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Research Article

Surface Modified Carvacrol-rich *Satureja khuzestanica* Essential Oil Nanoemulsion: A Novel Paclitaxel Formulation Induced Apoptosis on Paclitaxel-Resistant Breast Cancer Cells

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Abstract

Background: The ability of cancer cells to develop multidrug resistance (MDR) is a major challenge in modern chemotherapy. The current generation of commercially available paclitaxel formulations have not been designed to treat resistant tumours. In this study, a nanoemulsion-based delivery system was developed to enhance the efficacy of paclitaxel against resistant breast cancer cells.

Methods: The nanoemulsion was formulated using carvacrol-rich *Satureja khuzestanica* essential oil. Modification of nanoemulsion was performed by incorporating tocopheryl polyethylene glycol 1000 succinate (TPGS) which could inhibit drug resistance in cancer cells. Fabrication of paclitaxel nanoemulsion was performed by high speed homogenization. The cytotoxicity of prepared formulation against resistant breast cancer cells was investigated by MTT assay. Flow cytometry technique was used for cell cycle arrest analysis and examination of the apoptosis induction ability of prepared nanoemulsion.

Results: The nanoemulsion had a relatively small mean droplet diameter (93.6 ± 4.2 nm) and good long-term stability. The ability of paclitaxel to inhibit P-gp function in paclitaxel-resistant breast cancer cells (MCF-7/PTX) was synergistically enhanced by administering it within the nanoemulsion. The cytotoxicity of the prepared nanoemulsion on the HUVEC normal cells was much lower than that of MCF-7/PTX cells. Cell cycle analysis utilizing flow cytometry showed that the paclitaxel-loaded nanoemulsion promoted G_2-M arrest. Flow cytometry also demonstrated that this nanoemulsion induced apoptosis in MCF-7/PTX cells. Interestingly, apoptosis increased from 20.0% for the free paclitaxel treated group to 85.2 % for the paclitaxel-loaded nanoemulsion treated group.

Conclusion: This novel paclitaxel nanoemulsion efficiently suppressed the drug resistance of breast cancer cells and induced effective apoptosis in very low concentrations of paclitaxel.

Introduction

Paclitaxel is a potent antineoplastic drug widely used to treat various cancers such as breast, lung, and ovarian cancers.[1,2] However, in many cases, the efficiency of chemotherapy is compromised due to the establishment of multidrug resistance (MDR) in cancerous cells.[3,4] A common reason for paclitaxel resistance in cancer cells is the overexpression of P-glycoprotein, a drug efflux pump.[5] P-glycoprotein (P-gp) is a well-known member of the ATP-binding cassette (ABC) proteins, which pumps paclitaxel out of cancer cells via an ATP-dependent mechanism.[6,7] It has been suggested that P-glycoprotein inhibitors, such as verapamil, quinine, and cyclosporine A, could increase the efficiency of paclitaxel as a chemotherapeutic agent in MDR cancer cells. However, the application of these compounds is currently limited due to undesirable pharmacokinetic interactions and side effects.[8,9]

Several commercial formulations have been developed to enhance the solubility characteristics of paclitaxel for clinical applications. Taxol® is a well-known formulation of paclitaxel comprising polyethoxylated castor oil (Cremophor EL) as a solubilizing agent. Taxol chemotherapy often results in severe side-effects due to Cremophor EL, including hypersensitivity reactions,

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hyperlipidemia, aggregation of erythrocytes, abnormal lipoprotein patterns, and peripheral neuropathy.[10,11] For this reason, alternative formulations have been developed to overcome these drawbacks, including Abraxane® (albumin-bound paclitaxel nanoparticles), Genexol®-PM (paclitaxel polyethylene glycol-polylactide (PEG-PLA) micelles), Lipusu (liposomal paclitaxel),[12] and Onxol® (paclitaxel-loaded PCL-TPGS nanoparticles).[13] Despite reducing undesirable side-effects, the lack of efficiency against multidrug resistance in cancer cells is still recognized as the main challenge to the clinical application of the available paclitaxel formulations.[14]

Nanoparticle-based delivery systems have been shown to facilitate the administration, enhance the pharmacokinetic profile, increase the therapeutic efficiency, and reduce the adverse side-effects of various drugs.[15] This type of delivery system may also be effective at overcoming MDR in cancer cells, because the absorption of drug-loaded nanoparticles *via* endocytosis prevents the recognition of the drug molecules by efflux pumps.[16] The internalized nanoparticles could then release the drugs close to the perinuclear environment, which would inhibit drug efflux by membrane transporters.[17] Moreover, surface modification of nanoparticles can be used to target cancer cells, while receptor-mediated endocytosis can be used to overcome MDR.

Vitamin E TPGS (D-α-tocopheryl polyethylene glycol 1000 succinate) is a water-dispersible form of vitamin E,[18] that can be used as a solubilizer, emulsifier, stabilizer, permeation enhancer, absorption enhancer and nutrition supplement.[19] Moreover, this FDA-approved excipient could be utilized as an adjuvant in pharmaceutical preparations.[20,21] In addition, it can inhibit p-glycoprotein activity, which may circumvent MDR,[22,23] thereby leading to an increased concentration of this cytotoxic drug within the cancer cells.[24] It can, therefore, be considered to be a suitable alternative to other p-glycoprotein inhibitor compounds that are known to have undesirable pharmacokinetic interactions or side-effects. Moreover, it has been also reported that TPGS alone contained anti-cancer effects, which are associated with apoptosis induction in cancer cells.[25,26] In addition, there is some evidence that suggests modification of nanoparticles with TPGS could prolong the systemic circulation of formulation and improve the pharmacodynamic activity of chemotherapeutic agents.[27]

In the present research, we designed a TPGS-functionalized oil-in-water nanoemulsion for delivering paclitaxel and investigated its potential for inhibiting cancer using a paclitaxel-resistant breast cancer cell line (MCF-7/PTX). Prior to nanoemulsion formation, the paclitaxel was solubilized within an oil phase, which consisted of a carvacrol-rich essential oil isolated from a plant (*Satureja khuzestanica*) prevalent in Iran. A cytotoxicity assay was performed to evaluate the possible synergism between the essential oil and paclitaxel. Also, the efficiency of TPGS for suppressing the cell´s drug resistance and determination of therapeutic doses of paclitaxel were assessed. Finally, the anticancer effects of the nanoemulsion were evaluated using fluorescent microscopy and flow cytometry methods for apoptosis detection.

Methods

Materials

Paclitaxel (>97%) was obtained from Stragen Pharmaceutical Company (Switzerland), Tween 80 (hydrophilic) and Span 80 (hydrophobic) surfactants were purchased from Merck Millipore (Germany). *Satureja khuzestanica* essential oil (SKEO) was donated by Dr. J. Hadian of the Khorraman Pharmaceutical Company (Iran). TPGS was synthesized by pegylation of D-α-tocopheryl succinate via an esterification reaction.[28] 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and propidium iodide (PI) were supplied by Sigma–Aldrich (USA). 4′,6-diamidino-2-phenylindole (DAPI), Ethidium Bromide and Acridine Orange were obtained from Merck Millipore (Germany). The other reagents, chemicals, and solvents utilized in this research were of analytical or HPLC-grade.

