ORIGINAL ARTICLE

Apoptotic Effect of Bleomycin Formulated in Cinnamon Oil Nanoemulsion on HeLa Cervical Cancer Cells

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Abstract

Background: Using the essential oils with the cytotoxic agents in nanocarrier may have a potent effect on the cancer cells. **Materials & Methods:** A nanoemulsion (NE) consisting of the cinnamon oil (free NE) and bleomycin-loaded NE (BLEO-NE) was formulated, physically characterized by the zetasizer, and evaluated *in vitro* to determine its antineoplastic activity against HeLa cervical cancer cells. The cell viability, morphological changes, apoptosis, and DNA fragmentation of the cancer cells, subjected to the free NE and BLEO-NE, were examined by the cell counting kit-8 assay, light microscopy, annexin V-fluorescein isothiocyanate, and ELISA cell death detection, respectively. **Results:** It has been found that the dispersed nanodroplets of free NE had mean particle diameter and zeta potential of 119.60 ± 1.20 nm and −0.913± 0.001 mV, respectively, but they had got enlarged to 524.33 ± 1.10 nm and 0.537 ± 0.002 mV, respectively, when BLEO was loaded (BLEO-NE). The results of the cell viability, apoptosis, and cell death detection have demonstrated that BLEO-NE had a higher apoptotic effect than free BLEO. **Conclusion:** These findings suggest that the combination therapy of BLEO with NE-based cinnamon oil has a potential anticancer activity.

Key words: Antineoplastic activity, chemotherapy, cytotoxicity, DNA fragmentation, essential oils

INTRODUCTION

Cancer has long been a worldwide threat and is the second major cause of death. As one of the most prevalent cancer treatment methods, chemotherapeutic agents remain unfavorable due to non-selective nature and serious adverse effects.[1] One of the well-known anticancer drugs that have been used in chemotherapy against various types of cancer is bleomycin (BLEO), a glycopeptide antibiotic obtained from the bacterium *Streptomyces verticillus*. Many data indicate that DNA damage is a significant contributor to BLEO cytotoxicity.[2] The previous studies have verified that BLEO stops cancer cell proliferation by either breaking the double-helical of DNA or inducing apoptosis.[3] To ameliorate the bioavailability of BLEO and to lower its adverse effects, BLEO-loaded nanoparticles were reported.[2,4-7]

Nanoemulsion (NE) is one of the best approaches to increase the water solubility of the drugs, which in turns improves the bioavailability of the drug in the systemic circulation. As the droplets are in nanosize, the interfacial areas are increased, which in turn affects the drug's transport properties that area very significant factor in drug delivery.[8] NEs are defined as a thermodynamically stable system, in which two immiscible liquids phases are mixed to form a single-phase using an emulsifying agent, i.e., surfactant and cosurfactant. They are colloidal dispersion and characterized by their stability and clarity. Microfluid or ultrasonic emulsification methods are used to apply high shear to produce NE.[9]

Essential oils (EO's) with therapeutic potential can work in two ways: Chemoprevention and cancer suppression. Activation of detoxification enzymes, antimetastasis, modulation of DNA repair signaling, and antiangiogenesis are different mechanisms involved in cancer treatment.[10] Direct use of EO's is restricted due to their hydrophobicity

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and high sensitivity to external factors. Usage of EO's in the form of NEs protects them against the effects of oxidizing factors, increases their bioavailability, and improves their biocompatibility.[11]

Therefore, the present study was designed to formulate BLEO in NE based on cinnamon oil using energy generated by ultrasonication and evaluate its anticancer action on HeLa human cervical cancer cell line.

