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Cinnamon oil nanoemulsion as a novel nanocarrier for bleomycin amplifies its apoptotic effect on SKOV-3 ovarian cancer cells

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Loading the chemotherapeutic agents in nanoemulsions system has recently gained attraction in medicine due to their ability to improve the drug's efficacy and reduce its adverse effects. In this context, here, we loaded bleomycin (BLM) in nanoemulsion (NE) consisting of cinnamon oil in order to evaluate its antineoplastic effect on the SKOV-3 cells. The produced NE formulas were physically characterized by the zetasizer. The cytotoxic activities of BLM and NE formulas were examined by CCK-8 kit, Coomassie blue staining for the visualization of the morphological changes, Annexin V-FITC for identifying apoptosis and cell death detection ELISA plus kit for DNA fragmentation measurement. The average droplet diameter of the blank NE (450.90±1.57 nm) was increased when loaded with BLM (522.57±0.85 nm) while the magnitude of the negative zeta potential of the loaded formula (−0.381±0.003 mV) was less than the blank NE (−1.01±0.020 mV). The potential cytotoxicity of the BLM-NE was significantly greater than the toxicities of the free BLM and blank NE. The blank NE and BLM-NE have the greatest apoptotic effect and higher enrichment factor compared to free BLM. Loading BLM in NE based on cinnamon oil has considerably improved its efficacy as an anticancer drug on the SKOV-3 cells.

Keywords: Anticancer drug, Antineoplastic effect, Apoptosis, *Cinnamomum verum*, Cytotoxicity, DNA fragmentation, Drug delivery, Essential oils

Ovarian disease considered the fifth significant reason for cancer death and the main cause of gynecological malignancy death. In addition, it is considered as the second gynecological malignancy most commonly diagnosed[1]. Worldwide, over 0.314 million people are suffering from ovarian cancer constituting 1.6% of all the cites/types with a fatality reported as 0.207 million[2]. Nevertheless, the underlying pathophysiology remains delineated[1]. Cancer chemotherapy is a standard therapy to treat tumors. Chemotherapeutic drugs can kill cancer cells and inhibit tumor growth. However, the major drawback of chemotherapy that systemic distribution of these drugs cause adverse effects on normal cells and tissues[3,4].

Bleomycin (BLM) is an antibiotic antitumor commonly used in various types of cancer treatment[5]. BLM's therapeutic efficacy is limited because of its low diffusion into the cancer site as well as its short half-life[6]. BLM is unable to cross the plasma membrane to reach the site of action efficiently due to a relatively large molecular weight[5]. Moreover, a high level of BLM resulting in serious side effects of lung fibrosis[7]. Therefore, new formulations are needed to lower the side effects of BLM and improve their therapeutic efficacy. One of the most common applications of nanoparticles in oncology is to enhance the performance of chemotherapeutic drugs where bioavailability, safety, and specificity are concerned about taking benefit of nanoscale particulate properties[8].

Essential oils (EO's) based nanoemulsions have recently gained attention in medicine[9-12]. Firstly, EO's naturally exhibit excellent anticancer activities[13]. Secondly, nanoemulsions are capable of decreasing droplet size diameter to nanometric scale which increase the bioavailability and the solubility of drugs and thereby cause easier permeation of active compounds into blood vessels[14]. Saxena *et al.*[15] have recently used functionalized gold nanoparticles with cell penetrating peptide for effective delivery of drugs, particularly in cancer treatment. EO's and their components play a significant role in cancer protection and therapy[13,16,17]. Furthermore, these EO's have shown synergistic effects when combined with chemotherapeutic agents[18]. Therefore, in the current study we tried to formulate BLM in NE-based

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cinnamon oil and evaluated antitumor potential of BLM-loaded-NE against SKOV-3 cells *in vitro*.

