Coupling Ifosfamide to nanoemulsion-based clove oil enhances its toxicity on malignant breast cancer and cervical cancer cells

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Abstract

The anticancer effects of chemotherapeutic agents may be accentuated, and their side effects minimized by combining them with essential oils in nanocarrier systems. This study aimed to incorporate ifosfamide (IF) into nanoemulsion-based clove oil (IF-CLV). The nano-emulsion (NE) formulas were characterized with Zetasizer. The cytotoxicity of the formulated NEs against cervical (HeLa) and breast (MCF-7) cancer cell lines was determined using MTT assay, light microscopy, and DAPI staining. The z – average diameters of NE-CLV and IF-CLV were 63.1±1.00 and 89.4±2.64 nm, while Zeta potential values were – 4.39±0.4 and – 11.65±1.1 mV, respectively. Cytotoxicity studies revealed that relative to free IF, NE-CLV and IF-CLV were highly toxic on HeLa and MCF-7 cells, in a dose-dependent manner. The half-maximal inhibitory concentration (IC50) values of NE-CLV against HeLa and MCF-7 have decreased 38 and 27 folds, while the corresponding IC50 values of IF-CLV have decreased 57 and 35 folds, respectively. These results suggest that the incorporation of IF into NE-based clove oil produces potent therapeutic effects against cancer cell lines.

Keywords

Clove oil, Cytotoxicity, Ifosfamide, Nanoemulsion, Z-average diameter

Introduction

Ifosfamide (IF) is a chemotherapeutic alkylation agent belonging to the class of oxazaphosphorines. It is used for treating a wide range of human cancers, usually as a single anti-tumor agent, or in combination with other anti-cancer agents. The cancers treated with IF are osteosarcoma, advanced bladder carcinoma, small and non-small lung cell carcinoma, lymphoma, as well as ovarian, cervical, and thymic cancers (Kerbusch et al. 2001). Ifosfamide (IF) is metabolized and activated in the liver by CYP450 enzymes to phosphoramid mustard and acrolein, which attach to DNA and suppress DNA synthesis. Unfortu-
and Vittal 2019) leads to an increase in surface area and enhance the solubility of hydrophobic drugs (Thakur et al. 2013; Mahato 2017; Jain et al. 2018; Gurpreet and Singh 2018). NEs are passively accumulated in the tumor environment more than normal tissues due to their defective vasculatures and poor lymphatic drainage (Mahato 2017).

Previous studies have shown that essential oils prevent cancer cell proliferation and cytotoxicity through a broad range of mechanisms (Blowman et al. 2018; Russo et al. 2018; El-Abid et al. 2019; Rajivgandhi et al. 2020). Clove essential oil and its constituents have strong antioxidant, antitumor, and inflammatory properties (Batiha et al. 2020). Although their anticancer activity, the poor water solubility and instability of essential oils limit their use in clinical applications. Therefore, several attempts were conducted to encapsulate essential oils into NEs to improve their solubility and therapeutic effects (Thakur et al. 2013).

The current study aimed to load IF into oil-in-water NE after incorporation of clove essential oil and to evaluate its anticancer effect on cervical cancer cells (HeLa) and breast cancer cells (MCF-7).

Materials and methods

Preparation of NE formulations

A dispersed and transparent oil-in-water (O/W) NE formulation (NE-CLV) was produced after several attempts at generating the NE formula. This was successfully prepared by ultrasonic homogenization method through mixing tween 80/span 20 (Sigma, Missouri, US), clove oil (Sokar Nabat, Jeddah, KSA), and distilled water at a weight ratio of 0.055, 0.018, and 0.97, respectively. The tween 80 and span 20 were used at a 2:1 ratio. Mixing was carried out in a 5 ml screw cap Pyrex tube and heated up to 70°C in a water bath for 5 min until one phase emulsion formed. Then, transparent NE was produced from the resulted emulsion by using ultrasonication (Omni Sonic Ruptor 4000, Georgia, USA) equipped with a 3.8 mm diameter titanium probe tip and sonicated for 20 minutes at an amplitude of 40% and frequency of 20 kHz. The overheating was prevented during sonication by placing the emulsion under ice-coding. Ten replicates of clove oil-based NE formulations were produced. The stock solution (19mM) was prepared by dissolving 5mg of IF (Baxter, US) in 1 ml distilled water (IF) and 1ml of NE-based clove oil (IF-CLV).