Preparation of nanoemulsion

TPGS-modified paclitaxel-loaded nanoemulsion (PTX-NE) was prepared from a mixture of 3% oil phase and 9% surfactants (including tween 80 and span 80) by a high energy emulsification method using high-speed homogenizer. Paclitaxel was solubilized in SKEO in a concentration of 111 µM. The optimized hydrophilic-lipophilic balance (HLB) value for the preparation of stable nanoemulsions was determined based on the results of a previous study.[29] An aqueous solution of Tween 80 dispersed within deionized water was poured into an oil phase consisting of paclitaxel, SKEO, and Span 80. Then, TPGS (60 mg) was added and the mixture was sonicated using an ultrasonic bath for 5 min to form a primary emulsion. The paclitaxel-loaded nanoemulsion was then formed by blending 5 mL of the primary emulsion using a high-shear mixer (SilentCrusher M, Heidolph, Germany) operating at 15,000 rpm for 5 min using a 12F dispersion tool. For comparative studies, SKEO nanoemulsion without paclitaxel was prepared as blank nanoemulsion.

Physicochemical characterization

The mean particle size as well as particle size distribution of prepared nanoemulsions were measured using Dynamic Light Scattering (DLS) (Nanophox Sympatec GmbH, Germany). The storage stability of the nanoemulsion was assessed by monitoring variations in their particle size characteristics during three months' incubation in a refrigerator. The surface charge of nanodroplets was measured using Dynamic Light Scattering & Zeta potential analyzer (SZ-100, Horiba Jobin Jyovin).

Nanoemulsion microstructure was characterized using transmission electron microscopy (TEM). An aliquot of nanoemulsion (20 μL) was dropped onto a carbon film

coated with a 300-mesh copper grid (Agar) and then left for 2 min at ambient temperature. Afterwards excess fluid was removed, and the sample was then negatively-stained by adding a small aliquot (20 μL) of 2% w/v uranyl acetate solution onto the grid and allowing it to air-dry for one to two minutes. Images of the nanoemulsion samples were then obtained using a TEM (Zeiss EM900, Germany) with an accelerating voltage of 80 kV.

In vitro release study

TPGS-modified paclitaxel-loaded nanoemulsion was placed in a hermetically sealed dialysis bag (12000 Da molecular weight cut-off) and then immersed in a beaker containing 25 mL of phosphate buffer saline (PBS, pH 7.4) and 0.5% w/v of Tween 80 to inhibit drug precipitation. This system was then shaken at 100 rpm in 37 °C. Aliquots of the sample were periodically withdrawn and pre-warmed buffer solutions of the sample volume were added after each withdrawal to preserve sink conditions in the dissolution medium. Drug release of time was determined using a reverse-phase HPLC-DAD instrument with a binary well chrome K1001, a multiple wavelength UV-Vis (DAD)-2800 model (KNAUER, Germany). The separation process was carried out using a reverse-phase BODMAN-C18 column (250 × 4.6 mm × 5 µm) with a mobile phase composed of acetonitrile: water (50:50, v/v). The flow rate was 1.0 mL/min and detective wavelength for PTX was adjusted to 227 nm.

Cell culture

A human paclitaxel-resistant breast cancer cell line (MCF-7/PTX) was secured from the National Cell Bank of Iran (NCBI). The RPMI 1640 medium (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco), containing 100 units/mL penicillin, and 100 mg/mL streptomycin was utilized as the growth medium for cells which were kept at 37°C humidified air in 5% CO_2. The medium was changed twice a week and trypsin-EDTA (Sigma) 0.25%w/v was utilized for cell´s detachment. The number of viable cells was measured using a Trypan blue (Sigma) stain in combination with a haemocytometer.

Cytotoxicity study

The impact of various concentrations of paclitaxel (+TPGS), SKEO, their combination solution, and the nanoemulsion formulation (PTX-NE) to inhibit cell proliferation on the MCF-7/PTX cells was determined utilizing an MTT assay. The cells were seeded in 96-well plates at a density of 5×10^3 cells per well and incubated for 24 h. Then, the media were replaced by fresh one contained the mentioned treating samples and incubated for 24, 48 and 72 h. RPMI 1640 medium was used as the control. Subsequently, MTT solution (5 mg/mL) was included in each well and the plate was allowed to incubate for 3 h. Then, the media were removed and dimethyl sulfoxide was added to wells for dissolving formazan crystals which were produced by live cells. The optical density of the wells was measured at 490 nm by a microplate reader (BioTek, ELx800, USA). The viability of the cells was calculated by dividing the absorbance of the test samples by the absorbance of the control samples. The cytotoxicity of treatment groups on HUVEC cells (Human Umbilical Vein Endothelial Cells) as a model for normal cells was assessed by performing the same MTT method.

Determination of Synergistic Effects of Paclitaxel and SKEO

An MTT cytotoxicity assay was accomplished to establish the mass ratio of paclitaxel-to-SKEO that would maximize any synergistic effects. Various combinations of paclitaxel (+TPGS) and SKEO concentrations lower than their IC_{50} values were tested on MCF-7/PTX cells viability. Synergistic effects were defined by the Combination Index (CI) value equation ($CI_x=D_1/(D_x)_1 + D_2/(D_x)_2$) introduced by Chou and Talalay. According to this equation, CI < 1, CI = 1 and CI > 1 indicate the synergistic, additive and antagonistic effects, respectively. Both constant and non-constant ratio combinations of paclitaxel (+TPGS) and SKEO were tested for their cytotoxicity and CI values were established utilizing CompuSyn software.[30] The specific weight ratio of paclitaxel (+TPGS) and SKEO that led to the lowest combination index and the highest synergistic effect was selected for the formulation of the final nanoemulsion.

Fluorescence microscopy observations

Acridine orange (AO) / ethidium bromide (EB) dual staining

AO staining highlights both living and dead cells, but cell viability can be distinguished based on the color formed: in living cells, AO binds to DNA leading to an emission of green fluorescence; in dead cells, AO binds to RNA or single-stranded DNA leading to an emission of red fluorescence. Cells with disrupted membranes, including late apoptotic or necrotic cells, are stained by EB causing their DNA to emit red fluorescence. MCF-7/PTX cells were seeded into a 6-well plate at a density of 1×10^5 cells per well and incubated for 24 h. Paclitaxel (+ TPGS) solution, blank SKEO nanoemulsion, PTX (+ TPGS) and SKEO combination solution and PTX-NE (PTX concentration equivalent to 5 nM) was added to each well and the plate was allowed to incubate for 72 h. Afterward, the cells were trypsinized, washed three times with PBS (pH 7.4) and stained by the dye mixture (1 mg/mL AO and 1mg/mL EB). Digital images of the cells were obtained using a fluorescence microscope (Zeiss, Axioplan 2, Germany).

