PREPARATION AND EVALUATION OF ANTICANCER POTENTIAL OF BETULINIC ACID LOADED NANOLIPOSOMES

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Abstract

Betulinic acid (BA), a triterpene in nature shows significant antitumor activity. But, its low solubility in aqueous phase leads to its poor bioavailability. The utilization of liposomal nanoformulation is a promising way to deliver BA with enhanced solubility profile and bioavailability. In present work, BA nanoliposomes were prepared and their physicochemical properties were optimized and characterized. BA nanoliposomes displayed optimum properties, with a particle size of 60-125 nm, zeta potential of -42.5 mV, and percent entrapment efficiency of 65.2% for drug delivery applications. The cytotoxic activity of BA loaded liposomal nanoformulation against A-549, MCF-7 and Hela cell lines confirmed its potential to hinder cancer cell proliferation. Hence, the present research work demonstrated the applicability of BA nanoliposomes as promising nanoformulation for cancer therapy.

Keywords: Nanoliposomes, Betulinic acid, Liposomal Nanoformulation, Cancer therapy.

Introduction

Successful chemotherapy necessitates delivery of the anticancer drugs to their molecular sites of action. Random as well as non-targeted circulation of the anticancer drug inside the living system diminishes the therapeutic efficacy of the drug. At the same time, it augments the danger of undesirable adverse effects as well as toxicity. A major approach for site specific drug administration and to reduce its side effects is achieved by entrapment of anticancer drug in a specific carrier. But, carrier-mediated drug delivery to cancer site entails positioning a suitable agent on the particle's surface and ensuring its biocompatibility [1].

Nanotechnology has prospective to alter the cancer diagnosis as well as therapy [2]. The inherent limitations of the conventional chemotherapy resulted in the formulation and application of numerous nanotechnologies for more valuable and secure cancer treatment. A large number of nanotherapeutics (>40) have already reached the patients that include both chemotherapeutic and imaging agents. Nanomaterials have the ability to unite multiple therapeutic functions into a single platform [3]. It is for this reason they could be targeted to specific tissues. Effective systemic delivery of nanotherapeutics to solid tumors requires a deeper understanding of the biological factors and physicochemical properties of nanotherapeutics as well [4].

Nanoliposomes are well-established drug delivery systems that are biocompatible, biodegradable as well as non-immunogenic drug carriers [5]. Another favorable advantage of liposomal bioactive delivery is that these minuscule vesicle pockets transfer bioactive compound through digestive tract into the bloodstream with enhanced bioavailability. During the formulation of nanoliposomes hydrophilic material can be disintegrated into the watery center and hydrophobic material can be connected to the bilayer. Subsequently, nanoliposomes could be utilized for both hydrophilic and hydrophobic bioactive compounds. The development and improvement of phytonanoformulations have opened novel research endeavor to develop nanoformulation based advanced nano-herbal products [6]. Various herbal medications are used for the treatment of CVS disorders, respiratory diseases, diabetes, cancer etc.

Nanoformulations have enabled advancement in cancer treatment thus bringing about a reduction in cancer severity as well as death rates. Betulinic acid (BA), a triterpenoid compound has been accounted to possess anti-inflammatory [7] and

hepatoprotective [8] properties. In addition to this, BA exhibits anticancer activity towards a range of cancer cell lines with low toxicity [9-10]. But, BA has poor water solubility which limits its pharmacological potential. BA loaded novel liposomal nanoformulation could overcome such limitations and offers a promising strategy to deliver BA effectively.

Nanoliposomes of Betulinic acid might provide a potential formulation with enhanced solubility as well as bioavailability and therapeutic potential. In the present study, BA nanoliposomes were developed, characterized and were evaluated for anti-cancer activity against cancer cell lines.

Materials and methods

Drug and chemicals

Betulinic acid was procured from Sigma Aldrich, India. Cholesterol was obtained from Molychem, Mumbai. Cell lines A-549 (Human lung adenocarcinoma epithelial cells), MCF-7 (Human breast adenocarcinoma cells) and Hela (Human cervical carcinoma cell line) were procured from National Center for Cell Science (NCCS), Pune and Lecithin, Minimum Essential Eagle Medium, Foetal bovine serum (FBS), penicillin & streptomycin were purchased from Himedia, Mumbai. All chemicals utilized for research work were of analytical reagent grade.