MATERIALS AND METHODS

Materials

Polyoxyethylene-20-sorbitan monooleate (Tween 80) and Sorbitan laurate (Span 20) were purchased from Techno Pharamchem (New Delhi, 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 (×1 PBS, pH7.4) were obtained from UFC biotech (Riyadh, KSA). Fetal bovine serum (FBS) was purchased from Biochrom (Berlin, Germany). Dimethyl sulfoxide was purchased from Thermo fisher scientific (Washington, USA). BLEO (15 units) was purchased from Fresenius Kabi (Maharashtra, India). Cell counting kit-8 (CCK) (Lot. No LE612) was purchased from Dojindo Molecular Technologies (Tokyo, Japan). Annexin V-fluorescein isothiocyanate (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). Buffer solution (pH5) was ordered from AppliChem GmbH (Darmstadt, Germany). The HeLa human cervical cancer cell line was obtained from the Tissue Culture Bank at King Fahd Medical Research Center, King Abdulaziz University (Jeddah, KSA).

Preparation of the NE formulas

The free 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 cinnamon oil and 80% of buffer solution (0.5 mM, pH5). The mixture was directly emulsified by sonication using OMNI Sonic Ruptor 4000 (Georgia, USA) at 50% power until it became clear. The stock solution of 1 mg/mL of BLEO was formed by dissolving BLEO in sterile distilled water.

Physical properties of the cinnamon oil-in-water NEs

The z-average diameter of particles (nm), polydispersity index (PDI), and zeta potential (mV) of the NE formulations were determined by Zetasizer (Malvern Instruments, Malvern, UK). The tested samples were performed at 25 ± 0.2°C.

Cell culture

The HeLa cell line was cultured in a 25cm^2 cell culture flask containing DMEM, 10% (v/v) FBS, and 1% (v/v) penicillin-streptomycin in a humidified incubator at 37°C. The media were replaced every 48 h until cells reached confluence, followed by washing cells with 1 mL of PBS and detaching by adding 1 mL of trypsin. Finally, the cells were incubated at 37°C.

Scanning of cell toxicity using CCK-8 assay

The cytotoxicity of tested formulas against HeLa cells was evaluated by the CCK-8. Briefly, 100 µL of culture media containing cells was plated in each well of a flat-bottomed 96well plate and was incubated overnight at 37°C in a CO_2 incubator. After that, cells were treated with 200 µL of five different concentrations of BLEO (0.5–50 µM), seven concentrations of free NE (0.001–5 µM), and three combination formulas at different ratios (1:1, 1:2, and 2:1) by mixing the IC_{50} of each BLEO and free NE. Then, the plate was incubated for 48 h at 37°C in a CO_2 incubator. After that, 5 µL of CCK-8 solution was added to each well and incubated for 4 h. Finally, the absorbance for each well was measured at 450 nm by a microplate reader (Biotech, US). Wells, included complete culture media, were considered negative control while complete culture media with cells were considered as a positive control. The experiments of each sample were implemented in triplicate (n = 3). The percentages of cell viability for HeLa cells were obtained by the following equation: Cell viability (%) = ([A of treated cells - A of negative control])/(A of positive control) × 100.

Cell morphology characterization

The HeLa cell morphological changes were observed under the phase-contrast inverted microscope (Olympus ×1 51, Japan). Cultured HeLa 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. Then, cells were re-incubated with 200 µL of media containing tested formulas for 24 h. After that, cells were washed with 100 µL of PBS for 5 min and fixed by 4% formaldehyde for 5 min. Then, the fixation solution was removed, and the cells were stained with 100 µL of 10% Coomassie blue dye for 10 min. Finally, the stain solution was discarded, and cells were washed 5 times with tap water and left to dry at room temperature (25°C).