DAPI (4',6-diamidino-2-phenylindole) staining

DAPI is a DNA-binding fluorescent stain applied for detection of structural changes in DNA like chromatin condensation or DNA fragmentation. MCF-7/PTX cells were prepared according to the method described earlier. After 72 h, the cells were detached, washed with PBS (pH 7.4) and then incubated in pre-cooled methanol-PBS and cold methanol to be fixed. DAPI solution (0.5 mg/mL) was utilized for cell staining using fluorescence microscopy.

Cell cycle analysis by flow cytometry

Flow cytometry technique was applied for investigation of cell cycle utilizing propidium iodide (PI) as a fluorescent probe. MCF-7/PTX cells were seeded at a density of 1×10^5 cells and cultured for 24 h. Paclitaxel (+ TPGS) solution, blank SKEO nanoemulsion, PTX (+ TPGS) and SKEO combination solution and PTX-NE (PTX concentration equivalent to 5 nM) was added and incubated for 72 h. After this period, the cells were detached, washed with PBS (pH 7.4) and then fixed using pre-cooled ethanol (70%). The cells were washed twice with PBS (pH 7.4), and then 50 µL of RNase solution (100 µg/mL) was added to each sample. Subsequently, 200 µL of PI solution (50 µg/mL) was added and the cells were analyzed using a flow cytometer (BD FACS Calibur, BD Biosciences, San Joes, CA, USA).

Apoptosis analysis by flow cytometry

The assay was accomplished using a FITC Annexin V apoptosis detection kit with PI (BioLegend). The MCF-7/PTX cells were prepared in a similar manner as that used for cell cycle analysis. After 72 h treatment, the cells were trypsinized and washed with PBS (pH 7.4). Cells were washed twice with PBS (pH 7.4), centrifuged and resuspended in annexin V binding buffer. 100 µL of the cell suspension was transferred to a test tube followed by adding 5 µL of FITC Annexin V and 10 µL of PI solution and cells were incubated for 15 min in the dark. Subsequently, 400 µL of annexin V binding buffer was added to each tube and flow cytometry was utilized to discriminate between the stained cells.

Statistical analysis

The data were reported as mean ± standard deviation (SD), which were calculated using Microsoft Excel 2010. Statistical analysis of the results was carried out using student´s t-test and one-way analysis of variance (ANOVA) and p-values < 0.05 were taken to be statistically significant.

Results

Combinatorial effects of paclitaxel and SKEO

The results of a preliminary MTT assay indicated that paclitaxel alone did not induce appreciable toxicity to the MCF-7/PTX cells when applied in a concentration range from 10 nM to 10 µM, which confirmed the drug resistance phenotype of the cells. A relatively low TPGS concentration (26 µM) was used to prepare the paclitaxel-TPGS formulations tested so that any observed reversal in paclitaxel resistance could not be attributed to the surfactant alone. The cytotoxicity of TPGS alone as well as PTX in the concentration range of 2-50 nM (+TPGS) against MCF-7/PTX cells is represented in Figure S1. The cytotoxicity of the essential oil (SKEO) used to formulate the nanoemulsions was also tested to determine its potential contribution to the overall observed cytotoxicity (Figure S1). The *Combination Index* (CI) between paclitaxel (+TPGS) and SKEO was calculated utilizing CompuSyn software. Preliminary studies showed that constant mass ratio combinations of paclitaxel (+TPGS) and SKEO resulted in higher synergistic effects than non-constant mass ratios (data not shown) based on correlated cytotoxicity. The cytotoxicity and CI values of combinations of paclitaxel (+TPGS) and SKEO applied at constant mass ratios were measured (Table 1).

The IC_{50} values of the paclitaxel (+TPGS) and SKEO on the MCF-7/PTX cells were determined as 10 nM and 90 µg/mL, respectively. All combination indices were < 1, which confirmed the synergistic effect between these two components (Figure S2). Application of paclitaxel (+TPGS) and SKEO at a constant mass ratio of 0.5 IC_{50} (No. 3) and 0.25 IC_{50} (No. 2) resulted in the highest synergism, as indicated by the lowest CI values (0.45 and 0.48, respectively). The preparation of the paclitaxel-loaded nanoemulsions was therefore carried out using the constant mass ratio combination of paclitaxel and SKEO according to their IC_{50} values.

Characterization of paclitaxel loaded nanoemulsion

A TPGS-modified paclitaxel loaded-nanoemulsion was prepared by high-speed homogenization. The mean particle diameter of the nanoemulsion determined by DLS was 93.6 ± 4.2 nm, which was consistent with the dimensions of the particles observed in the TEM images (Figures 1a, b). No significant change in the mean particle diameter of the nanoemulsion was observed ($p>0.05$) after 3 months storage at 4 °C (refrigerated conditions), confirming their long-term storage stability (Figure 1c). The polydispersity index (PDI) of prepared nanoemulsion after 3 months storage was calculated as 0.004. Zeta potential of nanodroplets at preparation time was measured as -41.8 ± 0.9 mV. The physical properties of the prepared nanoemulsion indicating long-tern stability have been presented in Table S1.

The *in vitro* release of paclitaxel from the nanoemulsions increased only gradually over time, so that only 55.9% of

Table 1. MTT assay result of paclitaxel (+TPGS) and SKEO combination in constant mass ratios in the form of free drugs against MCF-7/PTX cells after 24 h incubation.

No.	[PTX] (nM) + TPGS (26 µM)	[SKEO] (µg/mL)	Percent Inhibition	Combination Index	PTX DRI	SKEO DRI
1	1.25	11.25	33.75 ± 3.15	0.65	1.99	6.54
2	2.5	22.5	50.98 ± 1.34	0.48	4.24	4.09
3	5	45	70.63 ± 0.43	0.45	12.35	2.69
4	10	90	83.54 ± 2.92	0.62	29.7	1.71

