Cytotoxicity Effect of the Combination of Doxorubicin and Pravastatin Loaded in Lipid Nanoemulsion on MCF-7 Breast Cancer Cells and HFS Human Foreskin Cells

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ABSTRACT

Mixing two antitumor agents in a nanocarrier is one of the potential suggested strategies in treating cancer. In this study, the anticancer activity of the combination of doxorubicin (DOX) and pravastatin (PRV) in a lipid nanoemulsion (DOX-PRV-NE) was evaluated in MCF-7 breast cancer cells and HFS human foreskin cells. According to the transmission electron microscopy, the droplets of DOX-PRV-NE were spherical and homogeneously distributed with a mean diameter of 34.8 ± 1.78 nm. It has been found that reducing the concentration of DOX from 15 to 7.5 µM by formulating it with 7.5 µM of PRV in DOX-PRV-NE has reduced the side effect of DOX on HFS cells and red blood cells while preserving its cytotoxicity against MCF-7 cells. The present study proved that the combination of the PRV and DOX in a nanodroplet improved the therapeutic potential of PRV as an anticancer drug and reduced the side effect of DOX on normal cells.

Key Words: : Nanoemulsions, Cytotoxicity, Apoptosis, DAPI assay, Hemolysis assays, MTT assay

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INTRODUCTION

Incorporating more than one chemotherapeutic agent in a nanocarrier would promote different mechanisms of actions on the cancer cells which could ameliorate the therapeutic index of the drugs and suppress their serious side effects [1]. Doxorubicin (DOX) is one of the common chemotherapeutic agents that is used to treat various cancers. However, its antitumor action is associated with acute and chronic side effects, such as myelosuppression, nausea, vomiting and arrhythmias [2].

Pravastatin (PRV), a hydrophilic statin, is a cholesterol-lowering drug found to have pleiotropic effects including cancer treatment and prevention [3]. Numerous in vitro experimental data validated that statins exhibit antiproliferation and apoptotic effects against various solid tumor cells of different origins [4]. Combining statins with other anticancer agents has attracted the attention of many pharmaceutical industries due to the possibilities of enhancing the efficacy of the drugs against the cancer cells while reducing their adverse effects on the healthy cells [5]. Jakobisiak and Golab [6] have demonstrated that mixing statins with anthracyclines has had a great antitumor effect on tumor bearing mice. Statins have also ameliorated the efficacy of many chemotherapeutic agents including cisplatin, paclitaxel, 5-fluorouracil and melphalan [7].

The objective of the present study was to in vitro evaluate a lipid nanoemulsions (NE) formulations designed by Alkhatib and Albishi [8] with some modifications as a drug

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carrier for the mixed PRV and DOX applied into human breast cancer cell line (MCF-7) and human foreskin cell line (HFS).

2. MATERIALS AND METHODS

2.1. Chemicals and subjects

DOX and PRV were purchased from the U.S. Pharmacopeia (Rockville, US). All of the constituents of the NE were obtained from Sigma Aldrich (Missouri, US). Chemicals used in the tissue culture were procured from Invitrogen life technologies (New York, US). Dialysis membrane (Molecular weight cut-off size 12,000 Da) was purchased from spectra lab (California, US). The human cell lines of MCF-7 breast cancer and the HFS human foreskin were obtained from American Type Tissue Culture Collection (Manassas, VA, USA).

2.2. Methods

2.2.1. Preparation of the drug-loaded nanoemulsion

The drug free-NE was produced by blending soya phosphatidylcholine (SPC), sodium oleate (SO) and polyoxyethylenglycerol trihydroxystearate 40 (Eumulgin® HRE 40, EU) at a weight fraction of 0.27, 0.32 and 0.34, respectively in a 98.88 (wt/wt) of 0.1 M Tris-HCl buffer (PH 7.22) containing 0.09 (wt/wt) of cholesterol and 0.1 (wt/wt) of 1-octanol. The mixture was vortexed and kept in a water bath at 90ºC for 7 days until the NE solution become clear and transparent. The produced NE formula was stored at room temperature.

2.2.2. Physical characterization of the drug formulas

Droplet morphology

The shape and droplet sizes of the NE formulas were determined by the transmission electron microscopy (TEM, Erlangshen ES500W, Gatan Company) at the unit of Electron microscope, Faculty of Sciences, KAU (Jeddah, KSA). A few drops of the desired NE formula were placed on carbon-coated grid and allowed to air dry after draining off the excess amount using a filter paper followed by fixation with 50 µl phosphotungstic acid. They were left for 2 min for drying purposes. The dried, coated grid was taken to a slide and covered with a cover slip for TEM observations. The tested formulas were the drug-free NE, 1mg/ml of DOX- loaded NE (DOX-NE), 1mg/ml of PRV-loaded NE (PRV-NE), and the mixture of 0.5 mg/ml of DOX and 0.5mg/ml of PRV loaded in NE (DOX-PRV-NE).

