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Formulation and evaluation of nanosized hippadine-loaded niosome: Extraction and isolation, physicochemical properties, and in vitro cytotoxicity against human ovarian and skin cancer cell lines

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ABSTRACT

Chemical compounds extracted from plants have been used in the development of many medicinal products. Hippadine is a compound extracted from *Crinum jagus*, a plant that has been widely used traditionally for the treatment of ailments such as asthma, malaria, depression, convulsions, and cancer. However, the therapeutic applications of hippadine is limited due to its poor solubility and bioavailability. In the context of drug delivery, nanotechnology has been employed widely to improve the bioavailability of poorly soluble compounds through the encapsulation and the delivery of these compounds to their target sites. This study aims to evaluate the cytotoxic activities on two human cancer cell lines of hippadine through its encapsulation into niosome nanoparticles which are a bilayer vesicle widely employed for drug delivery. Firstly, hippadine was extracted and isolated from *C. jagus* bulbs and encapsulated into niosome nanoparticles composed of span 60 (SP60) and cholesterol, prepared by microfluidic mixing. The prepared nanoparticles had spherical morphology, and were small, uniform, and monodisperse with an average particles size <150 nm when loaded or empty. The encapsulation efficiency was 36 ± 1%. Hippadine loaded niosomes showed superior cytotoxic effects over free hippadine as indicated by the significant ($p < 0.05$) decrease in the IC_{50} values when tested in vitro on human ovarian carcinoma (A2780) and human skin cancer cells (A375). This is the first study that reports the use of SP60 niosomes for successful encapsulation and delivery of hippadine into cancer cells.

1. Introduction

Plants and their extracts have been widely used in the preparation of various therapeutic products or as guide for drug discovery in the pharmaceutical industry [1]. Compounds originally derived from plant sources such as alkaloids, glycosides, flavonoids and terpenoids have been shown to exert therapeutic effects and are the corner stone in the development of several therapeutics such as anti-cancer and antibacterial agents. For example, Taxol® is a trade name of an approved product for the treatment of ovarian, breast, and lung cancer. The active ingredient in Taxol® is paclitaxel, a compound which was obtained from the bark of *Taxus brevifolia* tree [2,3].*Crinum jagus* (J. Thomps). Dandy is widely distributed in Africa, America, Australia and Southern Asia. Plants in this family are wild, bulbous, with beautiful flowers, hence their use as ornamental plants [4]. *C. jagus* bulbs are used in traditional medicine for the treatment of respiratory diseases, malaria, snake bite, infections, fever, wounds, epilepsy, anxiety, infantile convulsions, depression, psychoses, pain and inflammation, memory loss associated with aging, open sores, and as an antihelmintic [5]. *C. jagus* is a rich source of alkaloids and flavonoids which are mainly stored in the bulbs. Several alkaloids have been isolated from this plant including berberine, evodiamine, matrine, and tetrandrine [6]. The high distribution of

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alkaloids is said to be responsible for the broad spectrum of pharmacological activities associated with the plant. Pharmacological studies on compounds, fractions and extracts of the bulb have shown activities to treat cancer, bacteria, neurodegenerative disorders, viruses, malaria, oxidative stress, wound healing, inflammation, and snake venom [7–12]. Hippadine has also been extracted from the bulbs, it has poor water solubility, with limited clinical applications and thus has not been evaluated therapeutically. It has been reported that certain alkaloids such as hippadine and tazettine have a potential anticancer activity against several types of cancer such as solid tumours with malignant prognosis [13]. Hippadine appears to be functioning at the genetic level due to the lack of anti-mitotic activity [14]. For example, in the work of Ka et al., hippadine was shown to have inhibitory potential and cytotoxic activity on the breast cancer cell line MCF-7 at concentrations around 100 μM [15].