Annexin V-FITC apoptosis detection assay

Apoptosis was analyzed by flow cytometry using Annexin V-FITC kit. This kit had two staining protocols in which annexin V conjugated with FITC that stains the phosphatidylserine sites, which is normally located on the outer membrane surface of apoptotic cells. The necrotic cells

are stained with propidium iodide (PI) that binds the cellular DNA of the lysed cells. HeLa cells seeded into 6-well flat-bottomed tissue culture plate at a density of 2×10^5 cells per well-containing 1 mL of the growth media and incubated for 24 h at 37°C in a CO_2 incubator. Cells were treated with 2 mL of the tested formulas and were incubated for 24 h. Then, cells were washed with 1000 µL of PBS, dissociated by 300 µL of trypsin, and incubated for 3 min, followed by the addition of 2000 µL culture media. After that, cells were transferred to a centrifuge tube and spin down at 1400 rpm for 10 min. The supernatant was discarded, cells were re-suspended in ice-cold PBS cells, and spin down twice at ×400 g for 5min. Detached cells were re-suspended in 100 µL of the binding buffer. After that, the mixture was transferred to a flow cytometry tube followed by the addition of 5 µL of annexin V and 5 µL of PI and then incubated at room temperature for 15min away from light. Finally, 400 µL of the binding buffer was added, and the cell suspension was evaluated by BD FACSAria TM III Flow Cytometer (BD Biosciences, US). Data were analyzed using FACS Diva software version 6.1.3.

Cell death detection

Cell death detection ELISA plus kit allows specific determination of cytoplasmic histone release from the nucleus after inducing cell death. Briefly, 100 µL of growth media containing cells were plated in each well of a 96 well plate and were incubated for 24 h at 37°C in a CO_2 incubator. Then, the 96 well-plate was centrifuged at ×200 g for 10 min, followed by removing the supernatant. After that, 200 µL of lysis buffer was added to each well and incubated for 30 min at room temperature (25°C). The lysate was spun down at ×200 g for 10 min. Then, 20 µL of the supernatant was transferred to a streptavidin-coated microplate, and 80 µL of immune-reagent was added to each well. After that, the microplate was covered with adhesive foil and incubated at room temperature (25°C) on shaker under gently shaking (300 rpm). After 2 h, the solution was discarded thoroughly by tapping, and each well was rinsed thrice with 250 µL of incubation buffer. Then, 100 µL of ABTS solution was added to each well and incubated for 10 min on a plate shaker. Finally, the absorbance was measured at 405 nm using a microplate reader (BioTek, US). The enrichment factor was determined as absorbance of tested sample/absorbance of the control sample.

Statistical analysis

Using the MegaStat (version 10.3, Butler University, Indianapolis, IN), the differences between the samples were assessed statistically with the one-factor analysis of variance (ANOVA) test and the independent sample *t*-test. The variations between the samples were considered when $P < 0.05$.

RESULTS

Physical measurement of NEs

Mean droplet diameter, zeta potentials, and PDI of the tested NEs were analyzed by Zetasizer, as depicted in Table 1. Mixing BLEO with the NE formula had enlarged the nanodroplet size and changed the charge of the droplet from negative to positive ($P < 0.001$). The sizes of the dispersed nanodroplets of the tested formulas were homogeneously distributed since the PDIs, calculated through dividing the mean by the standard deviation, were <0.250.

Cytotoxicity screening of tested formulas

According to Figure 1, the IC_{50}'s of BLEO and Free NE were reported at 10 ± 1.12 µM and 0.2 ± 0.01 µM, respectively. As shown in Table 2, 1BLEO:2NE had the greatest toxicity among the combination NE formulas on the HeLa cells and the lowest ratio of beam loss monitoring (BLM). Accordingly, it was selected for the subsequent experimental studies.

Morphological changes in HeLa cells

Cell morphologies under the inverted microscope were examined for HeLa cells incubated for 24 h, as shown in