Absorption spectrum

The drug interaction with the nanocarrier was examined by identifying the change in the absorption at different wavelengths. The absorption spectrum, established by measuring the absorbance of the drug formula at different wavelengths, was performed in order to detect the wavelength at the maximum absorption (λ max) of the drug formula. All of the drug formulations, were scanned by using UV/Vis spectrophotometer (GENESYS 10S, Thermo Scientific™, USA) within the wavelength region of 200 – 600 nm.

2.2.3. Drug release test

The in vitro drug release test was performed as mentioned before [9]. 1 ml of the examined drug formula was introduced into a dialysis bag, sealed at both ends with clips, and suspended in a 250 ml beaker containing distilled water with stirring at 37ºC. A 1 ml of the sample was collected at regular time intervals (0, 1, 2, 3, 4, 5, 6, 7, 8 and 24 h) and then, replaced with the same amount of distilled water. The samples were analyzed by using UV/Vis spectrophotometer at λ_{max} of each drug formula determined by the absorption spectrum.

2.2.4. Hemolytic activity of drug formulas

The hemolysis assay was performed as described elsewhere [10]. In brief, a 5 ml of freshly collected human blood was centrifuged at 6000 rpm for 5 min followed by discarding the serum and collecting the precipitated red blood cells (RBCs) which were washed thrice by 150 mM sodium chloride (NaCl) and centrifuged for 5 min at 6000 rpm. After that, RBCs were suspended in 100 mM of sodium phosphate buffer (pH 7.40). Exactly 100 µl of 1mg/ml of the desired drug formula was mixed with 200µl of RBCs suspension. Following one-hour incubation of the mixture in a water bath at 37ºC, the mixture was centrifuged at 6000 rpm for 5 min. The absorbance of the collected supernatant was measured by a UV/Vis spectrophotometer at 541 nm. The sodium phosphate buffer served as a blank and deionized water served as the positive control. The experiment was done in triplicate and the percentages of hemolytic activities were calculated according to the following equation:

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

2.2.5. Anti-proliferation assay

The effect of the drug formulas on the proliferation of MCF-7 and HFS cells was measured by the MTT (3-(4, 5 Dimethylthiazole- 2-yl)-2, 5-diphenyl tetrazolium bromide) assay obtained from the Cayman's Chemical Company (Michigan, US). In each well of 96-well plate containing 100 µl of Dulbecco's modified eagle medium (DMEM), 5000 cells were cultured and kept in an incubator for 24 h at 37°C in a humidified 5 % CO_2. Cells were administered with 100 µl of different concentrations of the tested drug formula and incubated for 48 h at 37°C

in a humidified 5% CO_2. Following incubation, a 5 µl of MTT reagent was subjected to each well and kept for 4 h at 37°C in CO_2 incubator. After that, culture medium was removed from each well carefully and then 100 µl of the crystal dissolving solution was added to each well followed by shaking the 96-well plate for 10 min until a purple color was formed. Wells containing the untreated cells served as control. The absorbance of each sample was determined at 562 nm by ELISA plate reader (BioTek, US). The relative cell viability was measured by dividing the absorbance of the treated cells by the absorbance of the untreated cells.

2.2.6. Detection of cell death mechanism

Light microscopy

It was utilized to view the morphology of the treated cells subjected to the drug formula. A 1 x10^5 cells/500 µl of DMEM was cultured in each well of 24-well, flat bottomed culture plate and was administered with 500 µl of the tested formula. Following 48 h incubation at 37ºC in CO_2 incubator, cells were washed with phosphate- buffered saline (pH 7.22) and fixed with 4 % formaldehyde for 5 min. After the removal of the fixation solution, cells were stained with 10 % Coomassie blue for 10 min followed by washing twice with distilled water and drying for one hour at 25ºC. Cells were viewed with the phase contrast inverted microscope (1X2-SP Olympus, Japan).