DRI: Dose Reduction Index

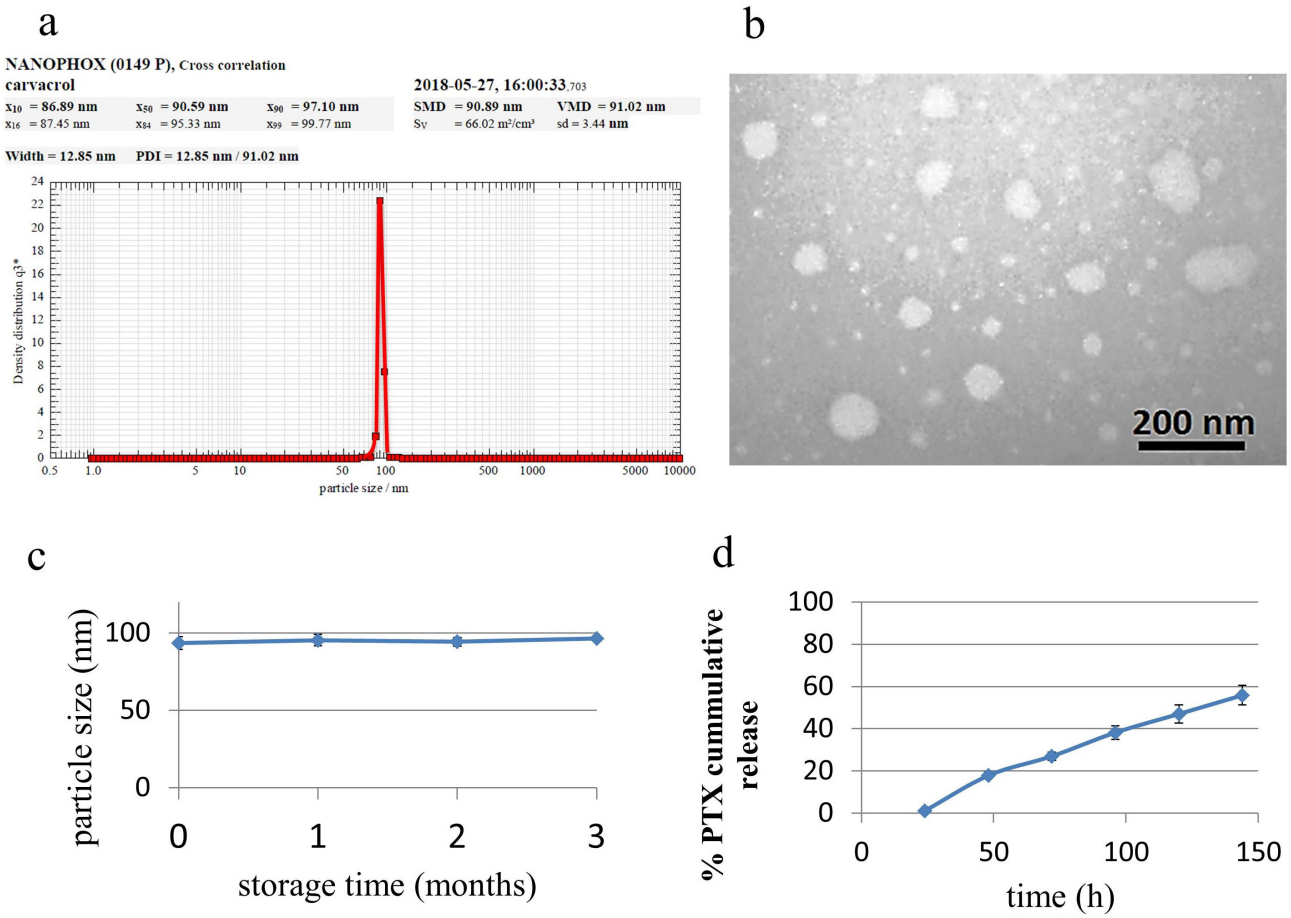

Figure 1. (a) Particle size distribution, (b) Transmission electron microscopy (TEM) image of PTX-nanoemulsions, (c) Changes in mean particle diameter of PTX-nanoemulsions over 3 months storage at 4°C, (d) *In vitro* release profile of paclitaxel from PTX-nanoemulsions.

the drug was released after 6 days in the release medium (Figure 1d). Our nanoemulsion formulation therefore appeared to be suitable for providing a delayed-release profile of paclitaxel, which may have been because this hydrophobic drug had a high affinity for the hydrophobic core of the oil droplets.

In vitro cytotoxicity of paclitaxel loaded nanoemulsion on MCF-7/PTX cells

An MTT assay was performed to evaluate the cytotoxicity of the nanoemulsion to MCF-7/PTX cells. The time-dependent toxicities of the paclitaxel-loaded nanoemulsion applied at mass ratios of 0.25 IC_{50} and 0.5 IC_{50} were measured (Figure 2). In addition, the toxicity profiles of the control nanoemulsion, paclitaxel (+TPGS), and paclitaxel (+TPGS)/essential oil combination were measured for the sake of comparison. At both constant IC_{50} values, the toxicity of the paclitaxel-loaded nanoemulsion and the combination of components in the free drug form increased with time. At 0.25 IC_{50}, the control nanoemulsion did not show any significant toxicity due to the low concentration of essential oil and surfactant present. Interestingly, the active component combination resulted in a higher cytotoxicity than the nanoemulsion formulation ($p<0.01$) for all three treatments. This effect could be attributed to the delayed-release profile of paclitaxel in the low concentration (2.5 nM) from the nanoemulsion formulation. At 0.5 IC_{50}, the active component combination also exhibited a higher cytotoxicity than the nanoemulsion formulation ($p<0.01$) in the 24 h and 48 h assays. In contrast, this pattern was reversed in the 72 h assay. In the case, the nanoemulsion formulation caused more inhibition of cell growth (80.9 ± 1.8 %) than the combination of active components (72.4 ± 2.8 %) ($p<0.01$). For this reason, the MCF-7/PTX cells were treated using the 0.5 IC_{50} formulation for 72 h in the remainder of the experiments.

A light inverted microscope image (Figure 3) represented the morphological changes of cells treated by different treatment groups. The morphological changes were much more pronounced in three treatment groups: paclitaxel (+TPGS), active component combination, and paclitaxel-loaded nanoemulsion. The cell membranes were disrupted, the cells became elongated, and the cells became detached from each other. These morphological changes appeared to be most obvious for the cells treated with the paclitaxel-loaded nanoemulsion, highlighting their potency at killing the model cancer cells.

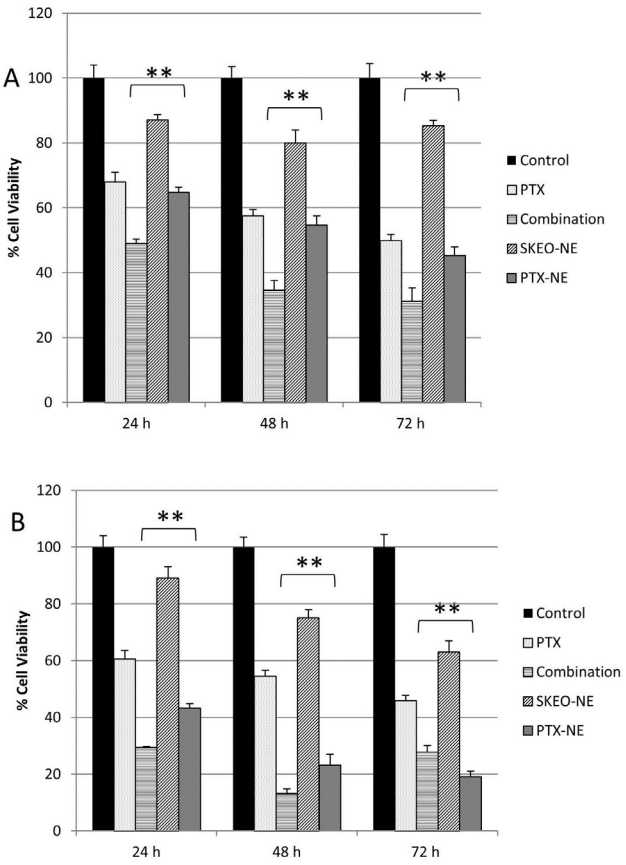

Figure 2. Time-dependent toxicity of treatment groups in the constant weight ratio of (a) 0.25 IC_{50} (Paclitaxel: 2.5 nM and SKEO: 22.5 µg/mL) and (b) 0.5 IC_{50} (Paclitaxel: 5 nM and SKEO: 45 µg/mL) against MCF-7/PTX cells (** p<0.01).