Nanotechnology is one approach currently employed for the improvement of physicochemical and pharmacokinetic properties of therapeutic agents that have limitations in terms of toxicity, solubility, absorption, or bioavailability [16]; [17] [18]. Nanoparticles can be used as drug delivery systems to encapsulate compounds for protection, reduction in toxicity, enhancing their stability and accumulation at specific targets in the body. Various types of nanoparticles are used for this purpose such as liposomes, niosomes, dendrimers, polymeric nanoparticles, and gold nanoparticles [19]. Niosomes are lipid nanoparticles developed for the cosmetic industry and have been investigated for their application as drug delivery systems. These spherical noisome nanoparticles are composed of non-ionic surfactants, cholesterol and lipid mixtures that are assembled into a bilayer structure encapsulating an aqueous compartment [20]. In these vesicles, water insoluble molecules (like hippadine) can be loaded onto the bilayer structure while hydrophilic molecules can be encapsulated into the aqueous compartment. Niosomes are prepared based on hydration of the surfactants and other components to enhance the self-assembly of these components into a bilayer structure. This includes the hydration of a thin film of lipids, the ether injection method, and microfluidic mixing techniques [21] [22].

This study aims to demonstrate that niosomes can be used to increase the therapeutic potential of hippadine against cancer cells through its encapsulation into the bilayer structure of niosomes. The niosomes used in this study were composed of span 60 (SP60) as the non-ionic surfactant and cholesterol at a molar ratio 50:50 and prepared by microfluidic mixing. The niosomes were evaluated for their ability to encapsulate and deliver hippadine to cancer cell lines to induce cytotoxic effects. The prepared nanoparticle formulations were characterised in terms of their physicochemical properties, encapsulation efficiency and improved cytotoxicity effects over the free drug.

2. Materials and methods

2.1. Materials

Silica gel 0.063–0.200 nm mesh size, Thin Layer Chromatography (TLC) plates, sorbitan monostearate (Span 60), cholesterol, resazurin (AlamarBlue®), phosphate-buffered saline tablets (PBS, pH 7.4), penicillin–streptomycin, ilica gel (mesh size 0.063–0.200 nm), cellulose dialysis membrane avg. flat width 10 mm, molecular weight cut-off 14000 Da, were purchased from Sigma-Aldrich (UK). Ethyl acetate, n-hexane, methanol, Roswell Park Memorial Institute (RPMI) 1640 medium and foetal bovine serum (FBS) were purchased from Fischer (UK). Sephadex LH20 was obtained from GE Healthcare (UK). Human ovarian carcinoma (A2780) and human melanoma cells (A375) were purchased from the European Collection of Authenticated Cell Cultures (ECACC, UK).

2.2. Extraction and isolation

C. jagus bulbs were collected from the botanical garden of the Forestry Research Institute of Nigeria, Ibadan, Nigeria. A voucher specimen was submitted to the Institutes' herbarium. The bulbs were air-dried and powdered (800 g). This was extracted using methanol in a Sohxlet apparatus and the solvent was removed to give 40 g of dried extract, which was fractionated by solid-liquid extraction method (trituration) using n-hexane, ethyl acetate and methanol successively. The ethyl acetate fraction (5 g) was further fractionated using column chromatography on silica gel and eluted with n-hexane: ethyl acetate (9.5: 0.5). Fraction 1 was subjected to column chromatography using silica gel and also eluted with gradient mixtures of n-hexane: ethyl acetate (8: 2) as the mobile phase. TLC enabled similar fractions to be combined and purified using gel permeation chromatography with Sephadex LH20 and eluted with methanol. The purified compound was analyzed for its molecular mass on a Thermoscientific®) Ultra High Performance Liquid Chromatography (UHPLC) composed of an Accela system coupled to an Extractive-orbitrap high resolution mass spectrometer Thermoscientific®. It was further subjected to Nuclear Magnetic Resonance on a Bruker Avance III 400 MHz spectrophotometer at the Department of Pure and Applied Chemistry, University of Strathclyde, Glasgow

2.3. Niosome preparation

Niosomes, composed of SP60: cholesterol at a 50:50 M ratio, were prepared by microfluidic mixing using a Neo-nano microfluidic platform (Neofluidics, USA) equipped with ISMATEC premp control software. To prepare the lipid phase of the niosomes, 20 mg/ml stock solutions of SP60 and cholesterol were prepared in ethanol and the required volumes from each stock solution were mixed together to prepare the lipid phase at a 50:50 M ratio. In formulations containing hippadine, the organic phase was prepared with the addition of hippadine at 1.8% wt/wt concentration compared to the total lipid weight. The final hippadine concentration in the lipid phase before mixing with the aqueous phase was 176 μg/ml. The lipid phase with and without hippadine was then mixed with PBS (pH 7.4) as an aqueous phase through the microfluidic chip. The mixing parameters were adjusted to a 3:1 ratio between the aqueous and the lipid phase and the two phases were mixed at a total flow rate of 12 ml/min. Both the aqueous and the lipid phases were heated to 50 °C using a water bath before mixing.

