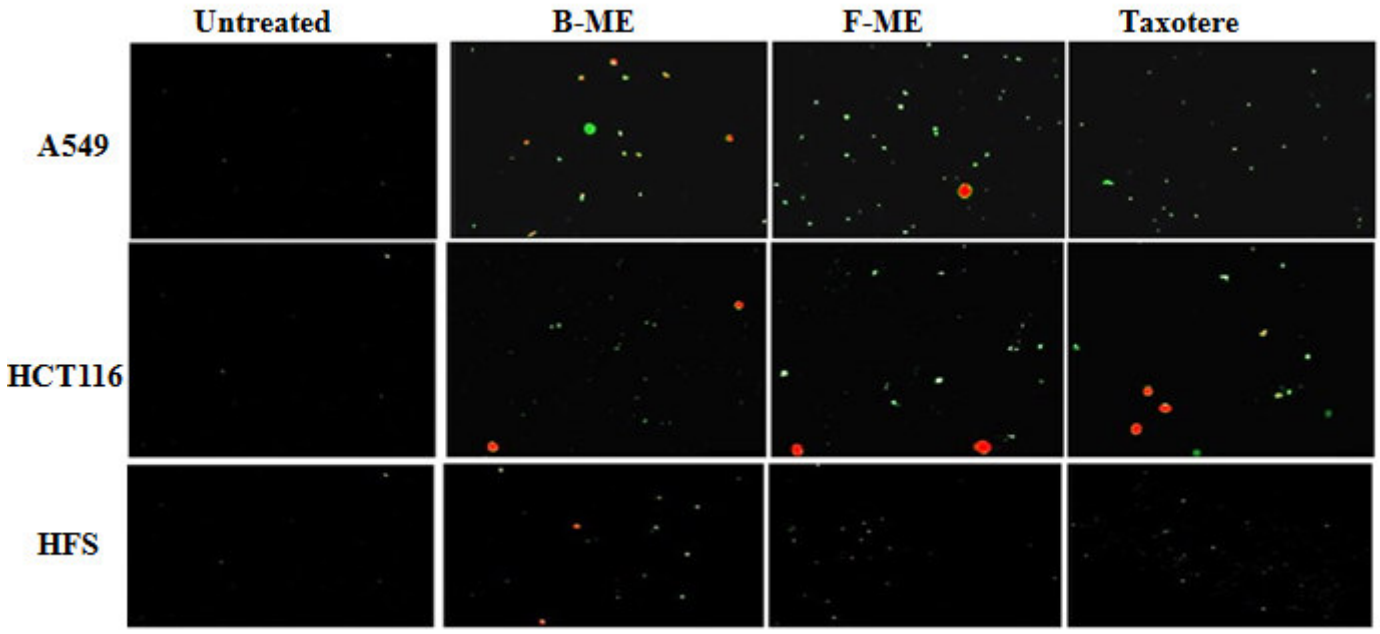

Figure 7

Fluorescent microscopic images of A549, HCT116 and HFS cells. Cells were labelled with Annexin-V-FITC and propidium iodide and were treated with 5 µM of B-ME, F-ME and Taxotere. Images were magnified a 200µl.

DISCUSSION

Cytotoxic drugs are still the major form of chemotherapy for the treatment of cancers. The actions of these drugs are primarily related to the cell cycle [27]. Docetaxel is mainly used for the treatment of breast, ovarian, prostate, and non-small cell lung cancer. It is more cytotoxic than doxorubicin, paclitaxel and fluorouracil. Currently, docetaxel is administered in a parenteral formulation for locally advanced or metastatic breast cancer with a first-line chemotherapy regimen. However, it is not convenient to apply it orally due its low bioavailability and high tendency to attach to the multidrug resistance protein 1. The microemulsions have recently attracted much attention in pharmaceutical research areas [28, 29, 30]. High thermodynamic and kinetic stability, low viscosity and optical transparency make them very attractive as a pharmaceutical application form to improve the solubility, the dissolution and the oral absorption of poorly water-soluble drugs [31]. Further advantages of using microemulsions as drug delivery systems include a better drug solubilization and the protection against enzymatic hydrolysis, as well as the potential for an enhanced absorption due to a surfactant-induced improvement in the permeability. In addition, microemulsions represent an interesting and potentially quite powerful alternative carrier system for drug delivery because of their high solubilization capacity, transparency, ease of preparation, and high diffusion and absorption rates, compared to solvents without the surfactant system [9, 32]. The combination of surfactants with oils to form microemulsion offers an advantage with a low free energy and a large surface area, which were considered to be responsible for transporting drugs to cancer tissue membrane for absorption [13]. A recent study has solubilized more docetaxel (30 mg/ml) by loading it in a microemulsion formula that composed of weight percentages of 37 of cremophore/transcutol mixture (2:1, w/w), 29 of capryol 90 and 34 of water [9]. The oral bioavailability of the microemulsion formula