Cytotoxicity effect of paclitaxel loaded nanoemulsion on HUVEC cells

The potential cytotoxicity of the nanoemulsion on normal cells was established by using an MTT assay with HUVEC (Human Umbilical Vein Endothelial Cells). The paclitaxel (+TPGS), control nanoemulsion, active component combination, and paclitaxel-loaded nanoemulsion had no significant toxicity on the HUVEC cells after 24 h incubation (data not shown). The paclitaxel-loaded nanoemulsion, however, did inhibit cell growth by 56.8 ± 1.7 % for MCF-7/PTX cells after 24 h treatment. A comparison of the cytotoxicity of the treatment groups on MCF-7/PTX cells and HUVECs after 72 h is presented in Figure 4. Although some cytotoxicity was observed in the HUVEC cells for all treatments, it was much less than that observed in the MCF-7/PTX cells (p<0.05).

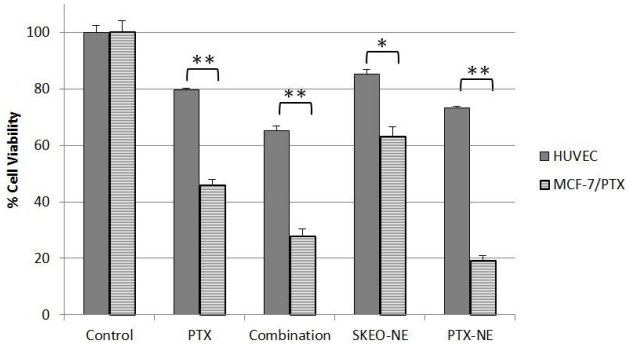

Figure 4. Cytotoxicity of treatment groups against MCF-7/PTX cells and HUVEC cells after 72 h (* p<0.05, ** p<0.01).

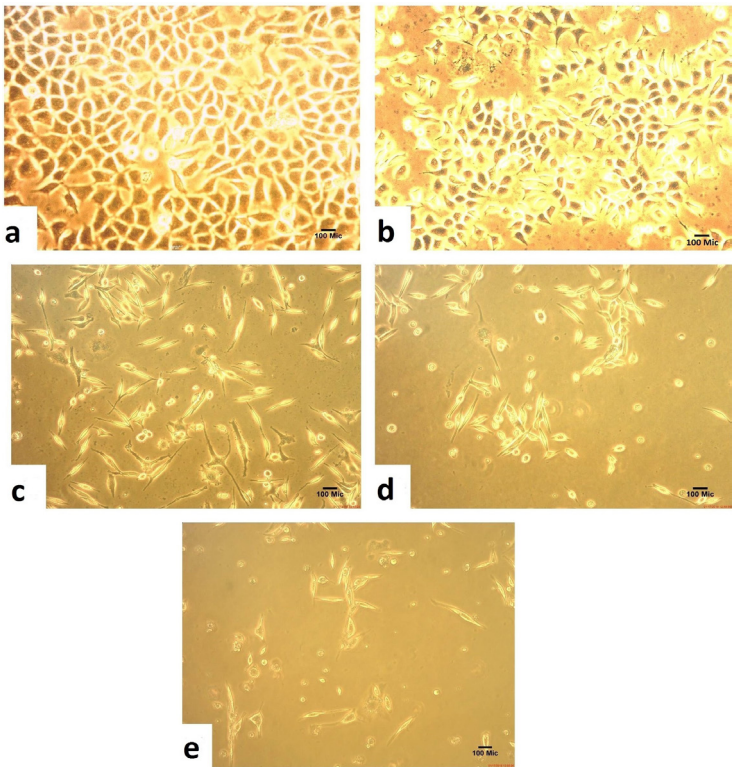

Figure 3. The effect of treatment groups on the morphology of MCF-7/PTX cells after 72 h (a) Control, (b) SKEO-NE, (c) PTX (+TPGS), (d) Combination, (e) PTX-NE. (25X magnification).

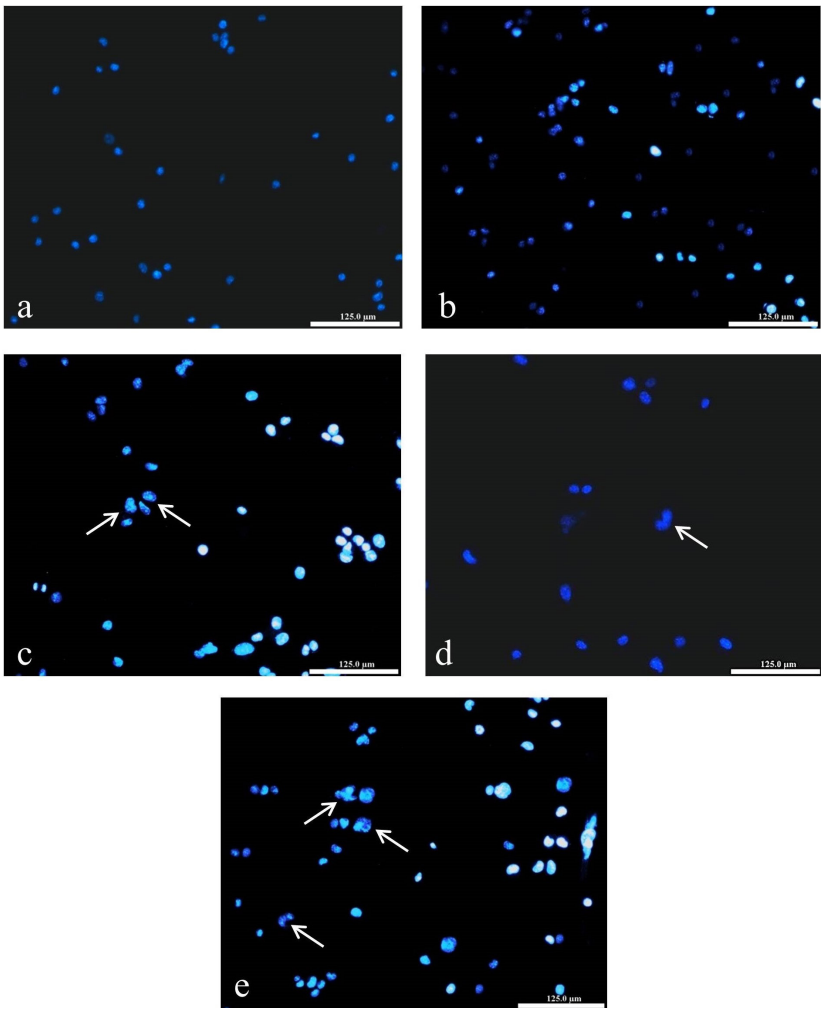

Figure 5. DAPI staining of the MCF-7/PTX cells nuclei after 72 h treatment (a) Control, (b) SKEO-NE, (c) PTX (+TPGS), (d) Combination, (e) PTX-NE. (20X magnification) arrows show condensed or fragmented chromatin.