Physical characterization of NE formulas

The nanodroplet properties of NE-CLV and IF-CLV were determined with a Zetasizer (Malvern Instruments, Malvern, UK). The sizes of the NE dispersed nanodroplets were measured as z-average diameters, while the charges were measured in terms of zeta potential. Measurements were performed in triplicate at room temperature.

Cell culture

The human cervical cancer cell line (HeLa) and human breast adenocarcinoma cell line (MCF-7) were purchased from American Type Tissue Culture Collection (Manassas, VA, USA). The cells were cultured in a 25-cm² cell culture flask containing Dulbecco’s modified eagle medium (DMEM) (Gibco Life Technologies, New York); 10% (v/v) fetal bovine serum (FBS) (Lonza Walkersville, USA) and 1% (v/v) penicillin-streptomycin, in a 5% CO₂/95% humidified atmosphere at 37°C. The media were refreshed every 48h until confluence, followed by washing in 2ml of 10 mM phosphate-buffered saline (PBS), pH 7 (Biодиагностик Company, Egypt). Thereafter, the cells were trypsinized with 2 ml of trypsin, followed by incubation at 37°C.

Cell viability screening using MTT assay

The toxicity of the tested formulations against the cancerous cells was determined using 3(4,5-dimethylthiazol e-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay (Biomatik, Ontario, Canada). The cells were cultured in 96-well plates at a density of 1x10⁴ cells per well in 100 μl of culture medium for 24h at 37°C in a CO₂ incubator. At cell adherence, the cells were treated with 100μl of several diluted concentrations were freshly prepared with culture medium from the stock solution (19mM) ranging between 100-500μM of each formulation, followed by incubation in a CO₂ incubator for 24h at 37°C. Thereafter, 5 µl of MTT reagent was added to each well and mixed gently, followed by incubation for 3 – 4h at 37°C in a CO₂ incubator. Then, the culture medium containing MTT reagent was discarded, and the resultant formazan crystals were solubilized with 100 μl of dimethyl sulfoxide (DMSO) (Biomatik, Ontario, Canada), followed by incubation for 2h. The absorbance of the formazan solution was read at 540nm in a microplate reader (BioTek, US).

Each formulation was tested in triplicate. Absorbance readings obtained from wells containing only culture media, and wells containing culture media + cells were considered as negative control and positive control, respectively. Cell viabilities were calculated as percentages using the following formula:

\[
\text{Cell viability} \cdot (\%) = \frac{(\text{Abs of treated cells} - \text{Abs of negative control})}{\text{Abs of positive control} - \text{Abs of negative control}} \times 100,
\]

where abs = absorbance

Determination of apoptosis using light microscopy

The cells were counted and seeded, each at a density of 1x10⁴ cells/well in a 96-well plate, and incubated for 24 h in 100μl of growth media. This was followed by incubation with 100μl of the tested formulation for 24 h. Thereafter, the wells were washed twice with 100μl of PBS, and fixed with 100 μl of 4% formaldehyde for 5 min. Then, the
fixation medium was discarded, and the cells were washed with 100μl of PBS, and stained with 100μl of 10% Coomassie blue (Biomatik, Ontario, Canada) for 10 min. The staining solution was then removed, and the cells were double-washed with 100μl of distilled water and left to dry at room temperature. Finally, the morphologies of the HeLa and MCF-7 cells were examined under the phase-contrast inverted microscope (Olympus IX51, Japan), and photographed.