have improved significantly, relative to the Taxotere, which was explained by the enhancement in solubility, the inhibition of P-gp efflux system and the increase in permeability. This microemulsion formula was produced in our study in order to evaluate it against A549 and HCT116 cells. The mean diameter of the microemulsion droplet, determined by SEM, was 89.32 ± 25.49 which is much higher than the droplet size, determined by Yin *et al.* [9] study, which was 35.1 ± 3.1, measured by the electrophoretic light-scattering (ELS) spectrophotometer. Actually, the specimens undergo drying in the SEM processing that would affect their droplet sizes, while the ELS technique correlate between the scattered light and the droplet diameter without changing the state of the sample. In contrast, SEM images display the morphology and the accumulation of the droplets in the microemulsion formulas which is not observed in ELS spectrophotometer. In addition, according to this research study, the droplet mean diameter of the microemulsion formula loaded with docetaxel, noting that it was not measured before, have increased significantly to 205.3 ± 13.10 but their morphology did not change as they were spherical, which indicates that the drug have got encapsulated in the droplet. The hemolysis activities of 1mg/ml of both of F-ME and O-ME were 36 ± 7.61 and 32.15 ± 6.32, respectively. At less than this concentration by 10 fold (100µg/ml), Taxotere has reported more than 40 % RBC lysis [33]. The main component in Taxotere is Tween 80, which has been reported to interact with cell membrane of RBC and cause significant hemolytic activity [34]. Upadhaya *et al.* [35] have formulated docetaxel in poly (g-benzyl L-glutamate)-block-hyaluronan polymersomes, which was non-hemolytic (less than 5% hemoglobin released) at concentrations ranged from 10 -100 µg/ml. However, they did not determine the hemolytic activity at higher concentration. The storage of the drug in the microemulsion formulation for around two weeks (O-ME) did not affect the toxicity of the drug formulations against the erythrocytes. Interestingly, B-ME has had less hemolytic activity (18.32 ± 1.64) at higher concentrations of 1mg/ml, which means that the cremophore have a slight effect on the cell membrane of RBCs, compared to Tween 80 [34].

The *in vitro* evaluation of the microemulsion formulations included identifying the cell toxicities against A549, HCT116 and HFS cells by using the SRB assay. The cytotoxicity screening revealed that among all the tested drug microemulsion formulations and Taxotere at all concentrations, subjected unto A549 non-small cell lung cancer cells, F-ME, O-ME and B-ME were the most cytotoxic at 5 µM (2 folds), verified by the light microscopy images which displayed late signs of apoptosis. Additionally, the fluorescence microscopic images labelled with Annexin-V-FITC and PI have exhibited condensed green and red flourecent, especially when treated with F-ME and B-ME, which indicate signs of apoptotic effect of the drug formulations. At 10 µM, Taxotere was more cytotoxic than all of the microemulsion formulations (F-ME, O-ME and B-ME). However, all of the microemulsion formulations (F-ME, O-ME and B-ME) and Taxotere were having similar antiproliferative effect at 1 µM. On the other hand, the average cytotoxicities of all of the microemulsion formulations (F-ME, O-ME and B-ME) against HCT116 cells were more than Taxotere by 2 folds. The effects of the microemulsion formulations (F-ME, O-ME and B-ME) at all micromolar concentrations (1, 5 and 10), except at 1 µM of B-ME, on the HFS cells were safe as revealed by light microscopy images and fluorescence microscopic images labelled with Annexin-V-FITC and PI. Docetaxel was encapsulated in a Tween 80-free liposomal formulation with better pharmacokinetic properties than free docetaxel, while maintaining the same in vitro cytotoxicity [4]. Additionally, drug plasma concentration as well as drug disposition up to 24 h was considerably increased in docetaxel liposomal formulation than in Tween 80. Moreover, docetaxel was recently formulated in a liposome that has a pH-dependent release behavior, which would be favorable for selectivity against tumor cells [13]. Compared with Duopafei®, the liposomal formula of