DAPI staining

Fluorescent microscopy with DAPI staining was used to evaluate the apoptotic effect of the treatment groups on the nuclei of the cancer cells. The untreated cells and control nanoemulsion-treated cells had rounded intact nuclei that were uniformly stained by DAPI (Figure 5). Conversely, the cells treated with either paclitaxel (+TPGS) or the active component combination exhibited pronounced nuclear changes, including condensed or fragmented chromatins, which are indicative of apoptotic effects. These effects were even more pronounced in the paclitaxel-loaded nanoemulsion: the number of apoptotic cells increased dramatically as demonstrated by the brighter nuclei and more fragmented DNA. These results indicate the ability of the paclitaxel-loaded nanoemulsion to induce apoptosis in resistant cancer cells, which is consistent with the results of the cytotoxicity test.

Acridine orange (AO)/ ethidium bromide (EB) dual staining

AO/EB dual fluorescent staining was used to provide insights into the apoptosis induction mechanism of the treatment groups on MCF-7/PTX cells. In this fluorescent staining method, four types of cells are detectable: i) viable cells that emit green fluorescence due to binding of AO to intact DNA; (ii) cells that are in the early apoptotic stage that emit bright green fluorescence due to binding of AO to fragmented DNA; (iii) cells in the late apoptotic stage that emit bright red fluorescence due to binding of EB to fragmented DNA; and, (iv) necrotic cells that emit red fluorescence due to their permeability to EB.[31] Untreated MCF-7/PTX cells had rounded intact nuclei that were uniformly stained green by AO (Figure 6). Treatment with the control nanoemulsion led to an increase in the number of necrotic cells. Treatment with paclitaxel (+TPGS) increased the number of cells in the early apoptosis stage. Treatment with the active component combination led to an increase in the number of both apoptotic and necrotic cells. Finally, treatment of the cells with paclitaxel-loaded nanoemulsion enhanced the number of cells in either the early or late apoptosis stages, which is advantageous because it shows that cell death was induced through an apoptosis-dependent mechanism.

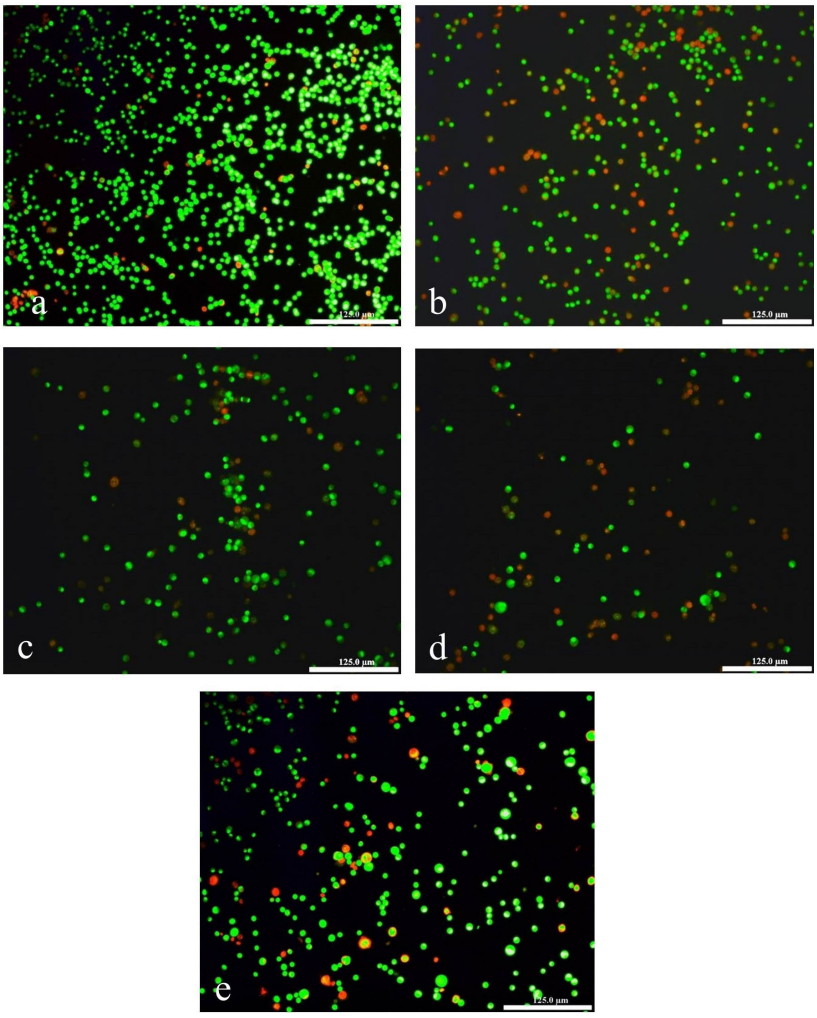

Figure 6. EB/AO dual staining of the MCF-7/PTX cells nuclei after 72 h treatment (a) Control, (b) SKEO-NE, (c) PTX (+TPGS), (d) Combination, (e) PTX-NE. (20X magnification).

Cell cycle analysis by flow cytometry

Flow cytometry was used to assess the impact of the treatment groups on the cell cycle of the MCF-7/PTX cells using propidium iodide (PI) as a fluorescent probe. Since PI attachment is proportional to the DNA content of cells, the cell number in each stage of the growth cycle can be quantitatively determined based on the fluorescence emission intensity. The analysis of the results of correlated histograms (Figure S3) is summarized in Table 2.

The cells treated with control nanoemulsion, paclitaxel (+TPGS) and the active components combination showed similar cell cycle percentage at each phase compared to that of the untreated group. Treatment of the cells with the paclitaxel-loaded nanoemulsion significantly enhanced G_2-M phase arrest compared to the untreated group (25.7% vs 9.3%, $p<0.05$).

Annexin V staining apoptosis detection

In the early stages of apoptosis, membrane symmetry is lost and phosphatidylserine (PS) transfers to the external leaflet of the cell. Annexin V possess a strong tendency to bind PS in a calcium-dependent manner. Consequently, it can be utilized to target and identify apoptotic cells. Staining with annexin V-PI enables one to distinguish between apoptotic and necrotic cells. Early-apoptotic stage cells exclude PI, whereas late-apoptotic stage cells and necrotic cells are permeable to PI, which can therefore enter them and stain their DNA. The apoptosis percentages calculated from the analysis of the resulted histograms (Figure 7) are summarized in Table 3. The results show that treatment with the control nanoemulsion (SKEO-NE) led to 9.3 % apoptosis (early and late). Treatment with paclitaxel (+TPGS) led to 20.0 % apoptosis (early and

Table 2. Cell cycle arrest analysis of treatment groups against MCF-7/PTX cells after 72 h treatment (percentage of cells in each phase).

