

Effect of vesicular encapsulation on *in-vitro* cytotoxicity of ciclopirox olamine

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Ciclopirox olamine (CPO), an antifungal has recently been cited as a drug repurposed for cancer treatment. Vesicular drug delivery systems like liposomes and niosomes have proven to increase the efficacy of anticancer drugs. The purpose of this paper was to evaluate the effect of two vesicular delivery systems liposome and niosome on the anticancer potential of CPO using *in vitro* cytotoxicity assays. CPO was encapsulated in liposomes (prepared from Phospholipon®90H) and niosomes (prepared from Span 60) by ethanol injection method. The cytotoxic effect of liposomal and niosomal CPO was evaluated on KB (oral cancer), PC3 (prostate cancer), Siha (cervical cancer) and Vero (kidney epithelial) cell lines using MTT assay. The IC₅₀ values were compared with free drug CPO and with standard anticancer drug doxorubicin. CPO exhibited cytotoxicity to all the cell lines studied. The niosomal encapsulation of CPO favored its cytotoxicity on the cancer cell lines. Much lower IC₅₀ values were obtained in comparison to the liposomal and free form of CPO. The enhancement in the cytotoxic effect on the non-cancer cell line Vero was not noted. CPO demonstrated marginal difference in the concentration required to produce cytotoxic effect on cancer and normal cell lines. The difference was enhanced by niosomal CPO as much lower concentration was required to produce cytotoxic effect on cancer cells while rendering no effect on normal cells. Enhanced cytotoxicity selectively to cancer cells in the present study demonstrates the pharmacological significance of niosomal drug delivery system of CPO.

Keywords: liposome, niosome, anticancer, ciclopirox olamine, cytotoxicity

Introduction

Ciclopirox olamine (CPO), a broad spectrum antifungal, is a hydroxypyridone derivative that has mechanism of action different from other marketed antifungal agents such as the azoles and the allylamines [1]. Ciclopirox does not affect sterol biosynthesis but its antifungal action involves chelation of polyvalent cations (such as Fe³⁺) with inhibition of metal-dependent enzymes like cytochromes responsible for the degradation of toxic peroxides in the fungal cells [2]. It has a broad spectrum of action against dermatophytes, yeasts, filamentous fungi and bacteria. A remarkable feature of CPO is that no single case of fungal resistance has been reported so far [3]. It was first introduced to the market in April 1975 and is now marketed as 1% cream and lotion in the United States for 15 years and worldwide for 24 years.

Recently CPO has been cited as a drug repurposed for treatment of cancer [4]. Developing a known drug for another clinical purpose is termed repositioning or repurposing. This approach has been very effective as many of the new oncology therapeutics have emerged from traditional or existing medicines that were until now not tested for anticancer efficacy [5]. It has also proved

advantageous to pharma industry as these repurposed drugs have been studied for their pharmacokinetics and safety profiles and often have already been approved by the regulatory agencies [6]. Previous reports investigating the mechanism of antifungal action of CPO too brought into light the indirect role of CPO in treatment of cancer. The anticancer property of CPO has been attributed to activation of hypoxia Inducible Factor pathway [7]. An induction of non-apoptotic programmed cell death characterized by chromatin condensation and DNA damage associated with the appearance of a sub-G0/G1 population and arrest in G2/M cell cycle phases has also been noted [8,3]. Of late, correlation between chelation of iron and cell death in leukemia and myeloma cells was reported by Eberhard et al (2009). They screened several off-patent drugs with previously unrecognized anticancer activity and found that CPO had anticancer activity at concentrations that are pharmacologically achievable. CPO also caused delay in tumor growth in mouse models in leukemia [4].

Sincere efforts towards advancing the cache of anticancer drugs and creating more effective treatments and targeted therapies are being taken by researchers worldwide. Use of vesicular drug delivery systems such as liposome and niosomes provide a key to increasing efficacy of the anticancer drugs [9]. Liposomes are



biodegradable, nontoxic, uni- or multilamellar vesicles, formed from naturally occurring phospholipids which have the ability to entrap and retain a wide range of drugs either in the aqueous or lipid phase [10]. Liposomes of certain sizes, typically less than 400 nm, can rapidly enter tumor sites from the blood, but are kept in the bloodstream by the endothelial wall in healthy tissue vasculature. They are continuing to evolve as tools for the delivery of potentially useful drugs to tumors [11]. Anti-cancer drugs such as doxorubicin (Doxil, Myocet, Caelyx), cytarabine (Depocyt), camptothecin and daunorubicin (Daunoxome) are currently being marketed in liposome delivery systems [12].