2.3.1. Removal of un-encapsulated hippadine

The prepared niosomes encapsulating hippadine were transferred to a dialysis tubing with a molecular weight cut-off of 14 KDa and the formulation was dialysed against a 10x volume of PBS overnight at room temperature with continuous stirring to remove un-encapsulated drug.

2.3.2. Determination of hippadine encapsulation efficiency

Following dialysis, 100 μl of the hippadine-loaded niosomes were mixed with 100% methanol to lyse the niosomes and release entrapped drug. The UV absorbance of the sample was measured at 298 nm using a HELIOS ALPHA ThermoSpectronic spectrophotometer (Thermo Fisher Scientific, UK). The concentration of the released hippadine was determined from a hippadine calibration curve prepared at concentration range from 2.2 to 52.5 μg/ml.

The encapsulation efficiency (EE) of hippadine was determined as a percentage of the initial hippadine concentration (44 μg/ml after mixing with the aqueous phase). The experiments were performed in triplicates and the average ± SD determined.

2.4. Physicochemical characterisation of niosomes

2.4.1. Particle size analysis

The average particle size and size distribution (polydispersity index,

PDI) of the empty and hippadine-loaded niosomes was measured by dynamic light scattering (DLS) using a Zetasizer Nano-ZS (Malvern Instruments Ltd., UK). Samples were diluted (1:10) with PBS before measurement and triplicate readings were taken at 25 °C.

2.4.2. Morphological analysis of niosomes

Morphological examination of the empty and drug-loaded niosomes was carried out using transmission electron microscopy (TEM) using a JEOL JEM-1200EX TEM (JEOL, Tokyo, Japan) operating at an accelerating voltage of 80 kV. For sample preparation, carbon-coated copper grids (400 mesh, agar scientific) were glow discharged in air for 30 s. Three microlitre samples were drop-cast on the grids and then negatively stained using uranyl acetate. Samples were then allowed to dry in a dust-free environment prior to TEM imaging.

2.5. Cell culture

Human ovarian carcinoma (A2780) and melanoma cells (A375) were cultured in sterile T25 flasks containing RPMI supplemented with 10% (v/v) FBS and 1% (v/v) penicillin/streptomycin at 37 °C, 5% CO_2, and 100% humidity. When the cells were approximately 70%–80% confluent, they were trypsinised from the culture flasks and 100 μl of cells were seeded in flat bottomed 96-well plates at concentration of 5×10^4 cells/ml. The plates were incubated at 37 °C, 5% CO_2 and 100% humidity for 24 h before treatment.

2.5.1. Cytotoxicity assay of empty niosomes

Cells incubated in 96 well plates were treated with a range of concentrations of niosomes starting from 1250 μg/ml and serially diluted 1:2. The cells were incubated for 24 h and then 20 μl of sterile 0.1 mg/ml resazurin was added to each well and incubated for a further 24 h. The transformation of resazurin into resorufin by the live cells was then detected by measuring the absorbance at 560–590 nm excitation/emission using a SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, CA, USA). Metabolically active cells following treatment were calculated as a percentage of the untreated control cells and results were expressed as a mean ± SD of triplicate readings.

2.5.2. Cytotoxic effects of hippadine alone and in niosomes

Free hippadine and hippadine-encapsulated niosomes were evaluated for in vitro cytotoxicity using the resazurin based test. After 24 h of incubation, cells were treated with hippadine dissolved in ethanol, and hippadine-loaded niosomes and then the plates were incubated at 37 °C, 5% CO_2 and 100% humidity for 24 h. The negative control consisted of cells treated with 100% ethanol only. Next, cells were treated with 20 μl resazurin (0.1 mg/ml) to each well and incubated for a further 24 h and then the fluorescence was measured as described in section 2.8. Viable cells following treatment with hippadine formulations were calculated as a percentage of the untreated control cells and results were expressed as a mean ± SD of triplicate readings.

2.5.3. IC_{50} determination

Originsoftware version 2022 using the Hill equation was used to calculate the IC_{50} of hippadine either as a free drug or loaded into niosomes using the results obtained from the cytotoxicity assays. Results were expressed as a mean ± SD of triplicate readings.