**Determination of nuclear DNA using DAPI stain**

Nuclear condensations in formulation-treated MCF-7 and HeLa cells undergoing programmed cells death were determined using 4',6-diamidino-2-phenylindole (DAPI) dihydrochloride stain (Invitrogen Life Technologies, New York, USA). The stain can penetrate cells undergoing apoptosis, where it binds to specific regions in DNA and is detected as a strong blue fluorescence. The cells were counted and seeded in 24-well plates at a density of 5×10⁴ cells per well in 500μl of 10% FBS–DMEM, and were treated with 500μl of each tested formulation. After one day of incubation, the cells were washed with 300μl of PBS and fixed in 200μl of formaldehyde (Fisher Chemical, UK), before staining with 300μl of 300nM DAPI solution. The stained cells were incubated for 1-5 min at room temperature, after which they were examined under an inverted fluorescent microscope (Leica CRTC6000, Germany). Quantitative analysis of fluorescent intensity was performed using Image J 1.43 n software (Rasband, W.S., ImageJ, National Institutes of Health, USA).

**Statistical analysis**

One-way analysis of variance (ANOVA) was used to analyze differences amongst the tested formulations. All statistical analyses were done with MegaStat Excel (version 10.3, Butler University, Indianapolis, IN). Statistical significance of difference was assumed at p < 0.05.

**Results**

**Characteristics of formulated nano-emulsions**

As illustrated in Figure 1A, a transparent, one-phase oil-in-water NE was located at the corner of the pseudo ternary phase diagram. This was successfully prepared after several attempts by mixing tween 80/span 20, clove oil, and water at a weight ratio of 0.055, 0.018, and 0.97, respectively.

The droplet sizes and zeta potential of the formulated nano-emulsions obtained using Zetasizer (Malvern Instruments, Malvern, UK) are shown in Figure 1. The droplet size index (PDI) values of the tested formulations were less than 0.05, which indicates a high quality of homogenization. The absolute value of negative zeta potential was increased significantly. The values of zeta potential were – 4.39±0.4 and – 11.65±1.1mV for NE-CLV and IF-CLV, respectively.

**NE – based clove oil inhibited cell proliferation in vitro**

The influence of NE-CLV and IF-CLV formulations on the proliferation of HeLa and MCF-7 cells, determined for 24h at various concentrations between 100 and 500 μM, is shown in Figure 2. All the studied clove oil-based NEs demonstrated dose-dependent cytotoxicity against HeLa and MCF-7 cell lines. Treatment with IF-CLV resulted in remarkably increased cytotoxicity against both cell lines, relative to the same concentration of NE-CLV (drug-free IF). A comparison between IF-CLV and NE-CLV showed that the highest inhibitions of cell proliferation in HeLa and MCF-7 cells were observed at lower concentrations (100-200 μM and 200-400 μM, respectively), while the highest toxicity values were detected at higher concentrations (300 μM and 500 μM, respectively). Therefore, the clove oil-based NE treatments resulted in markedly higher sensitization of HeLa cells than MCF-7 cells.

As exhibited in Figure 2, values of IC₅₀ for IF-CLV were highly and significantly lower than those of NE-CLV. Results for HeLa cells showed that IF-CLV formulation significantly decreased the IC₅₀ of NE-CLV by 1.5 folds, while for MCF-7 cells, there was a 1.5-fold reduction in IC₅₀ of NF-CLV. Moreover, as was reported recently, The IC₅₀ values of the free IF drug for HeLa and MCF-7 cells were 7.69 ± 1.88 mM and 9.20 ± 2.01 mM, respectively (Alkhathib, AlMotwaa and Alkreathy 2019). These IC₅₀ values indicate that the clove oil-based formulations NE-CLV and IF-CLV significantly reduced the viability of HeLa cells by more than 38 – and 57-folds, and that of MCF-7 cells by more than 27 – and 35-folds, respectively, when compared to free IF drug alone.