	Control	SKEO-NE	PTX	Combination	PTX-NE
G0-G1	56.75 ± 3.14	50.46 ± 2.83	42.70 ± 1.51	54.54 ± 0.80	43.12 ± 1.56
S	33.88 ± 4.30	38.88 ± 2.33	40.38 ± 0.70	33.04 ± 1.38	32.87 ± 0.27
G2-M	9.31 ± 0.31	11.34 ± 1.12	17.20 ± 2.40	13.50 ± 1.50	25.70 ± 1.03

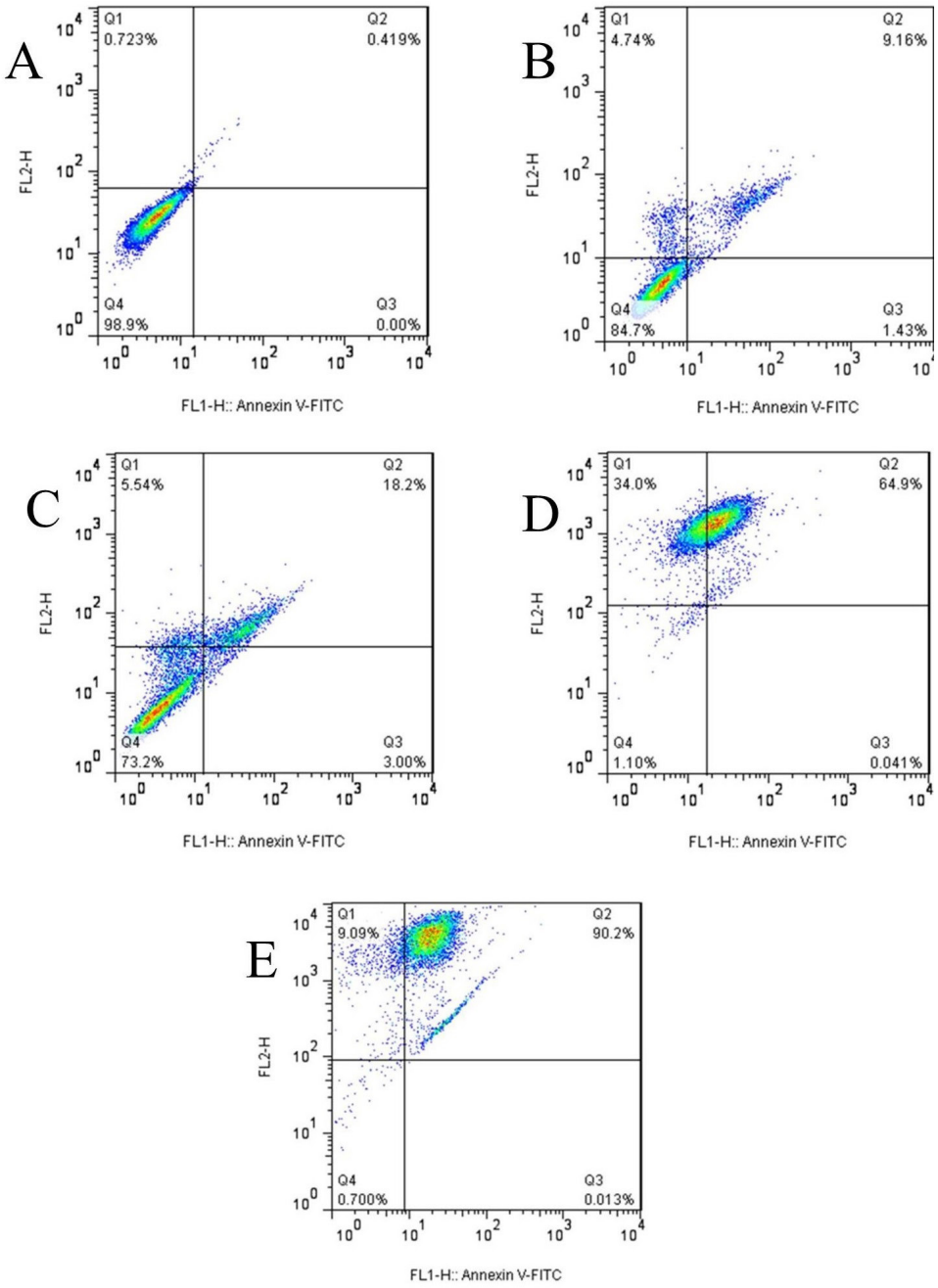

Figure 7. Annexin V-FITC/PI apoptosis detection of MCF-7/PTX cells after 72 h treatment (a) Control, (b) SKEO-NE, (c) PTX (+TPGS), (d) Combination, (e) PTX-NE.

Table 3. Percentage of apoptosis induction by treatment groups against MCF-7/PTX cells after 72 h treatment.

	Control	SKEO-NE	PTX	Combination	PTX-NE
% cells in early and late apoptosis stages	3.4 ± 0.7	9.3 ± 1.8	20.0 ± 1.7	61.5 ± 4.9	85.2 ± 7.1

late). Treatment with the active component combination led to an increase in the percentages of both apoptotic and necrotic cells. Treatment with paclitaxel-loaded nanoemulsion dramatically increased the percentage of apoptotic cells to 85.2 %, which confirmed the potential advantage of this system for efficient apoptosis induction.

Discussion

Chemotherapy is one of the most widely used methods of cancer treatment, but its efficiency is being compromised because of the expansion of drug resistance in many types of cancerous cells. Indeed, it has been reported that as many as 41% of breast tumor cells express P-gp.[32] Also, the expression of P-gp has been reported to increase after chemotherapy and is associated with a greater probability

of treatment failure.[7]

Paclitaxel is a highly cytotoxic natural compound that inhibits cancer cell proliferation in nanomolar concentrations. Cytotoxicity studies of paclitaxel against several cancer cell lines have reported that the optimal concentration range required to achieve anti-proliferative effects is between 10 and 100 nM.[33] The application of paclitaxel at levels greater than 500 nM has been reported to be unsafe for normal cells, such as fibroblasts.[34] The current generation of commercially available paclitaxel formulations have not been designed to treat resistant tumors. Consequently, the fabrication of novel formulations against MDR cancer cells would be advantageous. The aim of the present study was to develop a nanoemulsion-based formulation that could inhibit drug resistance cells, while also having a paclitaxel concentration below a level (20 nM) that could cause toxicity to normal cells.

TPGS, a water-soluble form of vitamin E, is known to be a potent P-gp inhibitor that has previously been shown to overcome drug resistance in some cancer cells. Indeed, TPGS has been shown to exhibit anticancer activity and to induce apoptosis of cancerous cells. Moreover, it does not exhibit toxicity on normal cells when used at sufficiently low levels.[22,25,35] It was therefore important initially to establish the optimum level of TPGS to use to formulate the anticancer nanoemulsions developed in this study. We showed that treatment of MCF-7/PTX cells with 26 µM of TPGS resulted in 19.7 ± 1.1 % toxicity.