Preparation of BA nanoliposomes

Betulinic acid nanoliposomes (BNLs) were formulated using thin film hydration technique. Lecithin and Cholesterol were used as key excipients. Lecithin (840 mg), Cholesterol (110 mg), Cetyl trimethyl ammonium bromide (10 mg) and 250 mg of BA were dissolved in mixture of 40 ml ethanol and 60 ml chloroform. Using rotary evaporator, the mixture was vanished (45˚ C for 4.5 h) for development of thin lipid film. The film was then hydrated with 50 ml deionized water containing 1ml 0.05% Tween-20 solution under constant stirring at 60˚ C for 1 h. The resultant large but stable & hydrated multilamellar vesicle suspension obtained was further processed under sonication followed by extrusion.

Characterization of BA nanoliposomes

Dynamic light scattering was used to quantify the average particle size of the nanoliposomes and its degree of heterogeneity (polydispersity index) of size-optimized nanoliposomes. Zetasizer Nano ZS-90 (Malvern Instruments, Malvern, UK) was employed to compute the electrokinetic potential in a colloidal dispersion of vesicles that signifies the stability of the liposomal nanoformulation at 25˚C. The unbound drug concentration in the supernatant was determined after centrifugation at 10,000 rpm, 4˚C for 30 min and analyzed by HPLC and percent encapsulation efficiency was computed. The morphological assessment of the optimized batch was done by transmission electron microscope (TEM-Hitachi-H-7501SSP/N-817-0520, Japan). One drop of optimized BNLs was initially loaded upon a copper grid, air-dried and scanned at 60,000 magnification factor and 80,000 V accelerating voltage to capture TEM micrograph. The morphology of BNLs was determined with fluorescent photomicroscope at 100X magnification before extrusion (LEICA DM 2500 M). The KBr pellet of powdered samples of BA, lecithin, cholesterol, and BNLs were analyzed in range of 4500–500 cm^{-1} using FTIR spectrophotometer Affinity-1 (Shimadzu, Japan). Differential scanning calorimetry-thermogravimetric analysis (DSC-TGA) analysis of BNLs and dummy nanoparticles was performed in order to determine the physical nature of drug and liposomes using TGA/DSC 3^{+} $Star^{e}$ System, Mettler Toledo AG, Analytical, Switzerland. Samples (5 mg) were taken in alumina pan and scanned in the range of 30 ˚C − 500 ˚C with a heat flow rate of 15˚C min^{-1}.

In vitro release profile of BA nanoliposomes

To study the release profile, the dialysis sac method was utilized. BNLs (10 mg) were kept in dialysis sac and positioned in water (10ml) and immersed in ethanol (25%) - phosphate buffer (0.1 M) saline having pH 7.4 and constantly stirred at 90 rpm at a steady temperature of 37 °C. One ml sample was withdrawn and collected at regular intervals of 1, 2, 3, 6, 12, and 24 h and examined by HPLC (Agilent 1200 Infinity Series) using ZORBAX SB C-18 column (5 µm, 150 x 4.6 mm) and mobile phase containing Acetonitrile : Water (80:20 v/v), 210 nm, **7**.07 min.

Antioxidant activity

The antioxidant activity was determined as a measure of the scavenging capacity of antioxidants towards DPPH. 1, 1-diphenyl-2-picrylhydrazyl (DPPH), a free radical was dissolved in methanol (3.9 mg/100 ml) and incubated with pure BA and BNLs for 30 min in dark. The absorbance was recorded using UV spectrophotometer at 517 nm. In order to determine the inhibition of DPPH, Blank nanoliposomes (Negative control), Betulinic acid (Positive control) and BNLs were used. The equation given under was utilized to ascertain the percentage inhibition of DPPH by pure BA and BA loaded nanoliposomes.