**Effect of NE-based clove oil on morphologies of HeLa and MCF-7 cells**

Morphological changes in HeLa and MCF-7 cells treated with the formulations were monitored under a light microscope. The formulas were used for 24h at their IC₅₀. As shown in Figure 3, the untreated cells (control) exhibited normal cellular characteristics, with normal cell membrane structure and no variation in nuclear size and shape. In contrast, treatment of the cells with NE-CLV led to damage to cellular structure. The HeLa cells appeared organized with larger nuclei and the presence of cytoplasmic vesicles. In contrast, the MCF-7 cells appeared disorganized in arrangement, with loss of normal cellular features.
such as membrane integrity and nuclei, and presence of extracellular vesicles. As shown in Figure 3, treatment of HeLa and MCF-7 cells with IF-CLV led to structural abnormalities. The Hela cells became enlarged, with larger and darker nuclei than normal cells, and the presence of cytoplasmic vesicles. The MCF-7 cells were also enlarged and displayed membrane blebbing. In contrast, treatment of MCF-7 and HeLa cells with drug-free IF resulted in lower cell count, normal plasma membrane integrity and normal nuclei, without any late sign of apoptosis.

**NE based clove oil induced apoptosis in HeLa and MCF-7 cells**

As shown in Figures 4 and 5, the fluorescence intensity of the nuclei decreased as the concentrations of the tested formulations increased. There were changes in nuclei and shape of HeLa cells treated with NE-CLV and IF-CLV, when compared to the control. In contrast, the shape of Hela cells treated with drug-free IF was comparable to that of the control group. The MCF-7 cells exhibited high fluorescence intensity when treated with NE-CLV and IF-CLV, relative to cells treated with drug-free IF. On the other hand, cell counts in the free IF-treated group were higher than those in MCF-7 and HeLa cells treated with NE-CLV and IF-CLV.

Figure 6 shows that there were marked decreases in the fluorescence intensity of the nuclei of cells treated with NE-CLV at all tested concentrations when compared to that of IF-CLV. The results obtained for HeLa cells showed the highest decrease from 2.25-fold to 0.52-fold at a concentration of 200µM, and the lowest decrease from 2.5-fold to 0.84-fold at a concentration of 100µM, relative to control. In MCF-7 cells, the highest reduction was from 1.27-fold to 0.89-fold at a concentration of 400µM, whereas the lowest reduction from 1.31-fold to 0.8-fold was at a concentration of 300µM, relative to control.
Figure 2. Cell viability of HeLa and MCF-7 cells after 24 h of incubation with clove oil-based NE at various concentrations. Data are expressed as mean ± SE (n = 3). Asterisks denote statistical significance (**p < 0.001 considered as very highly significant, *0.001 ≤ p < 0.01 considered as highly significant, and *0.01 ≤ p < 0.05 considered as significant).

Discussion

In this study, clove oil-based NEs was successfully prepared after several attempts by mixing tween 80/span 20, clove oil, and water at a weight ratio of 0.055, 0.018, and 0.97, respectively. The surfactant and co-surfactant (tween 80 and span 20) ratio in the NEs-based clove oil was 2:1. It has been demonstrated that a combination of the non-ionic surfactants tween 80 and span 20 enhances water solubilization and improves solubility when combined with oils. In addition, tween 80 improves the dispersibility of nanoparticles in aqueous media and prevents their aggregation (Porras et al. 2004; Silva et al. 2013; Zhao et al. 2010). The droplet size of the prepared formulations was within the nano range (less than 100 nm), with a low PDI value. Small diameters of NE droplets are indicative of increased surface area which leads to high absorption. The low PDI indicates the NE droplet was quite uniform and narrowly distributed (Pepper et al. 2017). The marked increase in average particle size after IF drug loading (IF-CLV) may be due to the distribution of IF inside NE droplet.