ApopNexin FITC/PI assay

The ApopNexin Annexin V FITC Apoptosis Kit, purchased from Millipore (MA, US), was utilized to detect the early stages of apoptosis by probing the translocation of the phosphatidylserine (PS) from the inner membrane to the outer surface through using the Annexin V conjugated with fluorescein isothiocyanate (FITC). Another stain, propidium iodide (PI), was used to discriminate the apoptotic cells from the necrotic cells. 5×10^4 cells per well of 24-well plates were grown and incubated at 37ºC in a CO_2 incubator for 24 h. After that, cells were subjected into 500 µl of the tested formula for 48 h followed by rinsing with 300 µl of 10 mM of phosphate-buffered saline (pH 7.4). After the removal of the buffer, cells were detached with 200 µl of trypsin (0.15 %) and a 500 µl DMEM was added to each well to be transferred to a flow cytometry tube for centrifugation at 1000 rpm for 5 min. The resultant precipitate was washed with cold 10 mM of phosphate-buffered saline (pH 7.4) followed by replacing the washing solution with a 200 µl of ice cold 1X binding buffer (10 mM HEPES (N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid) /NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) at a concentration of 10^6 cells/ml. Finally, a 3 µl of FITC and 2 µl of PI were added to the cell suspension and kept for 15 min in the dark at 25ºC for measurement by flow cytometer (BD Biosciences, US).

2.2.7. Statistical analysis

The statistical differences between the tested samples, examined by the one- way analysis of variance (ANOVA) test and implementing the post-hoc analysis using *t*-test, were considered when the *p*-value < 0.05. Data were presented as a mean ± standard deviation for sample size of 5. The statistical analysis was performed by the MegaStat Excel (version 10.3, Butler University, Indianapolis, IN).

3. RESULTS AND DİSCUSSİON

3.1. Morphology And Sizes Of Ne Formulas Droplets

The morphology and droplet sizes of the NE formulations were viewed under the TEM as displayed in Fig. 1 and illustrated in Table 1. The droplets of all of the drug formulas were spherical. The mean droplet diameter of drug-free NE (30.2 ± 1.05 nm) was reduced by a factor of 1.7 when it was loaded with PRV (18.1 ± 0.5 nm). On the other hand, the mean droplet diameters of DOX-NE (68.9 ± 1.72 nm) was approximately double the mean droplet diameter of the drug-free NE. Interestingly, the combination formula, DOX-PRV-NE, has a mean droplet diameter of 34.8 ± 1.78 nm that was slightly greater than the mean droplet diameter of drug-free NE.

It is noteworthy to mention that the droplets of the entire NEs formulations were homogeneously distributed with no aggregation or adhesion among them as percentages of the coefficient of variations were less than 10%. In a previous study, it has been found that nanoparticles, within 40 - 50 nm, could induce apoptosis and target cells more efficiently than larger nanoparticles [11].

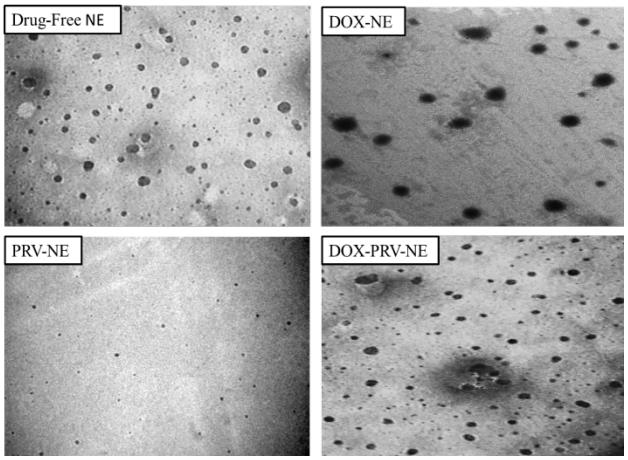


Fig. 1. The morphology of the NE formulas determined by the TEM. Images were magnified at 0.1 µm.

Table 1. The droplet sizes of the NE formulas determined by the TEM. There have been significant differences between all of the NE formulations in their sizes (n=15, P<0.05).

Formulations	Mean of droplet diameter (nm)	Range of droplet diameter (nm)	% CV*
Drug-free NE	30.2 ± 1.05	29.15-31.25	3.48
DOX-NE	68.9 ± 1.72	67.18-70.62	2.50
PRV-NE	18.1 ± 0.5	17.6-18.6	2.76
DOX-PRV-NE	34.8 ± 1.78	33.02-36.58	5.11

*%CV (coefficient of variation) is the ratio of the standard deviation to the mean multiplied by 100.

3.2. Absorption spectrum

The drug interaction with the nanocarrier was examined by determining the wavelength of the maximum absorption (λ_{max}) of the drug-free and drug formula. The absorption spectrum is constructed by plotting the absorbance of the sample at different wavelengths in order to detect the wavelength (λ_{max}) at which the absorbance is the greatest. As shown in Fig. 2 and illustrated in Table 2, formulating the DOX in NE formula resulted in decreasing the maximum absorption relative to DOX in the water without causing any shifting in the two λ_{max}'s, suggesting that the NE formula has suppressed the conjugation of DOX. Similarly, the maximum absorption of the combination formula, dissolved in NE, was less than the maximum absorptions of the water combination formula. In contrast, incorporating PRV into the NE did not cause any change in the structure of PRV as neither the maximum absorption nor the λ_{max} of the PRV-W has differed from the PRV-NE. In this study, the result of the absorption spectrum indicated that the NE formulations incorporated with DOX resulted in decreasing the maximum absorption relative to the water formulation of DOX, suggesting that the NE formula has suppressed the conjugation of DOX [12, 13].