Niosomes are non-ionic surfactant vesicles obtained on hydration of synthetic nonionic surfactants, with or without incorporation of cholesterol or other lipids. Similar to liposomes, this vesicular system can be used as a carrier of amphiphilic and lipophilic drugs. Niosomes, being non-ionic, are less toxic and can improve the therapeutic index of drug by restricting its action to target cells. Niosomes containing anti-cancer drugs possess potential to accumulate within tumors in a manner similar to liposomes. Improved tumor targeting was achieved by the delivery of doxorubicin in sorbitan monostearate niosomes, increasing the tumor to heart AUC₀₋₂₄ ratio from 0.27 to 0.36 and a doubling of tumoricidal activity [13, 14]. Formation and pharmacokinetic evaluation of methotrexate niosomes in tumor bearing mice has been reported previously [15]. Sustained and higher plasma levels of doxorubicin were detected using doxorubicin entrapped in polyethylene alkyl ether modified niosomes [16]. A niosomal formulation for brain targeting of doxorubicin has been developed [17]. Lesser toxicity and improved anticancer activity of niosomes of vincristine sulfate has also been reported [18]. A greater cytotoxic effect was observed for 5-FU-loaded PEG-coated bola-niosomes with respect to the drug solution at all the investigated incubation times [19].

Taking into account the enormous prospective of vesicular systems in cancer therapy, CPO, a potential anticancer compound has been formulated in liposome and niosome delivery systems in the present study. The possible benefits that vesicular entrapment can offer to the anticancer potential of CPO was investigated by evaluating their *in vitro* cytotoxicity on human cancer KB, PC3, Siha and non cancer Vero cell lines.

Material and Methods

Preparation of liposomes and niosomes

The blank and drug loaded liposomes as well as niosomes were prepared by ethanol injection method as mentioned elsewhere [20,21]. Liposomes consisted of Phospholipon[®]90H (20 mg), cholesterol (15 mg), CPO (10 mg) and diacetyl phosphate (8 mg). Niosomes were formed from Span 60 (90 mg), cholesterol (50 mg), CPO (10 mg) and diacetyl phosphate (8 mg). The prepared vesicles were centrifuged at 75000 rpm at 4°C in a Himac CS 150G X micro ultracentrifuge to separate free drug from the entrapped one. The separated vesicles were used for the study.

Visualization of vesicle by Transmission Electron Microscopy (TEM)

A drop of liposomal/niosomal dispersion was applied to a carbon film-covered copper grid. Most of the dispersion was blotted from the grid with filter paper to form a thin film specimen. The sample was then examined and photographed with a Zeiss EM 109 transmission electron microscope at an accelerating voltage of 80 kV.

Vesicle size measurement

The mean particle size of the liposomal/niosomal dispersion was determined by laser diffraction technique using Malvern 2000SM (Malvern, UK). Analysis was carried out at 30±2°C temperature keeping angle of detection 90°. The mean vesicle size was expressed in terms of d (0.9) nm.

Determination of entrapment efficiency

The untrapped CPO was separated from the niosomes/liposomes by minicolumn centrifugation. The dispersion (0.2 ml) was introduced into a Sephadex G-50 column and centrifuged at 2000 rpm for 2 min at 4°C. The free drug remained bound to the column while vesicles were eluted out of the column. The vesicles were disrupted with absolute ethanol to release the entrapped drug. This solution was suitably diluted and the concentration determined by measuring the fluorescence intensity. The percent entrapment efficiency was calculated using the following equation.

$$\% \text{entrapment efficiency} = (\text{amount of drug entrapped in the niosomes}) / (\text{total amount of the drug present}) \times 100$$

Zeta potential measurement

The zeta potential of the liposomes/niosomes was measured with the laser Doppler electrophoretic mobility measurements using Zetasizer 300 HSA (Malvern Instruments Ltd., UK) at a temperature of 25°C.