Materials and Methods

Chemicals and subjects

Polyoxyethylene-20-sorbitan monooleate (Tween 80) and Sorbitan laurate (span 20) were purchased from Techno Pharamchem (India). Cinnamon oil was ordered from the secret of Egypt (Sharm El Sheikh, Egypt). Dulbecco's modification of eagles medium (DMEM), 0.25% Trypsin- EDTA, Penicillin streptomycin and phosphate-buffered saline (1XPBS, pH7.4) were obtained from UFC biotech (Riyadh, KSA). Fetal bovine serum (FBS) was purchased from Biochrom (Berlin, Germany). Dimethyl sulfoxide (DMSO) was purchased from Thermo Fisher Scientific (USA). Dialysis bag was ordered from Spectrum Laboratories, Inc. (California, USA). Bleomycin (15 units) was purchased from Fresenius Kabi (India). Cell counting kit-8 (Lot. No LE612) was purchased from Dojindo molecular technologies (Japan). Annexin V-FITC apoptosis detection kit (Cat. No MBS668896) was purchased from MyBioSource (California, USA). Cell death detection Elisa plus (Cat. No 11774425001) was purchased from Roche (Mannheim, Germany). Phosphate buffer (pH7) and a buffer solution (pH8) were ordered from AppliChem GmbH (Germany). The human ovarian cancer cell line (SKOV-3) was obtained from the Tissue Culture Bank at King Fahad Medical Research Center, King Abdulaziz University (Jeddah, KSA).

Preparation of the NE formulas

The blank oil-in-water (o/w) NE formulation was prepared by mixing 13 % (v/v) of surfactant mixture of span 20 and tween 80 at a ratio of 1:2, respectively, 7 % (v/v) of the oil phase (cinnamon oil) and 80 % of aqueous phase (buffer solution pH8). The mixture was emulsified directly by sonication using OMNI Sonic Ruptor 4000 (USA) at 50% power until it becomes clear. Then, the resulting blank NE was stored at room temperature (25ºC). The stock solution of 1.0 mg/mL of BLM was produced by dissolving BLM in sterile distilled water. For formulation of BLM-NE, the IC_{50} of BLM was directly mixed with the IC_{50} of the prepared blank NE.

Physical characterization of BLM-NE formula

The analysis of the droplet size of the produced NE was performed by Zetasizer (Malvern Instruments, Malvern, UK) as described elesewhere[19]. The charges and sizes of the NE were expressed as z-average diameters and zeta potentials, respectively. Measurements were performed three times at 25°C.

In vitro drug release study

The drug release study was used to evaluate the impact of the nanocarrier on the drug release profile[20]. Briefly, 1.0 mL of the examined formula was introduced into a dialysis bag, sealed at both ends, and suspended in a 250 mL beaker containing phosphate buffer (pH7). The whole system was kept at 37°C with continuous shaking at 100 rpm/min on a magnetic stirrer (Thermo fisher scientific, USA). About 1.0 mL of sample was collected at a regular time interval (0, 1, 2, 3, 4, 5, 6 and 24 h) and then, replaced by the same amount of phosphate buffer. The absorbance of the samples was measured at 300 nm using a UV-Visible spectrophotometer (Thermo Fisher Scientific, USA).

Cell culture

The SKOV-3 cell line was cultivated in a 25 cm^2 cell culture flask containing DMEM which was supplemented with 10% (v/v) FBS and 1% (v/v) penicillin-streptomycin in a 5% CO_2 humidified incubator at 37°C[21]. The media was changed every 48 h until cells reached confluence. Then, cells were washed with 1.0 mL of PBS, detached by adding 1.0 mL of trypsin and finally incubated at 37°C.

Dose-response effect

The cell counting kit (CCK-8) was used to evaluate the cytotoxicity of drug and NE formulations against SKOV-3 cells[22]. 100 µL of culture media containing cells was seeded in each well of a 96-well plate and was incubated overnight at 37°C in a CO_2 incubator. Then, cells were treated with 200 µL of five different concentrations for single formulas of each BLM, blank NE, and three loaded formulas at different ratios (1:1, 1:2, and 2:1) by mixing the IC_{50} of each BLM and blank NE. Then, cells were incubated for 24 h at 37ºC in a CO_2 incubator. About 5 µL of CCK-8 solution was then added to each well and incubated in a CO_2 incubator for 4 h. Finally, the absorbance was measured of each well by a microplate reader at 450 nm (Biotech, US). Wells, included culture media, were considered negative control while culture media containing cells served as a positive control. The experiments of each sample were performed in triplicate (n = 3). The percentages of cell growth inhibition rate were determined by the following equation:

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

Characterization of cell morphology using a light microscope

Cultured cells were seeded at a density of 1×10^4 cells per well into a flat-bottomed 96-well tissue culture plates containing 100 µL of growth medium per well and incubated for 24 h at 37°C in a CO_2 incubator[23]. Cells were re-incubated with 200 µL of media containing drug and NE formulas for 24 h at 37°C in a CO_2 incubator. Then, they were washed with 100 µL of PBS for 5 min and fixed by the addition of 4% formaldehyde for 5 min. After that, the fixation solution was discarded and the cells were stained with 100 µL of 10% Coomassie blue dye for 10 min. Finally, the dye was discarded and cells were washed five times with tap water and left to dry at room temperature. Morphological changes were observed by phase-contrast inverted microscope (Olympus 1X51, Japan).

Detection of apoptosis

Annexin V-FITC kit enables quantitative determination and fluorescent detection by flow cytometry of annexin V linked to apoptotic cells. Briefly, annexin V conjugated with fluorescein isothiocyanate (FITC) that stains the phosphatidylserine sites, which are located on the membrane surface of apoptotic cells[24]. In addition, it includes propidium iodide (PI) that stains the cellular DNA in necrotic cells. All cultured cells plated into 6-well flat-bottomed tissue culture plates at a density of 2×10^5 cells per well containing 1.0 mL of the growth medium, and incubated for 24 h at 37°C in a CO_2 incubator. Cells were re-incubated with 2 mL of drug and NE formulations for 24 h at 37°C in a CO_2 incubator. Then, cells were washed with 1000 µL PBS, detached with 300 µL and incubated for 3 min followed by addition of 2000 µL of culture media. After that, cells were transferred to a tube and centrifuged for 10 min at 220×g. The supernatant was removed; pre-cooled PBS was added and cells were centrifuged at 400×g for 5 min (this step was repeated twice). Detached cells were re-suspended in 100 µL of the binding buffer after removing the supernatant. Thehe mixture was transferred to a flow cytometry tube followed by addition of 5 µL of annexin V and 5 µL of PI (without control) and incubated at room temperature (25°C) for 15 min away from light. Finally, 400 µL of the binding buffer was added and the cell suspension was applied to BD FACSAriaTM III Flow Cytometer (BD Biosciences, US). FACS Diva software (version 6.1.3) was used to analyze the data. The positive FITC identifies the release of PS, which occurs in the early stage of apoptosis and the positive of PI identifies necrotic cells.

DNA fragmentation detection

Cell death detection ELISA plus kit was used to measure histone release from the nucleus and DNA fragmentation during the apoptosis[25,26]. About 100 µL of culture media containing cells was seeded in each well of a 96-well plate and incubated overnight at 37°C in a CO_2 incubator. At first, the 96-well plate was centrifuged for 10 min at 200×g and the supernatant was discarded. Next, 200 µL of lysis buffer was added to each well and incubated 30 min at room temperature. Then, the lysate was centrifuged at 200×g for 10 min. After that, 20 µL of the supernatant was transferred to a streptavidin-coated microplate and 80 µL of immunoreagent was added to each well. The microplate was covered with adhesive foil and incubated on shaker under gently shaking (300 rpm) at room temperature. After 2 h, the solution was removed thoroughly by tapping and each well was rinsed three times with 250 µL of incubation buffer. 100 µL ABTS was added to each well and incubated for 10 min on a plate shaker. Finally, it was measured using a microplate reader at 405 nm (BioTek, US). The enrichment factor was determined as absorbance of test samples/absorbance of control cells, which was used as an apoptosis parameter.

Statistical analysis

Data were expressed as mean ± standard deviation ($\bar{X}\pm SD$). Statistical analyses were performed with a one-factor analysis of variance (ANOVA) and the independent sample *t*-test using MegaStat (version 10.3, Butler University, Indianapolis, IN). The statistically significant differences were considered when P-value <0.05.