$$\text{Percent antioxidant activity} = \frac{(\text{Control Abs.}) - (\text{Sample Abs.})}{(\text{Control Abs.})} \times 100$$

***In-vitro* cytotoxic assay**

The *in vitro* cytotoxicity activity of BNLs solutions was determined by MTT assay against three cell lines A-549 (Human lung adenocarcinoma epithelial cells), MCF-7 (human breast adenocarcinoma cells) and Hela (Human cervical carcinoma cell line). Tetrazolium dye-based assays are employed to quantify the cytotoxic or cytostatic effect of a therapeutic bioactive agent or toxic chemicals. Generally, the MTT reagent is photosensitive so assays are usually performed under dark condition [11]. Cell lines (both normal and cancer) were cultured in media supplemented with inactivated FBS (10%), 100µl/ml streptomycin & 100µl/ml penicillin and incubated at 37^{0}C and 5% of CO_2 in humidifier incubator. After attaining 70% confluence, the cells

were subcultured in 0.25% trypsin solution under hygienic conditions. The preparation of stock solutions of compounds and standard was done in DMSO (µM/ml) and further dilutions (1 µM, 10 µM, 20 µM, 50 µm and 100 µm per ml) were prepared in the medium. Seeding of cells was made in 96-well plates (5×10^3 per 100 µl per well). The density of every cell line was determined on basis of growth characteristics. Triplicate wells were treated after 8 h incubation with different concentration of BNLs (0.1-1000 µg/ml) and Betulinic acid for three days. After three days, the medium was replaced by 3 µl of MTT solution (5mg/ml) and incubated for 180 min and on the basis of the mitochondrial conversion of 3-(4, 5-dimethylthiazol-2-yl) 2,5 diphenyltetrazolium bromide (MTT) to Formazan crystals, the overall relative percent of metabolic active cells was compared with untreated controls,. Formazan crystals were dissolved in DMSO and its absorbance was calculated using microplate reader (BIORAD) at 570 nm. The anticancer activity of synthesized compounds was assessed using BA as a standard drug.

Results and Discussion

Synthesis of BA Nanoliposomes (BNLs)

Thin film hydration technique was employed for the preparation of BA nanoliposomes. Nanoliposomes have both lipid and aqueous phases and thus could be employed for the entrapment, delivery & release of lipid as well as water-soluble materials. In the present study, we observed the overall improvement in the pharmacological profile of hydrophobic drug Betulinic acid after its incorporation in the liposomes.

Characterization of BA Nanoliposomes

Particle size analysis and Zeta potential

Particle size is a significant characteristic of liposomal nanocarriers. It affects the stability, drug release profile, encapsulation efficiency, bio-distribution, and cellular uptake [12,13]. Nanoliposomes of a particular size (≤150 nm) are able to exit or enter the microenvironment of the cancer cells. The cancerous cells are bigger in size and are highly vascular [14]. As a result, vascular mediators will accumulate at the tumor sites. But, due to the leaky vasculature of cancer cells they permit the buildup of high molecular weight therapeutics which is called enhanced permeability & retention (EPR) effect. Drugs encapsulated with liposome up to the size of 400 nm can enter cancer cell sites by passively targeting them, but are restricted from the healthy tissues by the endothelial wall [15-20]. In the present work, the particle size of BNLs was found to be 211 nm suggesting their high vasculature and accumulation. Zeta potential estimations was done to assess the relationship between surface charge and stability [21-22]. The zeta potential of nanoliposomal formulations can help to control their aggregation, fusion & precipitation. More negative ζ value indicates higher stability of the preparation and higher cellular uptake. The zeta potential of BNLs was found to be -42.5 mV indicating their higher stability.

Percent encapsulation efficiency (% EE)

The encapsulation efficiency of the BNLs formulation was determined by estimating free drug concentration in the dispersion medium. The suspension was centrifuged for 30 min at 10,000 rpm (4 ˚C) and the amount of free BA in the supernatant was determined using HPLC (Fig 1). The equation used to evaluate encapsulation efficiency is given as under:

$EE(\%) = \{(C_{initial} - C_{final})/C_{initial}\} \times 100$

Where, $C_{initial}$ - initial drug concentration

and the C_{final} - free drug measured in the supernatant after centrifugation

The percent encapsulation was found to be 65.2% for Betulinic acid.