In vitro cytotoxicity assessment

Cell lines

Cell lines KB (carcinoma of nasopharynx), PC-3 (prostate cancer), Siha (squamous cell carcinoma; cervix), and Vero (African green monkey kidney epithelial cell line) were obtained from National Animal Cell Repository at National Center for Cell Science, Pune. Vero cells were used as representative of normal cells in this study [22, 23]. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (Invitrogen), 2mM L-glutamine, 50 IU/ml penicillin, 50 µg/ml streptomycin and maintained at 37°C in humidified 5% CO₂ atmosphere. For



cytotoxicity evaluation, cells were trypsinized and the cell suspension containing 1×10^4 cells were seeded into each well of 96 well microtitration plates. The plates were incubated at 5% CO₂ at 37°C and 80% RH for 24 hrs to allow the adherence of cells prior to administration of various drug samples for testing.

Drug application: After 24 hrs. of incubation, cells were treated with 0.06 μ M - 29.26 μ M concentrations of pure drug CPO, CPO loaded liposomes and CPO loaded niosomes in multiple wells of microtest plates. To evaluate possible effect of blank liposomes or blank niosomes on cell viability, cells were also treated with similar concentrations of blank liposomes or blank niosomes. The standard anticancer drug doxorubicin hydrochloride (VHB Medisciences Ltd. India) was inoculated as positive control at varying concentrations between 0.13 μ M – 17.24 μ M. Equal amount of plain PBS was added to wells which served as control. After addition of all test samples, plates were incubated in 5% humidified CO₂ atmosphere for next 48 hrs. The cytotoxic effect was analyzed using MTT assay.

MTT assay: 10 μ l of 5 μ g/ml MTT was added to all the wells of the test plates and plates were incubated in dark for 6 – 8 hrs. About 100 μ l of DMSO and 25 μ l of glycine buffer were then added to dissolve the formazan crystals resulting from the reduction of the tetrazolium salt by the metabolically active cells. The absorbance was measured at 540 nm using a micro-plate reader (BIO-RAD, Model 680). Since the absorbance directly correlated with the number of viable cells, cell survival was measured as absorbance (OD) of the mean of the replicate wells compared to that of control. IC₅₀ values, defined as the concentration of the drug that killed 50% of cells in comparison with the untreated cultures, were estimated by plotting OD readings versus the drug concentrations. Assays were repeated three times to confirm the results.

Statistical analysis

Regression analysis was used for determining IC₅₀ values. The software, Graphpad Instat version, was used to determine the significance of difference in cytotoxicity between cancer and normal cells through paired t-test. P-values less than 0.05 were considered to be consistent with statistical significance.

Results and discussion

Our previous work established the potential of liposomes and niosomes for dermal delivery of CPO in fungal skin infections [20, 21]. In the wake of CPO being investigated as a possible anticancer agent, the present study evaluates and compares the *in-vitro* cytotoxicity of its liposomal and niosomal forms with the free drug CPO and the anticancer drug doxorubicin. CPO loaded

liposome constructs were obtained by ethanol injection method. They possessed a mean vesicle size of 196 ± 1.73 nm, an entrapment efficiency of 44.89 ± 3.2 % and a zeta potential of -56.2 ± 1.4 mV. Niosomes loaded with CPO prepared using the same method displayed a mean vesicle size of 200.66 ± 6.1 nm, an entrapment efficiency of 67.89 ± 3.0 % and a zeta potential of -24.9 ± 0.8 mV. Both, liposomes and niosomes appeared spherical when visualized under a transmission electron microscope.

The cytotoxic effect of the pure drug CPO and its liposomal and niosomal forms were tested on three human cancer KB, PC3 and Siha cell lines and a non cancer Vero cell line. Blank liposomes and blank niosomes (not containing CPO) at vesicle concentrations same as the drug loaded ones were tested to rule out the effect of liposomal and niosomal composition. The blank liposomes and niosomes did not show any evidence of cytotoxicity on the cell lines chosen at the concentrations tested (Data not shown).

The effect of three test drugs on KB cell line is presented in Fig. 1. The figure shows the graph of optical density at 540 nm obtained after MTT assay of cells treated with 0.06 μ M – 29.26 μ M concentrations of the three test drugs. The absorbance in MTT assay is a measure of mitochondrial activity of viable cells obtained by the reduction of the tetrazolium salt, MTT (3-(4,5 – dimethylthiazol -2 -yl)-2,5-diphenyl tetrazolium bromide) to blue colored water insoluble product formazan. The absorbance thus, not only reflects the viability of cells but also the subtle metabolic perturbations if induced by the drug. All three test drugs demonstrated cytotoxicity to KB cell line. A dose dependent decline in their absorbance was noted. The IC₅₀ values of pure drug, its liposomal and niosomal formulation on KB cell line were 18.9, 15.9 and 5.21 μ M respectively. Although reduction in IC₅₀ values were noted in both liposomal and niosomal CPO formulation, the niosomal CPO demonstrated cytotoxic effect at significantly lower concentration (P < 0.01). Moreover, the residual fraction of cells usually noted even at higher concentration of most of the anticancer drugs was minimum in niosomal CPO treated cells.