Results

Physical measurements using zetasizer

According to Table 1, the z-average diameter of the blank NE was significantly increased when loaded with BLM. Interestingly, the variation coefficients among the nanodroplet sizes for both blank NE and BLM-NE

Table 1 — Zetasizer measurements of the produced NE formulas.

Formula	Zeta average diameter (nm)	Variation coefficient	Zeta potential (mV)
Blank NE	450.90±1.57	0.003±0.0001	–1.01±0.020
BLM-NE	522.57±0.85	0.002±0.0003	–0.381±0.003
P value	<0.001	>0.05	<0.001

[*P* value was measured by the pairwise t-test between Blank NE and BLM-NE. BLM, bleomycin; and NE, nanoemulsion]

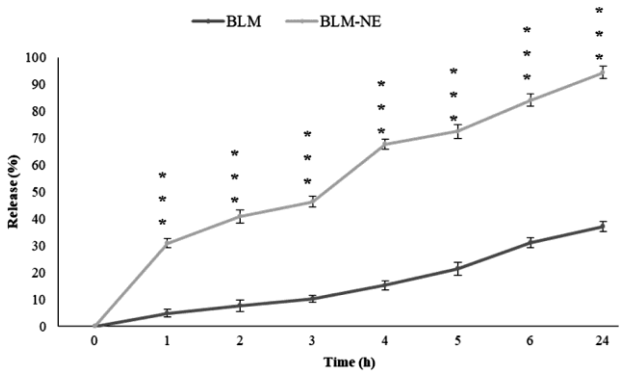

Fig. 1 —Release profile of BLM and BLM-NE using the dialysis method within 24 h at 37°C. [*Error bars* represent the standard deviation for n =3. (***) refers to very highly significant (P <0.01) differences between BLM and BLM-NE]

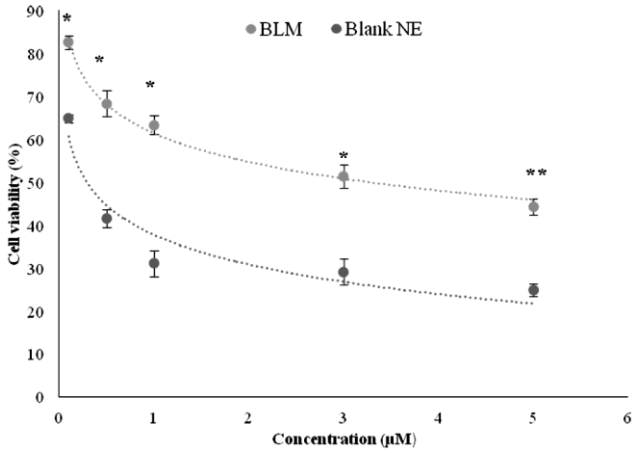

Fig. 2 —Percentage of cell viability of SKOV-3 cells treated for 24 h at different concentrations of the tested formulas. *Error bars* represent the standard deviation for n =3. (* & **) refer to the significant (P < 0.05) and highly significant (P < 0.01) differences between BLM and Blank NE.

were very small, indicating the homogeneous distribution of the droplet sizes. In terms of the zeta potential, the magnitude of the negative zeta potential of BLM-NE was less than blank NE.

Drug release study

Dialysis method was performed to determine the *in vitro* release of BLM and BLM-NE to examine the impact of nanoemulsion on the drug release. As illustrated in Fig. 1, loading BLM in NE was found to have a very highly significant release pattern compared to free drug. In other words, around 94% of BLM-NE was released within 24 h while only 37% of BLM was released in the same period of time.