The mean particle diameter of the designed formulation was calculated as 93.6 ± 4.2 nm by DLS and was confirmed by TEM image. The highly hydrophobic nature of Paclitaxel and its affinity for the hydrophobic core of the oil droplets caused the formulation as a reservoir of Paclitaxel that provides a delayed-release profile of paclitaxel.

In this study, we examined the potential of using *Satureja khuzestanica* essential oil (SKEO) to formulate the anticancer nanoemulsion. This type of essential oil has previously been reported to contain high levels of carvacrol, *i.e.,* up to around 94%.[36,37] We determined the carvacrol concentration of the essential oil used in our study to be 87.2%. The cytotoxicity effect of this essential oil on cancerous cell lines has not been investigated before. However, extensive cytotoxicity and anti-proliferative effects have been reported for carvacrol against various types of cancer cell.[38-40] Carvacrol ability to promote apoptotic body production and DNA fragmentation has been shown in metastatic breast cancer cell lines, such as MDA-MB 231.[41] The treatment of MCF-7 cells with carvacrol has been reported to induce apoptosis through p53-dependent and Bcl-2/Bax pathways. Also, caspase-3, -9, and -6 enzyme gene expression induction and DNA fragmentation were observed after MCF-7 cell treatment by carvacrol.[42]

In the previous study, we examined the *Satureja khuzestanica* essential oil (SKEO) to formulate Paclitaxel and utilized free form of TPGS to evaluate the cytotoxicity on MDA-MB-231 resistant breast cancer cells.[43] However, in recent study we have functionalized the surface of nanoemulsion using TPGS. Considering that the optimum PTX concentration in the previous study was calculated as 250 nM and in the present study is 5 nM, it can be reasonably understand that the functionalized nanoemulsion could induce apoptosis in resistant cancer cells more efficiently.

Paclitaxel can be easily dissolved in SKEO up to a concentration of 50 mg/mL. Therefore, nanoemulsion fabricated from SKEO can be considered to be efficient colloidal delivery systems for paclitaxel. Based on its oil solubility, the maximum concentration of paclitaxel in the nanoemulsion should be around 1.5 mg/mL, which is 50-fold higher than its aqueous solubility. Moreover, the utilization of SKEO to formulate the nanoemulsion appeared to synergistically amplify the cytotoxicity of paclitaxel, thereby reducing the dose required to have an anticancer effect.

The selectivity indices (SI) of treatment groups were calculated according to their correlated toxicities against HUVEC and MCF-7/PTX cells and are summarized in Table 4. The results indicate that circumventing drug-resistance by paclitaxel-loaded nanoemulsion reduces the required concentrations of paclitaxel and increases the safety of the formulation.

DAPI fluorescent staining confirmed the advantage of paclitaxel-loaded nanoemulsion in apoptosis induction compared to the free drug. Moreover, EB/AO dual staining showed that there was a large enhancement in the level of apoptotic cells observed after treatment of MCF-7/PTX cells with paclitaxel-loaded nanoemulsion than with the free drug. Moreover, the paclitaxel-loaded nanoemulsion induced more controlled cell death compared to the combination of active components in free drug form. The treatment of the MCF-7/PTX cells with the active component combination increased the number of necrotic cells. In contrast, treatment with the paclitaxel-loaded nanoemulsion gave a greater enhancement in the level of present apoptotic cells. The quantitative analysis of necrotic and apoptotic cells by flow cytometry confirmed the fluorescence microscopy observations. Previously, it has been reported that paclitaxel-loaded nanoemulsion stabilized by the same surfactant used in this study were capable of inhibiting MDR in breast cancer.[1] The IC_{50} of paclitaxel in the paclitaxel-loaded nanoemulsion against MCF-7/ADR cells was reported to be 5.39 µg/mL in that study [1] which is much higher than in the present study and may be unsafe for normal cells. Cell cycle analysis by flow cytometry revealed that the paclitaxel (+TPGS) and active component combination doesn't change the cell cycle percentage at each phase. A similar observation was reported by Bu. and co-workers.[1] However, the paclitaxel-loaded nanoemulsion induced potent G_2-M phase arrest.

Table 4. Selectivity index calculated for treatment groups based on cytotoxicity against HUVEC and MCF-7/PTX cells.

	SKEO-NE	PTX	Combination	PTX-NE
SI	1.80 ± 0.2	6.25 ± 0.3	4.00 ± 0.4	2.90 ± 0.2

The G_2-M phase arrested cells determined using the MTT assay have been reported to be dead cells.[31] These results suggest that a potential advantage of the paclitaxel-loaded nanoemulsion is their ability to arrest the cell cycle and induce cell death. Meng and co-workers reported co-encapsulation of paclitaxel and baicalein in nanoemulsions to overcome MDR in breast cancer. Treatment of MCF-7/Tax resistant cancer cells by 2 μg/mL paclitaxel-loaded nanoemulsion has been reported to result in 57.4 % apoptosis induction.[44] In the present study, however, a flow cytometry assay showed a significant improvement in the apoptosis induction on MCF-7/PTX cells treated with paclitaxel-loaded nanoemulsion containing very low paclitaxel concentrations (5 nM). This increased efficacy could be due to synergistic effects between paclitaxel and SKEO, as well as the potential for TPGS to act as a potent P-gp inhibitor.

Conclusion

In the present study, a TPGS-modified paclitaxel-loaded nanoemulsion was fabricated and its anticancer effects were investigated against paclitaxel-resistant breast cancer cells (MCF-7/PTX). Essential oil nanoemulsion was used to encapsulate and deliver the paclitaxel to the cancer cells. *In vitro* cytotoxicity assays implied that the essential oil showed significant synergistic effects with paclitaxel. The utilization of TPGS as a surfactant in the essential oil nanoemulsion may also have played an important role in suppressing the drug resistance of the cancer cells due to its ability to inhibit efflux mechanisms. The paclitaxel-loaded nanoemulsion significantly improved the apoptosis-induction potential of paclitaxel on MCF-7/PTX cells. In summary, the new formulation developed in this study may be an efficient delivery system for paclitaxel that can be used to combat the drug resistance of certain cancers, as well as reducing the effective dose required.

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Author Contributions

Zeinab Mazarei: Conceptualization, Formal Analysis, Investigation, Writing - Original Draft. Houri Sepehri: Methodology, Resources. Ladan Delphi: Methodology, Validation, Formal Analysis. David Julian McClements: Writing - Review & Editing. Hasan Rafati: Conceptualization, Methodology, Resources, Supervision, Writing - Review & Editing.

Conflict of Interest

The authors report no conflicts of interest.

Supplementary Data

Supplementary data, Figures S1-S3 and Table S1, are available at https://doi. org/10.34172/PS.2023.22.

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