Figures 2 and 3 shows the effect of three test drugs on PC3 and Siha cell lines. The IC₅₀ values of pure CPO, its liposomal and niosomal formulations were 14.09, 14.8 and 5.99 μ M on PC3 cell line and 13.7, 10.10 and 4.39 μ M on Siha cell line respectively. The results revealed the cytotoxic effect of pure CPO on all the three cancer cell lines studied at pharmacologically significant concentration. It supports the previous findings of Eberhard et. al.(2009) [4,7]. Of the three cancer cell lines tested, the Siha cell line appeared more sensitive to pure CPO as relatively lower concentration of the drug (IC₅₀: 13.7 μ M) produced toxicity to cells. The liposomal encapsulation provided modest benefit to the



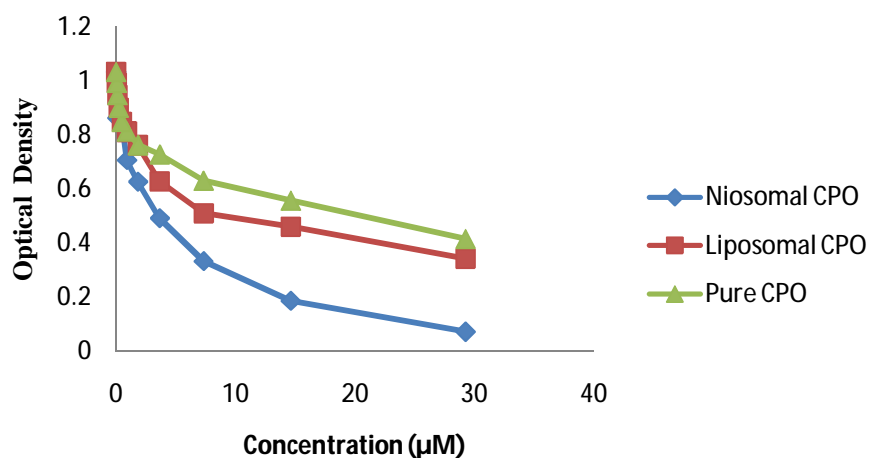


Figure 1: Effect of pure CPO, its liposomal and niosomal formulations on KB cell line using MTT assay, Effect of varying concentrations (0.06 µM – 29.26 µM) of the pure, liposomal and niosomal CPO on KB cell line eliciting IC₅₀ values of 18.9 µM, 15.9 µM and 5.21 µM respectively

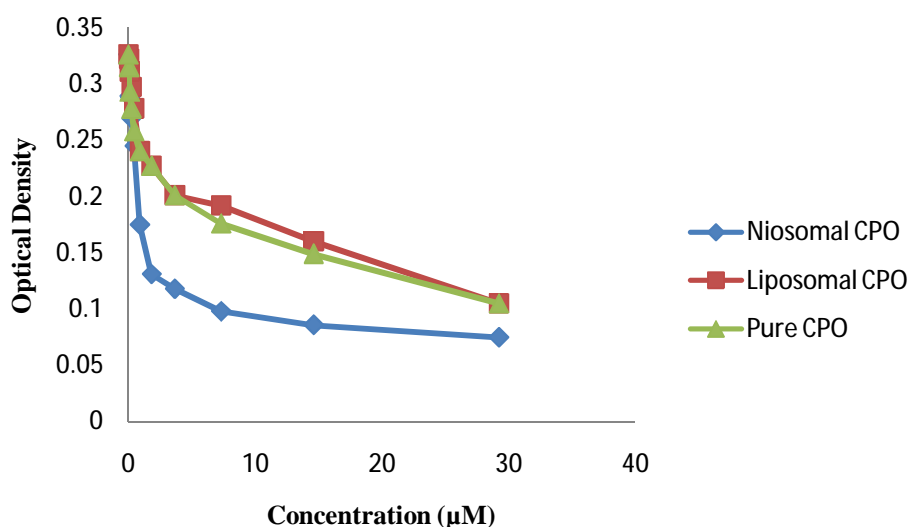


Figure 2: Effect of pure CPO, its liposomal and niosomal formulations on PC3 cell line using MTT assay, Effect of varying concentrations of the pure, liposomal and niosomal CPO on PC3 cell line showing IC₅₀ values 14.09 µM, 14.8 µM and 5.99 µM respectively