2.6. Statistical analysis

Statistical analysis was carried out using GraphPad Prism 5.0, via two-way Analysis of Variance (ANOVA). Each sample was repeated in triplicate and the mean ± SD were analyzed. $P < 0.05$ was considered significant.

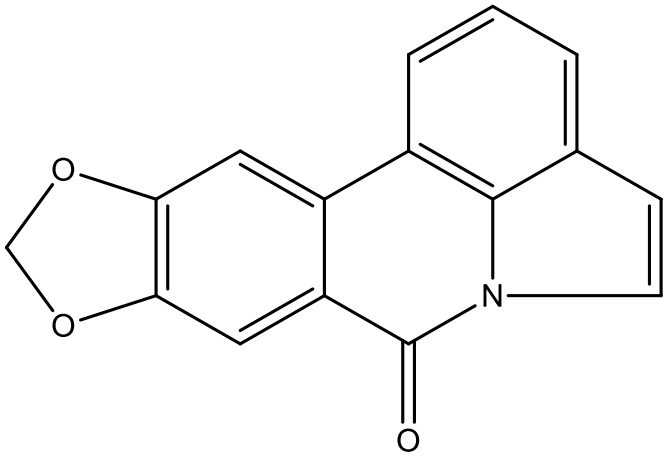

Fig. 1. Hippadine.

3. Results and discussions

3.1. Hippadine extraction and isolation

Following the extraction using methanol in a Sohxlet apparatus, the yield of pure hippadine was 17.9 mg.

3.2. Structural elucidation of hippadine

HRMS revealed the composition of the compound as $C_{16}H_{10}NO_3$ (264.0658 *m/z* $[M+H]^+$, calc. 264.0661). NMR analysis and comparison of its chemical shift data with the literature reports [26], gave the identity of the sample as hippadine which is presented in Fig. 1.

3.3. Hippadine encapsulation efficiency (EE)

The use of microfluidic mixing method for the preparation of hippadine-loaded niosomes resulted in EE% of 35.98 ± 0.99%. Hydrophobic molecules like hippadine will be incorporated into the bilayer membrane of niosomes. This means that the level of encapsulation of these molecules will depend on factors like the number of layers forming the niosomes along with the membrane thickness. A high level of encapsulation of hydrophobic molecules can be achieved when niosomes are prepared with a traditional thin film hydration method which is known to produce multilamellar nanoparticles [23]. Microfluidic mixing produces unilamellar vesicles which means that the level of encapsulation of hydrophobic molecules is reduced. Several factors control the process of microfluidic mixing such as the total flow rates (TFR) the flow rate ratio (FRR), the type of aqueous media, preparation temperature, non-ionic surfactant used and concentration [24]. In this study, these factors were initially evaluated and have been optimised to increase the level of hippadine encapsulation. The microfluidic mixing conditions reported here were the optimised conditions which resulted in the maximum level of hipaddine encapsulation of around 36%. This level of hippadine encapsulation indicates that microfluidic mixing can be used for the preparation of hippadine-loaded niosomes, which can be used for large scale industrial applications. The level of hippadine encapsulation reported here is comparable with the level of hydrophobic molecule encapsulation reported in literature. For example, the hydrophobic compound balanocarpol was encapsulated into niosomes at around 35% [25]. Moreover, the encapsulation of propofol was around 50 mol% when encapsulated into liposomes using the same method of preparation [26].

Although the level of encapsulation reported here was not very high and higher levels of encapsulation could be achieved using the thin film hydration method, still the microfluidic mixing is a reliable and fast

Table 1
Properties of empty and loaded SP60 niosomes.