Currently, the use of chemotherapeutic drugs is the major treatment method for arresting the growth and spread of cancer cells. Unfortunately, these agents (including IF) have undesirable side effects and they need to be administered repeatedly within short intervals (Klastersky 2003). In the current study, incorporation of IF into NEs-based clove oil resulted in stronger anti-cancer effects than when the free IF was used alone. Using MTT assay, IC_{50} values of NE-CLV and IF-CLV for HeLa cells were reduced by 38 and 57 folds, respectively. For MCF-7 cells, the IC_{50} values were reduced by 27 and 35 folds, respectively. Moreover, for the two cell lines, the IC_{50} of the IF-CLV formula was lower than that of NE-CLV. In addition, the changes in cellular morphology revealed different signs of cellular damage due to treatment with NE-CLV and IF-CLV formulations. These changes included loss of cell membrane, and disappearance of extracellular and intracellular vesicles when compared with IF drug alone, which preserved cell membrane integrity and produced very minimum cell damage. Furthermore, the nuclei fluorescence intensities due to NE-CLV and IF-CLV treatments were greater than that from IF alone. This indicates a high degree of apoptotic effect of NEs-based clove oil on HeLa and MCF-7 cells. The significant reduction in the viabilities of cancer cells enhances the cell apoptosis suggested that the NE contai-
Fluorescence microscopic images of changes in nuclear morphology of HeLa cells treated with various concentrations of the tested formulas for 24h, as revealed using DAPI stain (n = 3).

Figure 4. Fluorescence microscopic images of changes in nuclear morphology of HeLa cells treated with various concentrations of the tested formulas for 24h, as revealed using DAPI stain (n = 3).

The high anticancer effects of IF-CLV and NE-CLV were due to the combination of NE and anticancer essential oil, but the growth inhibition was higher in IF-CLV than in NE-CLV due to the anti-cancer properties of IF. It has been found that the NE system increases the absorption and bioavailability of drugs and protects oils from oxidation and hydrolysis when applied as an oil-in-water dispersed system (Gurpreet and Singh 2018).

Moreover, it has been reported that clove essential oil exerted an anti-cancer effect against several cancer cell lines including MCF7 cells, prostate cancer cells (PC-3) and
Figure 5. Fluorescence microscopic images of changes in nuclear morphology of MCF-7 cells treated with various concentrations of the tested formulas for 24h, as revealed using DAPI stain (n = 3).

Liver cancer cell Hep G2 (Ogunwande et al. 2005; Yoo et al. 2005; Salama et al. 2018). It has been demonstrated that clove oil extract inhibited cancer growth and induced cell cycle arrest through upregulation of protein expression of p21\(^{WAF1/Cip1}\) and \(\gamma\)-H2AX, and reduced expressions of cell cycle-regulated proteins, in addition to induction of apoptosis by enhancing G0/G1 cell cycle arrest (H. Liu et al. 2014). Indeed, a recent study found that an active fraction of clove oil extract promoted apoptosis of human colorectal cancer HCT-116 cells via phosphorylation of members of the PI3K/Akt/mTOR signaling pathway (M. Liu et al. 2018). Studies have reported that eugenol is the most active component of clove essential oil that is responsible for the strong antitumor effects against a broad spectrum of
Cancers through the regulation of oncogene and the caspase-dependent pathway (Batiha et al. 2020). Eugenol has been shown to inhibit several oncogenes that correlate with breast cancer, including NF-κB and cyclin D1, and it promoted the intrinsic apoptotic pathway through potent reduction of E2F1 expression and inhibition of the anti-apoptosis target (Raja et al. 2015).

Conclusion

The results obtained in this study have demonstrated that NE-based clove oil formulation (IF-CLV) strongly induced cytotoxicity and apoptosis in HeLa and MCF-7 cells, resulting in enhanced damage of cell morphology and nuclei, relative to when the free IF drug was used. MTT assay demonstrated a sensitivity of the HeLa cells more than MCF-7 cells when treated with NE-CLV and IF-CLV formulas. Moreover, IF-CLV formulas were highly effective than NE-CLV formulas. Cellular and nuclear morphologies approved that the IF-CLV formula was more affected than the NE-CLV formula. The variation in the inhibition of the cell viability, cell membrane damage, and nuclear alteration, that's perhaps because of the different mechanisms of action of NE-CLV and IF-CLV in killing the HeLa cells and MCF-7 cells. Further studies are needed to understand the mechanism behind the cytotoxic effect of NE-based clove oil.

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