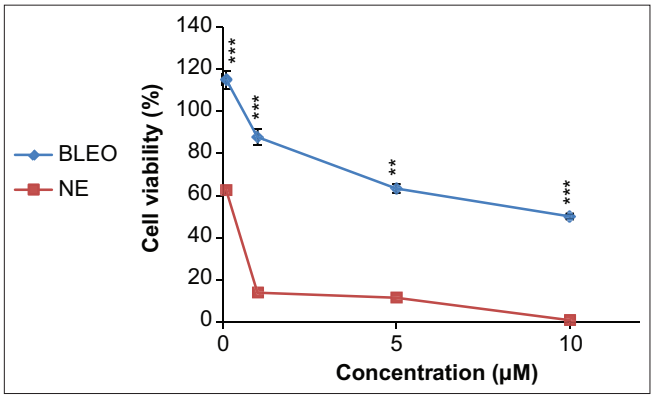

Figure 1: Growth inhibitory rates in response to the various concentrations of beam loss monitoring and nanoemulsion (NE) on HeLa cells. *P*-values were measured using the one-factor ANOVA and the independent sample *t*-test to assess the statistical differences between bleomycin and the NE formula. **$P < 0.01$ and ***$P < 0.001$

Table 1: Zetasizer measurements of the tested formulas

Formulas	Z-average diameter (nm)	Polydispersity index	Zeta potential (mV)
Free NE	119.6±1.20***	0.010	−0.913±0.001***
BLEO-NE	524.33±1.10	0.002	0.537±0.002

***Very highly significant difference between free NE and BLEO-NE ($P<0.001$). BLEO-NE: Bleomycin-nanoemulsion

Figure 2. The nuclei of the untreated cells (control) showed homogenous color with no evidence of fragmentation after staining. Among the loading formulas, DNA fragmentation, and formation of apoptotic cells, membrane blebbing, and chromatin condensation were observed. The nuclei of HeLa cells showed moderate apoptotic bodies formation when treated with BLEO.

Detection of apoptosis

The effect of the BLEO and 1BLEO:2NE on the HeLa cells was demonstrated in the flow cytometry plots displayed in Figure 3a, while the percentages of apoptotic cells (Q2 + Q4) are displayed in Figure 3b. It has been found that the loaded formula was significantly more apoptotic ($P < 0.001$) than the single formula as the sum of Q2 and Q4 was 70.40%.

Table 2: The cytotoxicity of tested formulas subjected to HeLa cells at different ratios of (10 ± 1.12 μM) BLM in (0.2 ± 0.01 μM) NEs for 24 h

Formulas	Cell viability	*P*-value
BLEO	50.01±1.12	-
1BLEO:1NE	55.88±2.40	<0.001*** (very highly significant)
1BLEO:2NE	47.17±1.34	>0.05 (no significant)
2BLEO:1NE	56.55±2.51	<0.001*** (very highly significant)

The percentages were expressed as $\bar{X}$±SD. *P*-values were measured using the one-factor ANOVA and the independent sample *t*-test in order to assess the statistical differences between BLEO and the other combination formulas.
BLEO-NE: Bleomycin-nanoemulsion

Evaluation of DNA fragmentation

Cell death was observed in HeLa cells when treated with single and loaded formulas. There was a very highly significant increase ($P < 0.001$) in DNA fragmentation, in which the enrichment factor of1BLEO:2NE was (16.04 ± 0.12) compared to BLEO (8.42 ± 0.04).

DISCUSSION

The EO's are hydrophobic concentrated liquids possessing aromas generated by aromatic plants.[12] EO's and their components are well known for anticancer activity.[13] Junhong *et al*.[14] reported that cinnamon oil has shown a cytotoxic effect against HaCaT (human epidermal keratinocytes) cell lines. Similarly, the cinnamon EO was one of the most toxic oils in MCF-7 and MDA-MB-231 breast cancer cells.[12]

Over the past few years, NEs have gained more attention due to their unique physicochemical and functional characteristics.[15] Therefore, the present study commenced formulating BLEO in NE based on cinnamon EO by a high energy method. Mixing BLEO with the NE formula has enlarged the nanodroplet size and changed the charge of the droplet from negative to positive. The previous study reported that the z-average diameter of the NE formula has significantly increased when loaded with the drug.[16] Ghosh *et al*.[17] demonstrated that increasing surfactant concentration resulted in a decrease in droplet diameter, which may explain the larger NEs droplet diameter in our study due to low surfactant/co-surfactant ratio. It was suggested that the delivery efficiency of nanocarriers defined from −10 to