3.3. Drug release test

In vitro drug release assays was implemented at regular time intervals (0, 1, 2, 3, 4, 5, 6, 7, 8 and 24 h) to make quantitative comparisons of drug leakage between the different solution and NE drug formulations. The shape of the curves, shown in Fig. 3, demonstrates that all types of drug formulas loaded in the NE were controlled release (zero-order) with fast leakage from the dialysis bag compared to the water formulations. The maximum percentages of drug release of DOX-NE, DOX-PRV-NE, and PRV-NE were within 4, 6 and 7 h, respectively, whereas the drugs loaded in water formulations have got released within 10 h. This result indicated that the NE have controlled release that help in the maintenance of constant drug level in the therapeutic range and thus an improvement of the circulation and efficacy of drug [14].

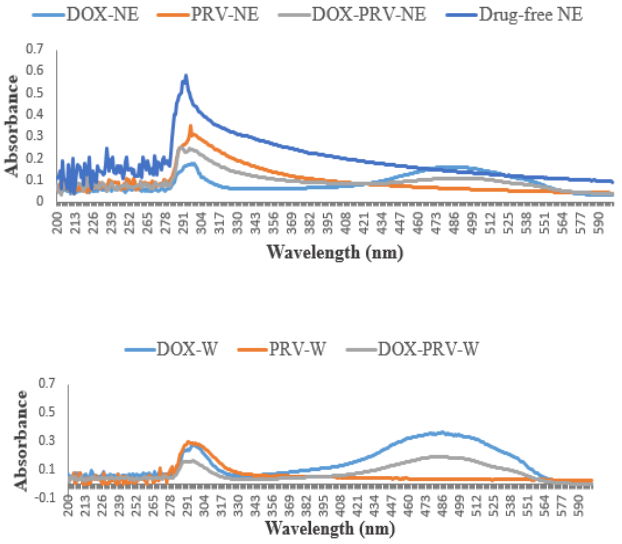


Fig. 2. The absorption spectrum of the solution and NE formulations

Table 2. The maximum absorption (λ_{max}) of different formulation of 1mg/ml of DOX, PRV and their combination loaded in water or NE formula as determined from the absorption spectrum

Formulation	λ_{max}	Absorption
DOX-W	486	0.367
DOX-NE	299	0.179
PRV-W	291	0.308
PRV-NE	296	0.3492
DOX-PRV-W	482	0.1924
DOX-PRV-NE	289	0.2498

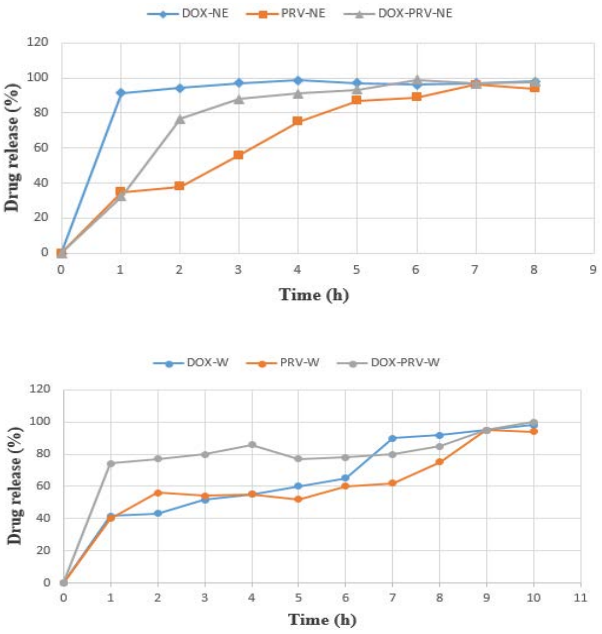


Fig. 3. The in vitro drug release profiles of the tested drug formulas

3.4. Hemolytic activity of drug formulas

It is very essential to *in vitro* evaluate the effect of chemotherapeutic drugs like DOX on the RBCs as they may cause anemia [15]. The hemolysis assay was employed to assess the damage of RBCs caused by 15 µM of different solution and NE drug formulations as illustrated in Fig. 4. The water drug formulations, DOX-W has the largest effect on the RBCs by revealing the maximum percentage of hemolysis activity of 23.70 ± 0.02, whereas PRV-W has the least effect on the RBCs as the hemolysis activity was 3.77 ± 0.01. Therefore, combining PRV with DOX in water has reduced the effect of DOX on the RBCs as the percentages of hemolysis activity of DOX-PRV-W was 13.07 ± 0.009. Further, the effect of the drug-free NE and DOX-NE were having a comparable hemolytic effect to the water combination formulas as their percentages of hemolysis activity were 13.08 ± 0.03 and 10.48 ± 0.03, respectively. In contrast, DOX-PRV-NE had revealed less hemolytic effect than DOX-PRV-W as the percentage of the hemolysis activity was 6.37 ± 0.03. It is noteworthy to mention that encapsulating PRV in NE formula has significantly increased its percentage of hemolytic effect to 9.01 ± 0.003.