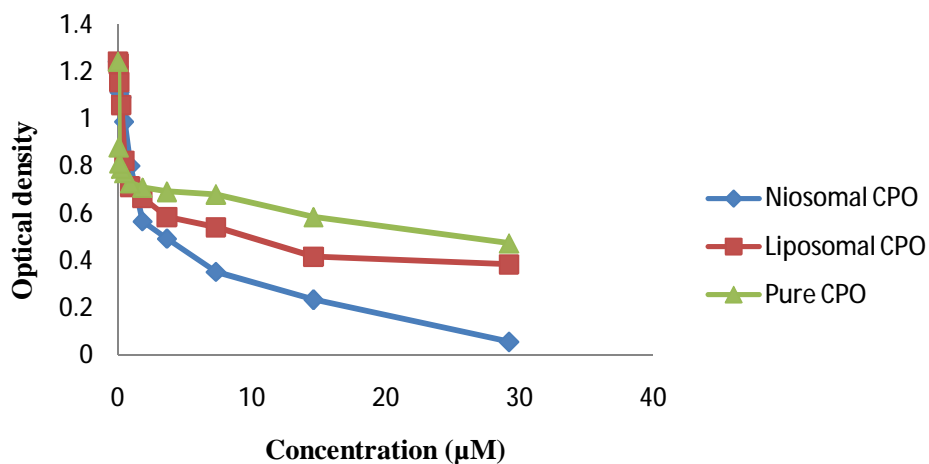


Figure 3: Effect of pure CPO, its liposomal and niosomal formulations on Siha cell line using MTT assay, Effect of varying concentrations of the pure, liposomal and niosomal CPO on Siha cell line showing IC₅₀ values 13.7 µM, 10.1 µM and 4.39 µM respectively

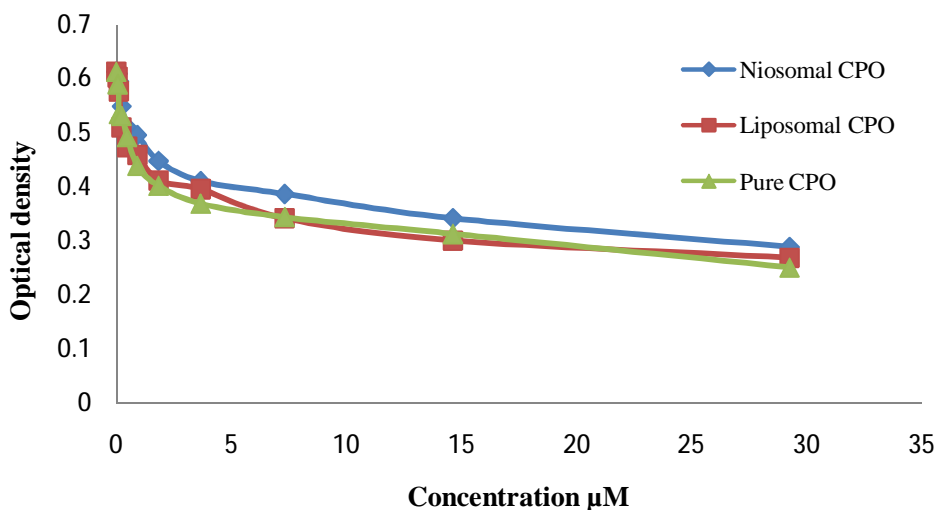


Figure 4: Effect of pure CPO, its liposomal and niosomal formulations on Vero cell line using MTT assay, Effect of varying concentrations of the pure, liposomal and niosomal CPO on Vero cell line showing IC₅₀ values 17.77 µM, 18.86 µM and 22.55 µM respectively