Determination of cell viability using CCK-8

The % of cell viability and the half-maximal inhibitory concentration (IC_{50}) of BLM and blank NE were evaluated for 24 h at different concentrations. As displayed in Fig. 2, there was a steady decline in the SKOV-3 viabilities as the concentration of the subjected formulas increase. In fact, the viabilities of cells displayed a sharp drop with increase concentrations when subjected to blank NE. On the other hand, the viabilities of cells were gradually decreased when subjected to BLM. It was clearly shown that the reduction of cells was significantly higher when subjected to blank NE than BLM. It has been found that the IC_{50} of the tested blank NE was less than the IC_{50} of BLM. In particular, the IC_{50}'s of blank NE and BLM were estimated 0.40±0.001 µM and 3.5±0.003 µM, respectively.

In terms of the combination formulas, loading BLM in NE at different ratios had more cytotoxicity than the single formulas (Table 2) and 2BLM:1NE, which consisted of 2.33 µM of BLM and 0.133 µM of blank NE, was selected for further studies because it has the best toxic effect on the viabilities of SKOV-3 cells.

Table 2 — Cytotoxicity effect of BLM incorporated into the NEs at different ratios of (3.5±0.003 µM) BLM in (0.40±0.001 µM) NEs subjected to SKOV-3 cells for 24 h.

Formulas	Formulas conc. (µM)	% Cell viability	P value
BLM	3.50	50.00±1.20	-
NE	0.400	50.00±1.51	(>0.05) no significant differences
1BLM:1NE	1.75BLM +0.200NE	46.35±2.49	(>0.05) no significant differences
1BLM:2NE	1.20BLM +0.267NE	44.85±2.58*	<0.01* (significant difference)
2BLM:1NE	2.33BLM +0.133NE	37.38± 0.36***	<0.001*** (very highly significant difference)

[The percentages of cell viabilities are expressed as $\bar{X}$± SD. P value was measured by the pairwise t-test between BLM and the other tested formulae. BLM, bleomycin; and NE, nanoemulsion]

Morphological examination of SKOV-3 cells

As shown in Fig 3, cells exhibited morphological changes after treatment for 24 h at the IC_{50}'s of the single and NE formulas. Cells demonstrated changes in morphology, membrane blebbing and chromatin condensation when treated with BLM. Cells treated with blank NE and loaded formulas have shown changes in shape, the formation of apoptotic bodies, chromatin fragmentation and membrane blebbing. It should be noted that the intercellular spaces were observed in all of the cells treated with the tested formulas and increased in the ratio of 2BLM:1NE.