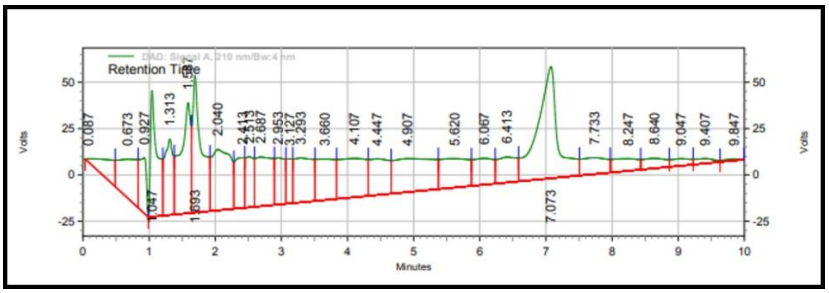


Figure 1. HPLC Chromatogram for supernatant

Morphological studies using Photomicroscopy* and *TEM

The multilamellar vesicles containing BA were found to be stable [23]. The multilamellar vesicles were observed under photomicroscope at 100X magnification prior to extrusion. The transmission electron microscope (TEM) is an analytical technique utilized to observe very fine morphological features of liposomal vesicles at the nanometric scale. Size of nanoparticles influences release rate, solubility rate & dissolution rate of a molecule/drug [24-26]. Nanoliposomes pass through different body organs, according to their shape & size [27, 28]. BNLs nanoformulation was observed to be segregated and uniformly spherical (Fig 2). The size of prepared BNLs was 60-150 nm as revealed by TEM.

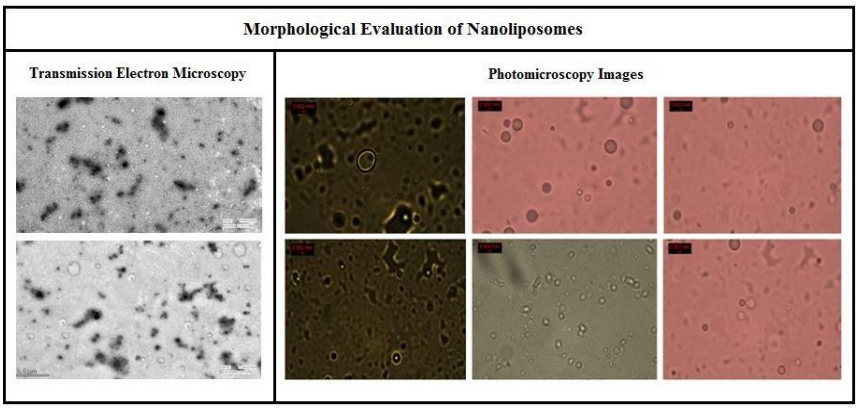


Figure 2: TEM and Photomicroscopic images of Betulinic acid loaded nanoliposomes

FTIR spectroscopy

FTIR can distinguish a wide range of functional groups and provide data in the form of peak or spectra that give structural information and is sensitive to changes in molecular arrangement, due to significant interaction between drug and excipients. The peaks of FTIR that are characteristic to Betulinic acid at 3447 cm^{-1} is for –OH stretch. The FTIR peak at 2929 cm^{-1} is for – CH_2 group vibrations. The FTIR peak at 1700 cm^{-1} infers C=O functional group. The FTIR peak at 1458 cm^{-1} correspond to –OH group(Stretch).The peak at 1376 cm^{-1} for -CH_3 and 1043 cm^{-1} for Stretch i.e., C-O group(Fig 3A). The peaks of FTIR characteristic to cholesterol are hydroxyl group at 3432 cm^{-1}. The peak at 2936 cm^{-1} is attributed to aromatic stretch of CH=CH and peak at 1465 cm^{-1} is to carboxylic acid C=O group and for ester stretch at 1023 cm^{-1} (Fig 3B). The peaks of FTIR characteristic to lecithin are peak of the amide group at 3346 cm^{-1}. The peak observed at 3012 cm^{-1} has a vibration for –OH carboxylic stretch, at 1236 cm^{-1} for P=O stretch vibration and vibration of a P-O-C stretch at 1068 cm^{-1} (Fig 3C).