Formulation	Size (nm)	PDI	ZP (mV)
Empty niosomes	143.13 ± 0.45	0.17 ± 0.02	−31.00 ± 2.79
Niosomes loaded with Hippadine	138.40 ± 1.40	0.15 ± 0.01	−32.80 ± 2.50

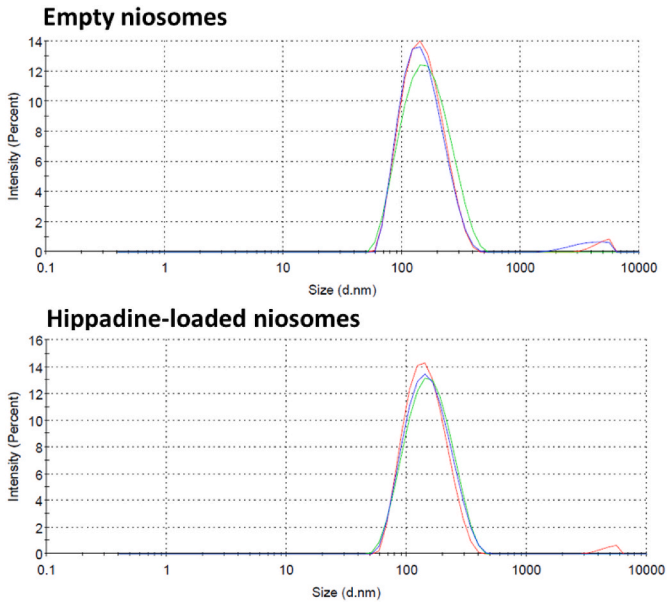

Fig. 2. The size distribution for empty and loaded niosomes as measured by DLS. The results are for three different experiments.

method which can prepare small and uniform nanoparticles with narrow size distribution. This can be useful when considering the preparation of these formulations at larger scale where the translation of this method from small bench scale to larger industrial scale was reported to be feasible [27,28].

3.4. Particle size analysis

The average particle size, PDI, and zeta potential (ZP) for empty and hippadine-loaded niosomes are presented in Table 1 and Fig. 2. Niosomes, either loaded or empty, were small in size (<150 nm) with a monodisperse size distribution as indicated by the PDI values. Hippadine-loaded niosomes were slightly smaller in size than the empty niosomes and this can be explained by the presence of the hydrophobic molecule in the bilayer structure causing the particle layers to be more packed which with slightly smaller size. However, this size difference was not significant ($p > 0.05$) and is similar to other hydrophobic compounds loaded into niosomes such as curcumin and balanocarpol [25,29]. In terms of surface charge, there was no significant difference in the ZP values between the empty and the loaded particles which supports the concept that hippadine is located within the bilayer structure of the niosomes and not decorating the surface. In both formulations, the ZP values were negative and less than −30 mV. This means that the chance for aggregate formation is minimised and they will be stable as a ZP value less than −30 mV will enhance the stability of the nanoparticles [30,31].

3.5. Morphological analysis of niosomes

Fig. 3 shows the morphology of the niosomes as measured by TEM at 200 nm scale. Particles shown are spherical in shape irrespective to whether they are empty or loaded. No differences can be seen between the two formulations in terms of surface properties which indicates that the compound is loaded in the bilayer structure and not on the surface of niosomes. The size of the particles is comparable with the particle size

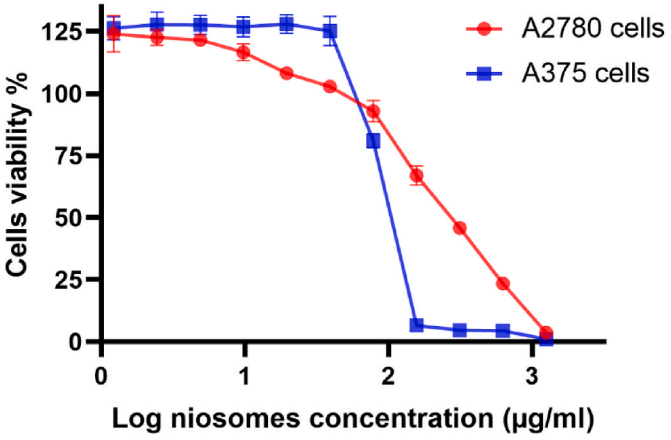

Fig. 4. Cell viability of A2780 and A375 cells treated with different concentrations of empty niosomes, ranging from 0.6 to 1250 µg/ml. Results are expressed as a percentage of the viability of untreated cells and reported as mean ± SD.