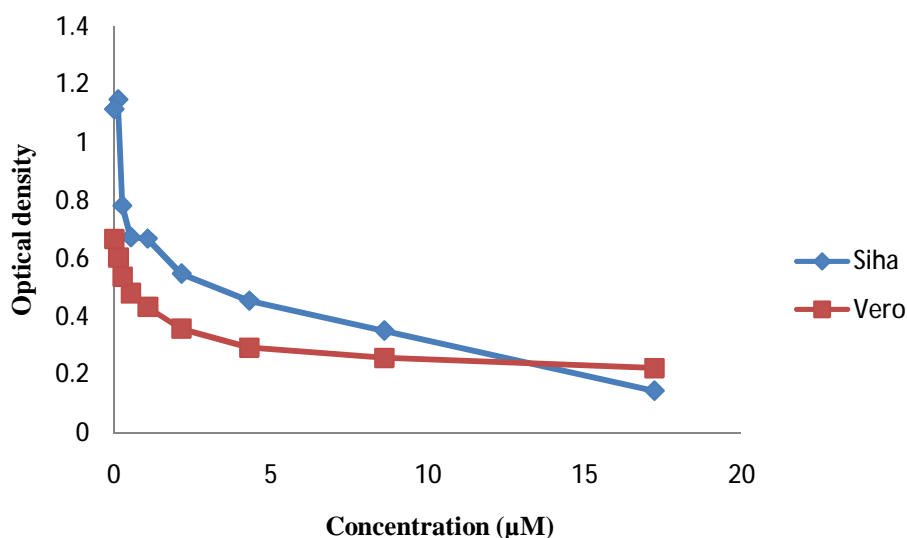


Figure 5: Effect of doxorubicin on Siha and Vero cell lines using MTT assay, Effect of different concentrations (0.13 μM – 17.24 μM) of doxorubicin on Siha and Vero cells lines. The IC_{50} values were 3.89 μM and 6.7 μM respectively for 2 cell lines.

cytotoxic effect of CPO on all the cancer cell lines tested in the present study. Investigations involving niosomal CPO revealed significant influence of encapsulation on the cytotoxic effect of CPO on all three cancer cell lines. The IC_{50} values 5.21, 5.99 and 4.39 μM on KB, PC3 and Siha cell lines respectively demonstrated significant reduction as compared to pure drug CPO ($P < 0.01$). Similar enhancement in cytotoxic effect of niosomal CPO was however not observed on the normal Vero cell line (Fig.4). The IC_{50} values 17.77, 18.86 and 21.55 μM observed for pure CPO, its liposomal and niosomal forms respectively on Vero cell line appeared more or less same. The results state that the drug CPO although shows pharmacologically significant cytotoxic activity on cancer cell lines, at slightly elevated concentration it also displays toxicity to normal cell line. However, when encapsulated in niosomal delivery system, much lower concentration is required to produce cytotoxic effect on cancer cells while rendering no effect on normal cells. Similar effect of niosomal drug delivery has also been demonstrated for 5-fluorouracil and vincristine sulfate [24,18]. The enhanced cytotoxic effect of niosomal CPO selectively to cancer cell lines probably could be co related to the differences in charge dependent uptake of particles by the interacting cells [25, 26]. Particle size and surface charges are known to affect the efficiency of cellular uptake of vesicles by influencing their interaction with cells [27]. Although of the same size, the niosomal CPO possessed less negative zeta potential (-24.9 ± 0.8 mV) in comparison to liposomal CPO (-56.2 ± 1.4 mV). This difference in surface charge may have been responsible for the enhanced interaction of the niosomes with cancer cells.

The anticancer drug Doxorubicin hydrochloride was used as positive control in this study. The cytotoxic effect of Siha and Vero

cells after treatment with this drug is shown in Fig 5. The IC_{50} values 3.9 μM and 6.7 μM on Siha and Vero cell lines respectively were found to be lower than even the niosomal formulation of CPO, but displayed only marginal difference in the toxicity to cancer and normal cells ($P = 0.72$). The drug CPO entrapped in niosomal delivery system attained IC_{50} value closer to doxorubicin and displayed much better ability to distinguish between cancer and normal cells in the present study. CPO entrapped in niosomal delivery system thus merits further investigations to explore the possibility of developing improved anticancer drug.

Conclusion

The possible benefits of liposomal and niosomal encapsulation of CPO, a drug possessing anticancer potential was evaluated in the present study. The CPO loaded liposomes and niosomes expressed significant differences in their cytotoxic behavior towards the cancer and normal cell lines tested. Niosomal CPO in comparison to liposomal and pure CPO demonstrated cytotoxic effect at significantly lower concentration selectively on cancer cells. The niosomal delivery system thus improved the anticancer potential of CPO.

Author's contribution

KS and AP conceived the study and were involved in the formulation development. AM intended cell based study, performed data analysis. SA carried out cytotoxicity testing. KS and AM were involved in drafting of manuscript.

Conflict of Interest

There is no conflict of interest

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