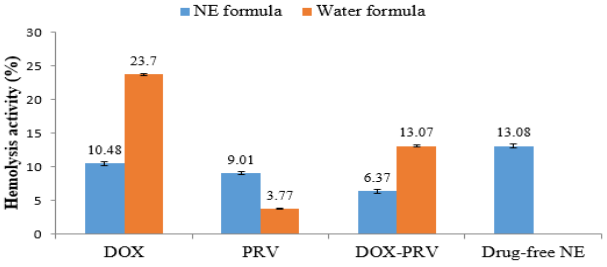


Fig. 4. The percentages of hemolytic activity of the drug formulas. Error bars represent the standard deviation. It should be noted that there have been a very highly diffferences between the NE and water formulas (n=3, P < 0.001).

3.5. Cytotoxicity screening using MTT assay

MCF-7 cells

As demonstrated in Fig. 5, the viability percentages of MCF-7 breast cancer cells varied significantly by increasing the concnceration of the various solution and NE formulations from 1 -15 µM (P < 0.05). At 1 µM, both of DOX-W and DOX-PRV-NE have the most cytotoxic effect as the % of cell viabilities were the least and comparable, whereas both of DOX-NE and DOX-PRV-W have similar and less cytotoxicity. The drug-free NE formula has the least antiprolifareative effect at all concntrations when compared to the other tested formulas (P< 0.05). At 10 µM, the least % of cell viabilities were recorded when the cells were subjected into the DOX-W and DOX-NE. On the other hand, the viabilities percentages of the cells subjected into the the DOX-PRV-NE were considerably greater than the viabilities of the cells administered with DOX-PRV-W (P < 0.05). At 15µM, DOX-NE has the best cytotoxic effect while the other drug formulas have similar toxicity. Regarding the single treatment of PRV formulation, PRV-W have showed low cytotoxic effect at 1, 10 and 15 µM as the % of cell viabilities were 97.76±7.97, 87.88±4.79 and 78.06± 6.81, respectively. On the other hand, incorporating PRV into NE increased its cytotoxicity significantly (P < 0.001) since the % of cell viabilities subjected into 1, 10 and 15 µM of the PRV-NE were 86.66±4.95, 67.18±2.63 and 59.20±1.87, respectively.

It should be notified that the NE formula composition could help the nanocarriers to permeate the cells more efficiently [16]. In agreement with our study, Werner et al. [17] found out that the combination of simvastatin and DOX resulted in a considerable improvement in the suppression of topoisomerase II and disruption of DNA double strand. Other studies showed that cerivastatin has bolstered the anticancer effect of cisplatin against T4-2 cells and of DOX against both MDA-MB-231 and T4-2 human breast cancer cells [18]. In addition, Coimbra et al. [19] found out that PRV-loaded in liposomes has suppressed the growth of murine B16F10-melanoma in mice by greater than 70% when compared to free PRV.

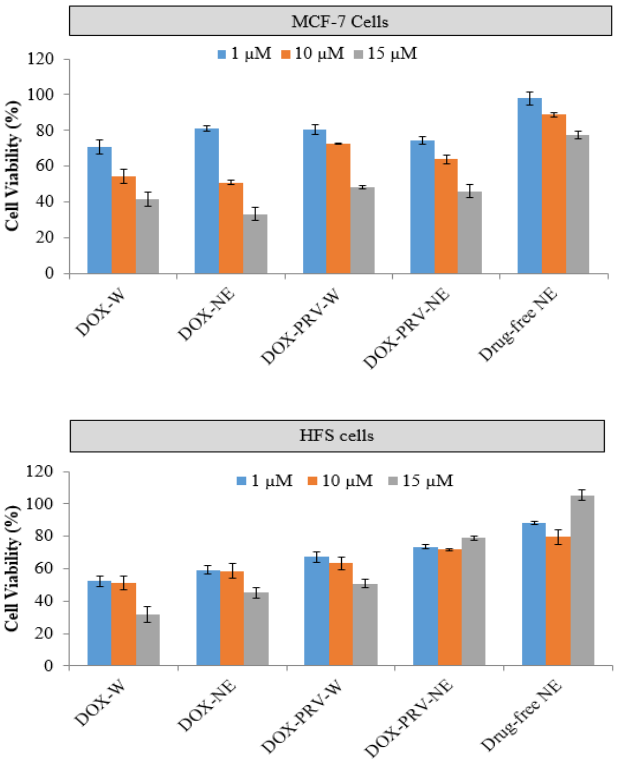


Fig. 5. The cytotoxicity effect of the drug formulas when subjected into the MCF-7 and HFS cells determined by the MTT assay. Error bars represent the standard deviation.