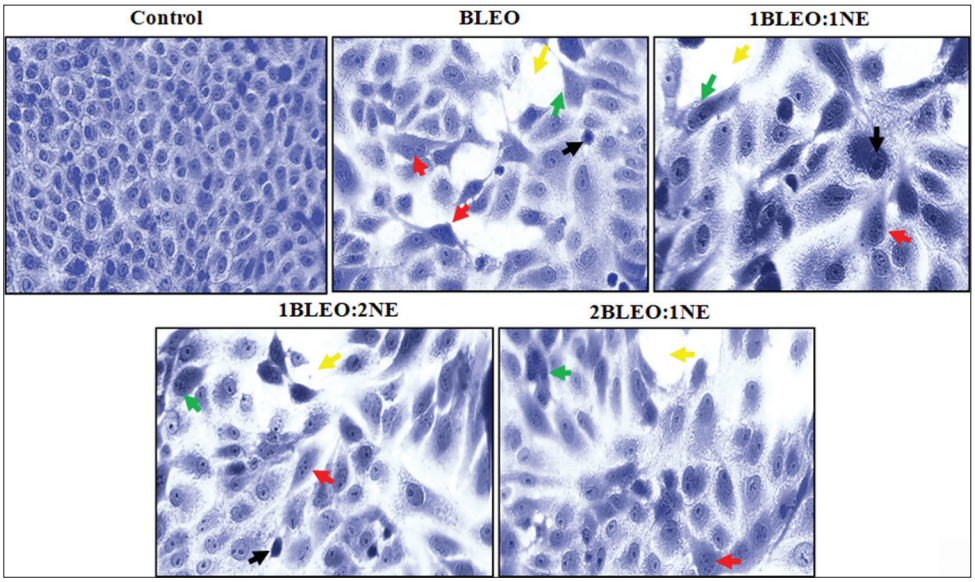

Figure 2: Morphological changes in the untreated (control) and treated HeLa cells with different concentrations of the tested formulas mixed at different ratios of 10 ± 1.12 μM beam loss monitoring in 0.2 ± 0.01 μM NEs for 24 h. Images were magnified at ×40. Signs of apoptosis are represented by the black arrows (chromatin condensation), yellow arrows (intercellular space), the green arrows (membrane blebbing), and red arrows (chromatin fragmentation)