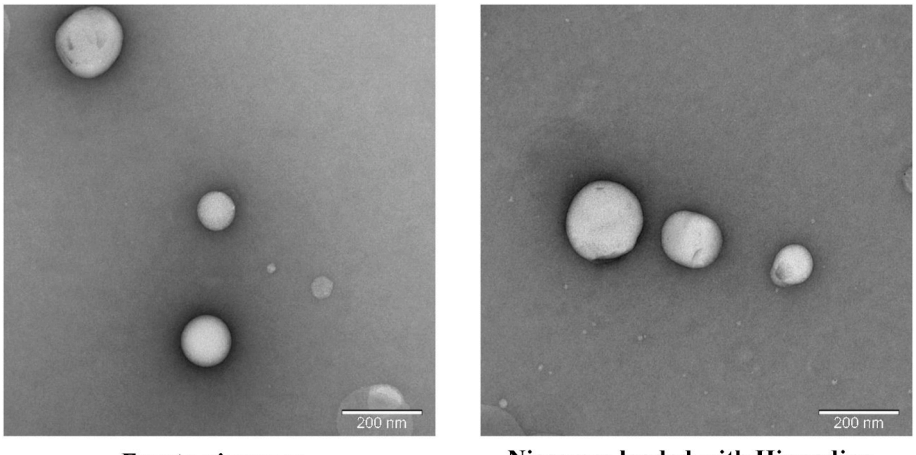

Fig. 3. The TEM images presenting the morphology of empty niosomes compared to hippadine-loaded niosomes.

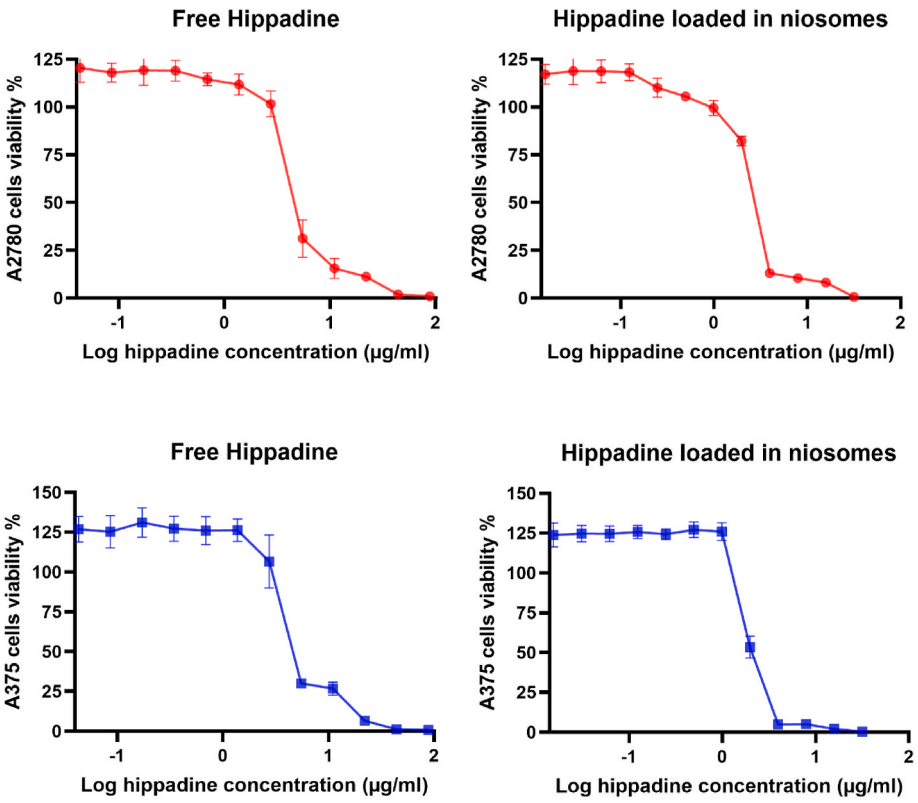

Fig. 5. Percentage cell viability of Hippadine and Hippadine-loaded in niosomes on A2780 and A375 cells. Results are expressed as percent of cell viability of untreated cells. The results represent mean ± SD from three independent experiments.