HFS cells

The effect of the different concentrations of the solution and NE formulation on the percentages of cell viabilities of HFS cells is displayed in Fig. 5. All DOX formulas were having comparable cytotoxicity at 1 and 10 µM, DOX-W was the most cytotoxic formula while the DOX-NE was the lowest cytotoxic formula at 15 µM ($P < 0.05$). Regarding the single treatment of PRV formulation (PRV-W and PRV-NE) at 1, 10 and 15 µM, the cell proliferations were similar since the % cell viabilities were more than 98. Previous studies demonstrated that statins ameliorate the normal endothelial, fibroblast, and smooth muscle cell growth [20, 21]. Another study showed that lovastatin protected human endothelial cells from the geno- and cytotoxic effects of the anticancer drugs DOX and etoposide [22]. In addition, Alkhatib and Albishi 2013 [8] showed that DOX- loaded- NE was having a reduced side effect on the HFS cells relative to the Drug-free NE and free DOX.

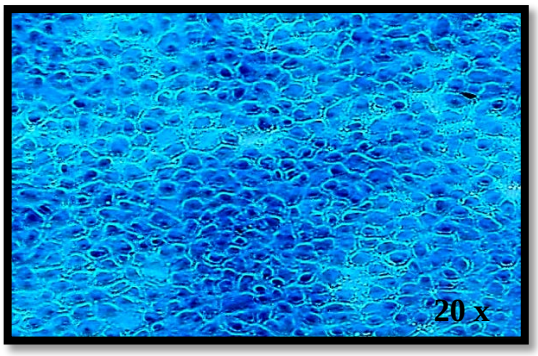
Among the combination formulas at 15 µM, the viability percentages of cells were the highest when treated with DOX-PRV-NE while the least viability percentages of cells were observed when they were treated with DOX-PRV-W. Based on the results of the anti-proliferative MTT assay, it has been found that the best inhibitory effect of the combination formulas in either water or NE was observed at 15 µM. However, the NEs formulations were much safer on the HFS cells as their cytotoxicities were significantly less than the water formulations.

3.6. Characterization of cell morphology using light microscope

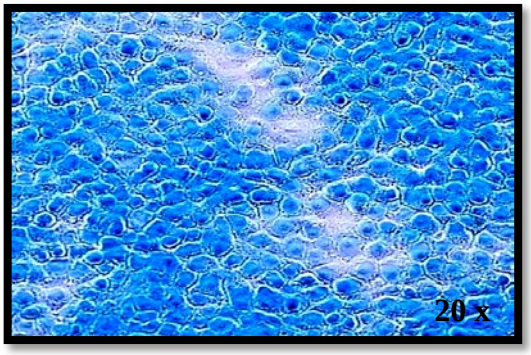
The mechanisms of cell death and cell morphological changes were analyzed by the light microscopy. In general, the signs of apoptosis, induced by the anticancer agents, are the intracellular spaces between the cells, membrane blebbing, chromatid condensation, fragmentation and formation of apoptotic bodies. As exhibited in Fig. 6, MCF-7 cells treated with Drug – free NE, PRV-W and PRV-NE were having slight changes in their morphology. In contrast, cells treated with DOX-W has shown chromatid fragmentation, extremely increased intracellular space and clearance of cells. On the other hand, cells subjected into DOX-NE, DOX-PRV-W and DOX-PRV-NE have shown membrane blebbing, chromatid condensation and fragmentation, extremely increased intracellular spaces and clearance of cells.

The safe effect of the drug formulations was examined in the HFS cells as shown in Fig. 7. The least effect was noticed when the cells were treated with Drug-free NE, PRV-W and PRV-NE. Drug formulations that incorporate DOX were having a toxic effect on the cells as they have slightly decreased in their total number and less intracellular spaces have displayed between them. It should be mentioned here that combining PRV with DOX in NE have had less effect than DOX-W, DOX-NE and DOX-PRV-W.