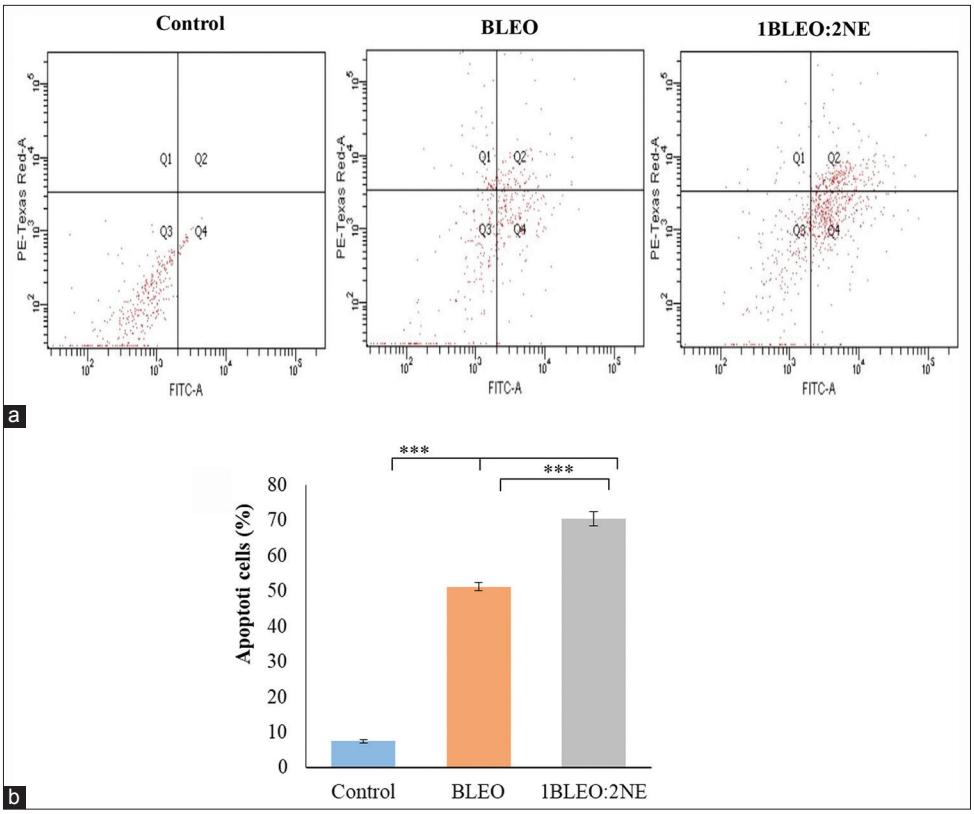

Figure 3: (a) Fluorescein isothiocyanate/propidium iodide flow cytometry plots of HeLa cells subjected to the tested formulas for 24 h. (b) Histogram presentation of the apoptosis percentages (Q2 + Q4) of the HeLa cervical cancer cells treated with 10 ± 1.12 μM of bleomycin (BLEO) and 1BLEO:2 nanoemulsion (NE) (10 ± 1.12 μM BLEO: 0.4 ± 0.01 μM) for 24 h. *P*-values were measured using the one-factor ANOVA and the independent sample *t*-test to assess the statistical differences between BLEO and the NE formula. ***$P < 0.001$

+10 mV, tends to be higher than the delivery efficiency of nanocarriers with positive (>10 mV) or negative (<−10 mV) zeta potentials.[18] It was reported that the negative charges on the cell membranes of the tumor vasculature might have potentiated the electrostatic interaction between positively charged nanoparticles and tumors. In addition, tumors are preferentially taken up nanoparticles with a positive charge and retained them for a longer time when compared to negatively charge or neutral particles.[19] The lower value of PDI for NE can be correlated with the use of ultrasound and also comparatively more stable than emulsions prepared by other mechanical devices.

In the present study, the cytotoxicity screening of both NE and BLEO formulations was assessed in the HeLa cells by CCK-8 assay. 1BLEO:2NE showed better antitumor activity than 1BLEO:1NE and 2BLEO:1NE. In agreement with our study, Ujhelyi *et al*.[20] have shown that the inhibitory effect of BLEO in self-microemulsion was higher than the applied anticancer agent alone.

Interestingly, it has been demonstrated that the apoptotic effect of BLEO was significantly ameliorated when loaded in NE based on cinnamon oil, which was found to have greater antiproliferative activity than the free BLEO. Koshkaryev *et al*.[2] have reported that loading BLEO in octaarginine-modified fusogenic liposomes led to significantly stronger cell death and DNA damage *in vitro* relative to all controls. In addition, it demonstrated a prominent anticancer effect in the BALB/c mice bearing 4T1 tumors. According to the study by Shatskaya *et al*.,[21] delivery of BLEO into cells using TiO_2 nanoparticles appeared to be 7–10 times more efficient than free BLEO in the fragmentation of intracellular nucleic acids and enhance the condensation of chromatin. Moreover, it may allow reducing the therapeutic dose of BLEO.

CONCLUSION

The resulted formula of BLEO:2NE exhibited cell growth inhibition and apoptotic effects *in vitro* when compared to Free BLEO. It is recommended to apply the formula to other cancer cells. In addition, further studies are necessary to determine the antitumor effect of the new formula and its adverse side effects *in-vivo*.

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