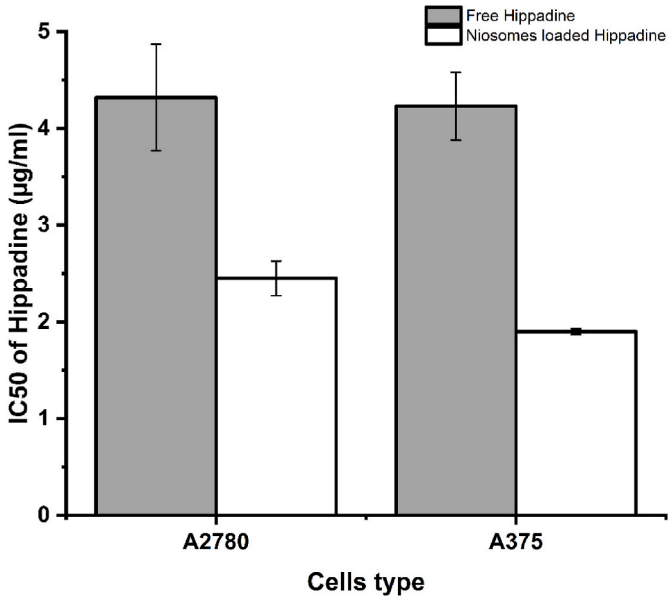

Fig. 6. IC_{50} values of hippadine (free and loaded into niosomes) on A2780 and A375 cell lines.

indicated by DLS.

3.6. Cytotoxicity of empty niosomes

Fig. 4 shows the cytotoxicity results of the empty niosomes on the human ovarian (A2780) and human skin cancer (A375) cell lines. This experiment aims to evaluate the non-toxic concentration of the prepared empty niosomes on these two cell lines. This is important to make sure that any cytotoxic effect of the hippadine-loaded niosomes will be an effect of the hippadine rather than just a toxic effect of the vehicles themselves.

Here, we have found that the IC50 of the empty niosomes was 189 ± 13.2 µg/ml and 87.5 ± 1.49 µg/ml on A2780 and A375 cells, respectively. At concentrations less than 80 µg/ml, niosomes were non-toxic to the A2780 cells and the cells were more than 90% viable. For A375 cells, the viability was more than 90% at niosome concentrations equal to or less than 60 µg/ml. These results were taken into consideration when analysing the cytotoxicity effect of the Hippadine-loaded niosomes in the following experiments.

3.7. Cytotoxic effects of hippadine formulations

The cytotoxicity of hippadine alone in comparison with hippadine-loaded in niosomes against the A2780 and A375 cell lines after 24 h was assessed with resazurin and showed in Fig. 5. The highest concentration of hippadine in the niosome formulation was 31.68 µg/ml, based on the 35.98 ± 0.99% EE of the initial hippadine concentration (88 µg/ml). Niosome-loaded hippadine exhibited significantly ($p < 0.001$) higher cytotoxicity compared to free hippadine for both cell lines (Fig. 5). In A2780 cell, the IC_{50} value was significantly ($p < 0.05$) reduced from 4.32 ± 0.55 µg/ml for the free hippadine to 2.45 ± 0.18 µg/ml for hippadine-loaded into niosomes (Fig. 6). Similarly, for A375 cells, the IC_{50} value was significantly ($p < 0.05$) reduced from 4.23 ± 0.35 µg/ml for the free hippadine to 1.9 ± 0.03 µg/ml for hippadine-loaded into niosomes (Fig. 6). Cytotoxicity of hippadine-loaded niosomes against A375 appeared to be slightly better compared to A2780 as can be seen from the IC_{50} values (Fig. 6). For example, at 3.96 µg/ml hippadine loaded in niosomes caused a 95% decrease in A375 cells viability, compared to 87% decrease in the viability of A2780 at the same concentration.

4. Conclusion

Hippadine is a hydrophobic compound with promising cytotoxic effects on cancer cells. However due to its poor solubility in aqueous solution, low bioavailability and hence therapeutic activity would be expected. This limits the use of this compound in the development of future therapeutics for cancer treatment. The use of niosome nanoparticles has been extensively investigated for the delivery of similar hydrophobic molecules in an attempt to improve the physicochemical properties which are thought to improve the pharmacokinetics properties of such compounds. Here, niosomes were shown to significantly improve the characteristics of hippadine by increasing its cytotoxic properties. Overall, compared to free hippadine, hippadine-loaded niosomes exhibited significantly improved cytotoxicity for both cell lines, which can be attributed to the niosomes resulting in improved molecule solubility and enhanced drug uptake by the cells at a higher rate. This is the first report of the cytotoxic effects of hippadine on an ovarian and skin cancer cell lines.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

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