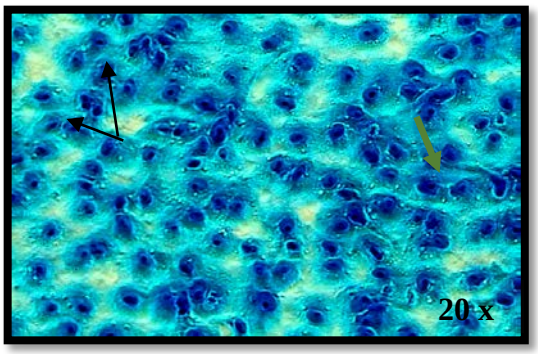
Untreated



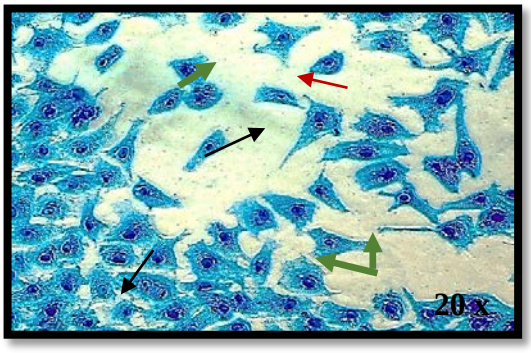
Drug-free NE



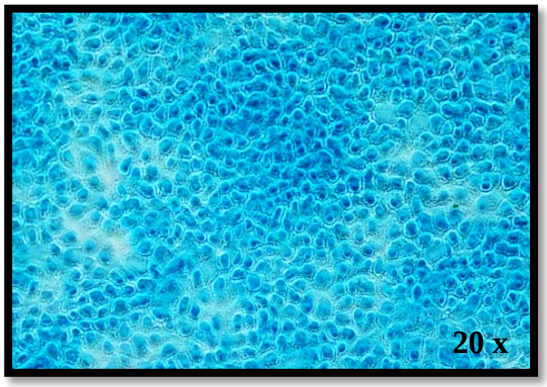
DOX-W



DOX-NE



PRV-W



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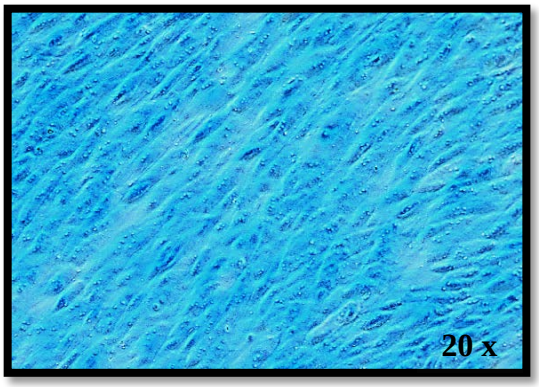
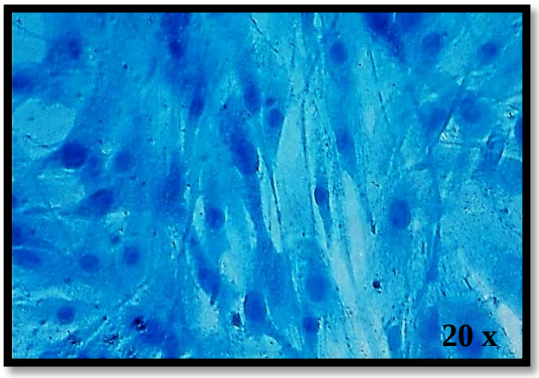
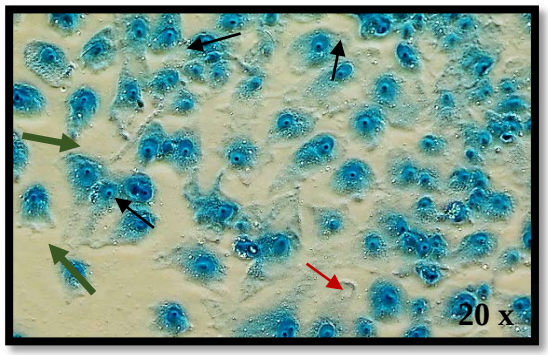