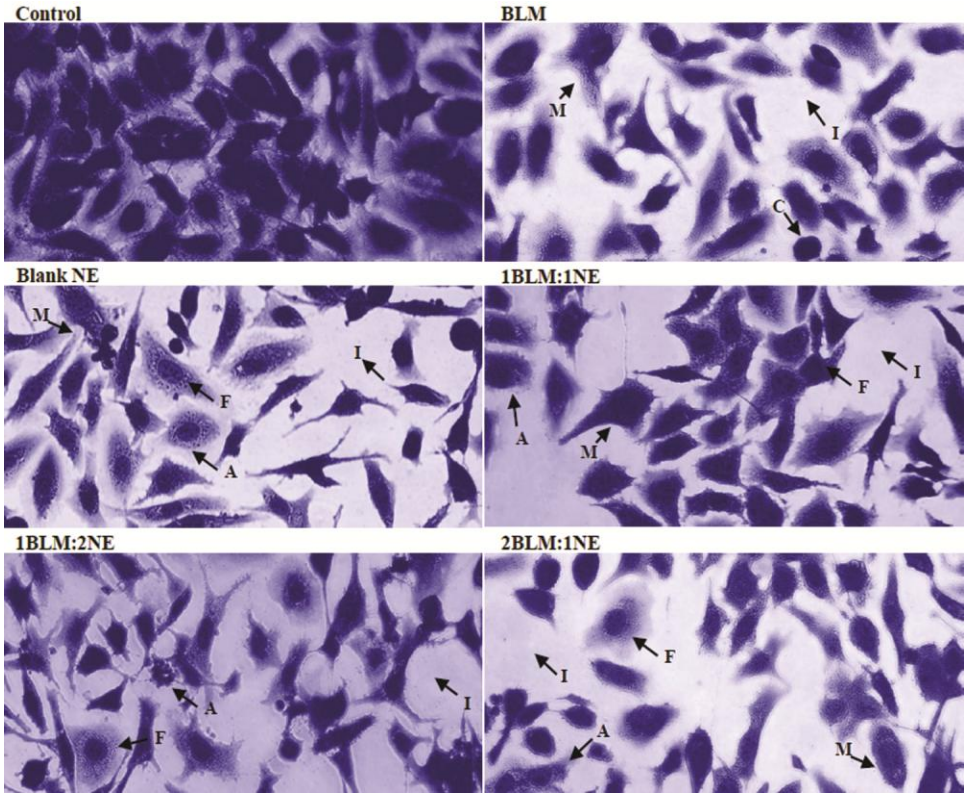

Fig. 3 — Light microscopy images of SKOV-3 cells treated with BLM and NEs formulas for 24h. [Coomassie blue was used for staining and images were magnified at 40×. Signs of apoptosis are represented by arrows as chromatin fragmentation (F), chromatin condensation (C), intercellular space (I), membrane blebbing (M) and apoptotic bodies (A)].

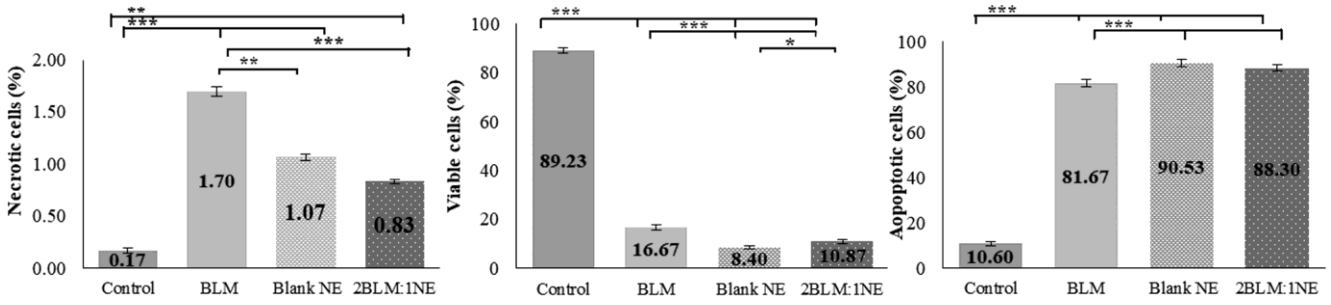

Fig. 4 — Flow cytometry measurements of the percentages of the viable (Q3), necrotic (Q1) and apoptotic (Q2+Q4) SKOV-3 cells treated with the tested formulas for 24 h and double-stained with Annexin V FITC and PI. *Error bars* represent the standard deviation for n=3. (*, ** & ***) refer to the significant (P <0.05), highly significant (P <0.01) and very highly significant (P <0.001) differences between the tested formulas.

Annexin V-FITC apoptosis detection assay

Double staining with FITC/PI can differentiate between necrotic (Q1), viable (Q3), early apoptotic (Q4) and late apoptotic (Q2) cells. As exhibited in Fig. 4, both of blank NE and BLM-NE had a markedly more apoptotic effect than BLM (P <0.001). In contrast, BLM had a more necrotic effect than the NE formulas. The percentages of viable cells subjected to the blank NE and BLM-NE were considerably less than those subjected to BLM.

Cell death detection

The extent of cellular DNA fragmentation was estimated by measuring the enrichment factor. According to Fig. 5, cells treated with the blank NE and BLM-NE had greater enrichment factors than those treated with BLM.