docetaxel gave a prolonged residence time of the drug in mice and an improved efficiency to reticuloendothelial system (RES) organs, such as the liver and spleen, but not to non-RES organs, which might potentially contribute to decrease the risk of toxicity. Furthermore, a 0.8 mg/ml of docetaxel was encapsulated in the emulsion that composed of 10% oil phase (Soya oil and Miglyol 812), 1.2% soybean lecithin and 0.3% Pluoronic F68 [36]. The submicron lipid emulsion showed promising intravenous carrier in place of the available commercial ones with more efficiency. Zhao *et al*. [10] have also formulated docetaxel in lipid emulsion that exhibited higher plasma concentrations in rats than Taxotere but bioequivalent to it in beagle dogs. The docetaxel loaded-lipid emulsion displayed safe effect and exhibited antitumor activities against the A549, BEL7402 and BCAP-37 cell lines in nude mice, similar to Taxotere. Hwang *et al.* [6] have produced a nano-sized drug carrier, consisted of hydrophobically modified glycol chitosan, which easily loaded the docetaxel. The docetaxel-loaded nanocarriers showed higher antitumor efficacy such as reduced tumor volume and increased survival rate in A549 lung cancer cells-bearing mice and strongly reduced the anticancer drug toxicity compared to that of free docetaxel in tumor-bearing mice. Moreover, formulating docetaxel in lecithin nanoparticles have enhanced the drug oral bioavailability by 3.65 folds, compared to the free docetaxel [5].

Ma *et al.* [12] have incorporated Tween 80 into poly-ε-caprolactone (PCL) to produce a novel nanoparticle composed of PCL-Tween 80 copolymer, which could encapsulate 10% of docetaxel. The docetaxel-loaded PCL-Tween 80 nanoparticles showed better *in vitro* cytotoxicity towards C6 cancer cells than commercial Taxotere at the same drug concentration. Some researches attempted to encapsulate docetaxel in micelles in order to improve its efficiency and reduce its side effects. Liu *et al*. [7] have evaluated the novel docetaxel-loaded micelle which was based on the biodegradable thermosensitive copolymer poly(N-isopropylacrylamide-co-acrylamide)-b-poly(dl-lactide). Docetaxel-loaded- micelle showed reduced toxicity and higher antitumor efficacy in mice as well as enabling a prominent higher docetaxel concentration in tumor than conventional docetaxel formulation. It also caused less body weight loss in mice. Another recent study have incorporated docetaxel into mixed micelles that comprised of methoxy poly(ethylene glycol)–poly (lactide) polymer and Pluronic copolymers. The micelle formula has enhanced the bioavailability (3.6 fold) and overcome the multidrug resistance of docetaxel in rats [11]. Docetaxel was recently formulated in a solid lipid nanoparticle. Xu *et al.* [8] produced a new docetaxel-loaded hepatoma-targeted solid lipid nanoparticle (tSLN) that was prepared with galactosylated dioleoylphosphatidyl ethanolamine. The anticancer activity of tSLNs against liver cancer cells was improved.

CONCLUSION

In this study, the microemulsion formula, consisted of weight percentages of 37 of cremophor/transcutol mixture (2:1, w/w), 29 of capryol 90 and 34 of water, was produced. The mean diameter of the droplet, determined by SEM, was 89.32 ± 25.49 nm, while the microemulsion formula loaded with docetaxel, noting that it was not measured before, have increased significantly to 205.3 ± 13.10 nm. The morphologies of both droplets were spherical. The storage of the drug in the microemulsion formulation for around two weeks (O-ME) did not affect the toxicity of the drug formulations against the erythrocytes as the hemolysis activities of 1mg/ml of both of F-ME and O-ME were 36 ± 7.61 and 32.15 ± 6.32, respectively. Interestingly, the cremophore have slight effect on the cell membrane of RBCs, compared to Tween 80, as B-ME has had less hemolytic activity (18.32 ± 1.64) at higher concentrations of 1mg/ml. The cytotoxicity screening using SRB assay revealed that among all of the drug microemulsion formulations and Taxotere at all concentrations, subjected unto A549 non-small cell lung cancer cells, F-ME, O-ME and B-ME

were the most cytotoxic at 5 µM, verified by the light microscopy images which displayed late signs of apoptosis. Additionally, the fluorescence microscopic images labelled with Annexin-V-FITC and PI have exhibited condensed green and red flourecent, especially when treated with F-ME and B-ME, which indicate signs of apoptotic effect of the drug formulations. At 10 µM, Taxotere was more cytotoxic than all of the microemulsion formulations (F-ME, O-ME and B-ME). However, all of the microemulsion formulations (F-ME, O-ME and B-ME) and Taxotere were having similar antiproliferative effect at 1 µM. The average cytotoxicities of all of the microemulsion formulations (F-ME, O-ME and B-ME) against HCT116 cells were more than Taxotere by 2 folds. The effects of the microemulsion formulations (F-ME, O-ME and B-ME) at all micromolar concentrations (1, 5 and 10), except at 1 µM of B-ME, on the HFS cells were safe as revealed by light microscopy images and fluorescence microscopic images labelled with Annexin-V-FITC and PI. In conclusion, encapsulating docetaxel in a microemulsion formula enhanced its efficacy and decreased its side effect.

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