PRV-NE

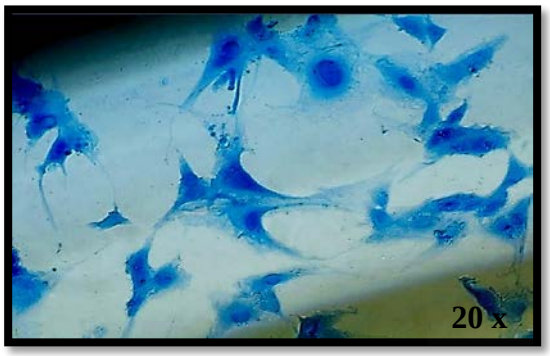
Drug-free NE



DOX-PRV-W



DOX-W



DOX-PRV-NE

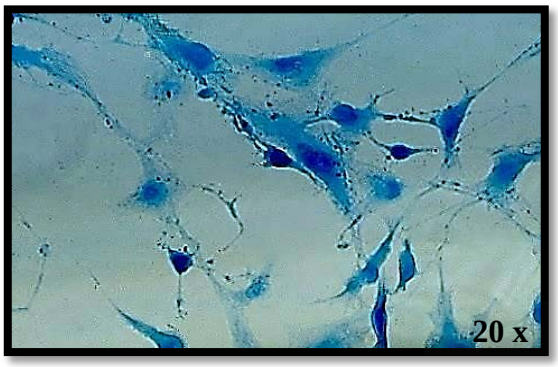
DOX-NE

Fig. 6. Light microscopy images of MCF-7 breast cancer cells treated with 15µM of the drug formulas. Black arrows represent chromatin fragmentation; green bold arrows represent membrane blebbing; and red arrows represent chromatin condensation.

PRV-W

PRV-NE

PRV-NE



DOX-PRV-NE

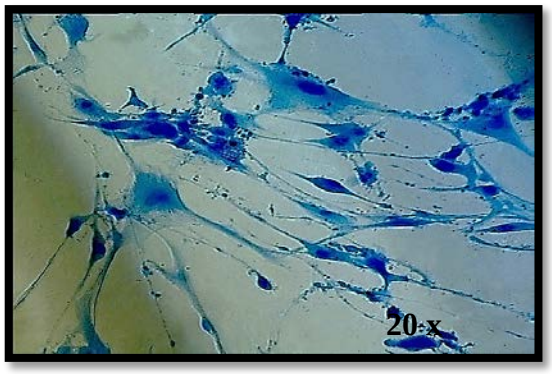


Fig. 7. Light microscopy images of HFS human foreskin cells treated with 15µM of the drug formulas

3.7. ApopNexin FITC apoptosis detection assay

In order to investigate the apoptotic effect of the drug formulations, ApopNexin FITC apoptosis detection kit was utilized. Double staining with two fluorescents, FITC/PI, can distinguish between necrotic (Q1), viable (Q3), early apoptotic (Q4) and late apoptotic (Q2) cells. The percentages of the cells undergoing necrosis and different stages of apoptosis are illustrated in Table 3. High percentages of the necrotic cells were observed when the MCF-7 cells were subjected into PRV-W and PRV-NE. Interestingly, more than 40% of the late apoptotic cells were detected when the MCF-7 cells were treated with DOX and DOX-PRV, formulated in either water or NE. The percentages of the viable cells, administered into all of the formulations, were in the range of 19.6- 28.6 while the percentages of the early apoptotic cells were in the range of 0.7 -15. Regarding the HFS cells, higher percentages of the viable cells (>60%) were recorded when cells were treated with PRV-W, PRV-NE, DOX-PRV-NE and Drug-free NE. The most toxic formula was DOX-W followed by DOX-PRV-W.

4. CONCLUSİONS

In this study, an oil-in-water NE formulation was in vitro evaluated as a drug carrier for the combination of PRV and DOX. The combinations treatments, loaded in either water or NEs, contained lower compositions of DOX and PRV when compared with the single treatments. It has been found that incorporating PRV into the combination formula improved the cytotoxicity effect of DOX since DOX-PRV-NE formula, which contained less amount of DOX by half than the single treatments, has a similar toxic and apoptotic effect to the DOX-W formula on the MCF-7 cells. Aditionally, it has been found that incorporating the drugs into the NE has reduced the side effect on the HFS cells relative to the water drug formulations. Furthermore, it has been found that mixing DOX and PRV in NE formulations have shown decreased cytotoxicity against the RBCs. Based on this study, it is recommended to establish further in vivo researches in order to give a complementary study of the NE combination formula effect on the body tissues.

Table. 3. The percentages of the MCF-7 cells distinguished between necrotic (Q1), viable (Q3), early apoptotic (Q4) and late apoptotic (Q2) when subjected into different formulations.

Formula	Quadrant of MCF-7 cells				Quadrant of HFS cells			
	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4
Control	0.1	0	99.8	0.1	0	0	98.7	1.2
Drug-free NE	32	44.4	19.6	4	3.7	21.2	61.2	14
AD-W	15.1	45.1	24.8	15	1.5	25.8	37.8	35
AD-NE	12	45.5	28.5	14	2.5	10.6	47.3	39.7
PV-W	69.8	8.6	20.9	0.7	0	0	68.5	31.5
PV-NE	50.5	21.8	21.8	6	3	17.9	62.6	16.4
AD-PV-W	17.7	46.5	27.6	8.1	1.5	15.2	45.2	38.1
AD-PV-NE	13.5	45	28.6	12.9	1.6	15.3	66.2	16.9

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