Discussion

In the present study, oil in water (O/W) NE formulations based on cinnamon oil were developed

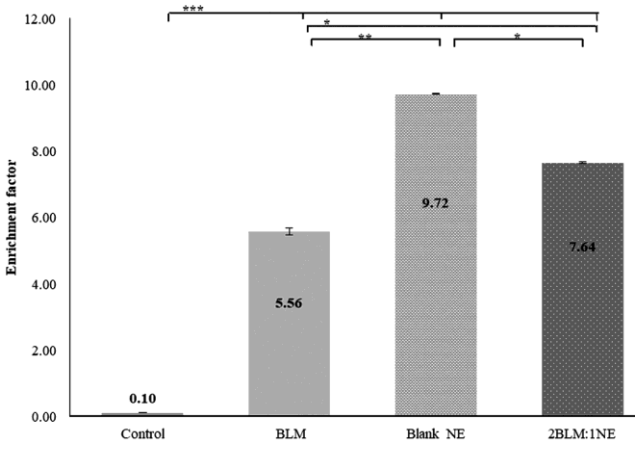

Fig. 5 — Quantification of nucleosomal DNA fragmentation of cells treated with BLM, blank NE and 2BLM:1NE in SKOV-3 cells after 24h incubation. *Error bars* represent the standard deviation for n=3. (*, ** & ***) refer to the significant ($P< 0.05$), highly significant ($P <0.01$) and very highly significant ($P <0.001$) differences between the tested formulas.

to deliver BLM into SKOV-3 cells. It was reported that the administration of BLM in W/O emulsion showed poor potency as there was a slight accumulation of BLM. On the other hand, the administration of BLM in O/W emulsion led to a noticeable accumulation of the drug[27]. The clinical application of BLM has been limited due to its severe side effects and impermeability to the cell membrane[28]. Thus, O/W nanoemulsions might control the drawback of this drug by improving the bioavailability, drug stability, and lower adverse effects[29]. Moreover, O/W NEs are one of the most promising colloidal delivery system suitable for encapsulating Eos[30,31] which they have the ability to decrease the volatilization and degradation of Eos[32].

As it appears in the results, the average droplet diameters of blank NE have increased when loaded with BLM implying that BLM was incorporated into the core of the nano-suspension droplets[9,12]. In addition, the NE formulations have a negative zeta potential which implies that the negatively charged nanocarriers accumulated more efficiently inside the cells and thereby could be potentially more toxic[33]. The release rate of BLN from NE was significantly higher than that of free BLM. Similar results showed that the release rate of the drug from the self-nanoemulsifying drug delivery system was markedly higher compared with a drug suspension[34].

The cytotoxic activity of both NE and drug formulations was assessed in SKOV-3 cells for 24 h by CCK-8 assay. The results indicated that the cells showed an increase in their sensitivity when subjected to blank NE and BLM- loaded NE based on cinnamon oil when compared to cells treated with free BLM. Our findings are in agreement with several studies which previously confirmed that the cytotoxicity of BLM and various EO's were enhanced when included in different nanocarriers system[35-40]. It has been reviewed that cinnamon oil and its active constituents exert antiproliferative activity and cytotoxic effect against various types of human cancer cells[41,42]. Our finding revealed that the IC_{50} of BLM decreased when loaded in NE. Previous study demonstrated that the IC_{50} of BLM loaded nanostructured lipid particle was lower than the IC_{50} of BLM solution[36].

Within 24 h, the best level of apoptosis was observed in blank NE and BLM-NE followed by free BLM. In agreement with our study, previous reports revealed that Bleomycin sulfate loaded nanostructured lipid particles induces a significantly greater degree of apoptosis in comparison to BLM[36]. Moreover, it has been found that using nanoliposomes as a carrier for BLM leads to an increase in apoptotic cells in comparison to conventional BLM[28].

According to DNA fragmentation analysis, there was an increase in DNA fragmentation of BLM when loaded in NE compared to free BLM. A similar study exhibited that there was an enhancement in DNA double-strand breaks in cells treated with gold nanoparticle loaded BLM when compared with Free BLM[43].

Conclusion

The results reveal that Blank NE had the best cytotoxic effect among all the tested formulas and the efficacy of BLM was improved when formulated in NE. Our finding data evinced that BLM-NE based on cinnamon oil may have the capability to prolong the drug release rate, improved its cytotoxic and apoptotic effects on SKOV-3 cells. It is recommended to apply the new formulas in animal models in order to investigate the adverse side effects of the NE formulas on the organs and tissues.

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Conflict of Interest

Authors declare no competing interest.

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