An FTIR spectrum of BNLs shows the characteristic peaks for Betulinic acid, cholesterol, and lecithin. The peak of the hydroxyl group at 3401 cm^{-1}, the peak at 2947 cm^{-1} is attributed to an aromatic stretch of CH=CH and peak at 1420 cm^{-1} is characteristic of carboxylic acid C=O group and for cholesterol ester stretch at 1019 cm^{-1}(Fig 3D).

The peak at 2985 cm^{-1} is for amide, while the peak at 2911 cm^{-1} has a vibration for –OH carboxylic stretch, at 1210 cm^{-1} for P=O stretch vibration and vibration of a P-O-C stretch at 1081 cm^{-1}. The peaks of FTIR are characteristic for Betulinic acid. The FTIR peak at 2911 cm^{-1} is for –CH_2 group vibrations and peak at 1420 cm^{-1} of –OH group(Stretch).The peak at 1282 cm^{-1} for -CH_3 and 1045 cm^{-1} for Stretch i.e., C-O group. Betulinic acid – Lecithin interaction shows a significant shift of P=O and P-O-C i.e for P=O 1236 cm^{-1} to 1282 cm^{-1} and for P-O-C at 1068 cm^{-1} shift to 1081 cm^{-1}.

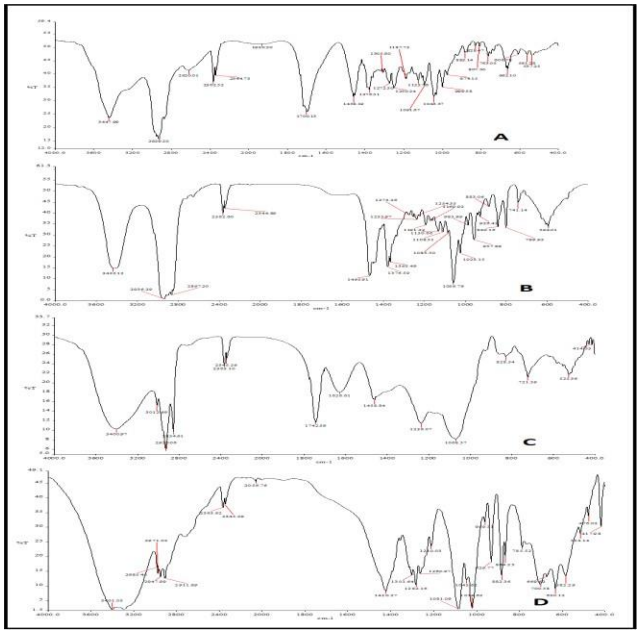


Figure 3. FTIR spectra of (A) BA, (B) Cholesterol, (C) Lecithin, (D) Betulinic acid loaded liposomal nanoformulation

Differential Scanning calorimetric and Thermo-gravimetric analytical studies

The DSC thermogram of free Betulinic acid displayed two endothermic peaks. The first endothermic peak at 286ºC is of low intensity which confirmed the presence of Betulinic acid whereas the other peak is of high intensity that affirmed its crystalline nature (29-30). In case of BNLs thermogram, endothermic peak was observed at 375ºC followed by disintegration which began after 370 ºC (Fig 4A). The peak was of low intensity confirming the amorphous nature of BNLs. In blank nanoliposomes, two endothermic peaks were observed i.e. low intensity peak at 170 ºC whereas the second peak at 365ºC which undergo decomposition after 365 ºC (Fig 4B). The peaks observed were not sharp which can be attributed to its distinct nature.

TGA was conducted to determine the weight loss with respect to temperature. In dummy nanoliposomes, the maximum weight loss was observed at 340 ºC whereas in BNLs there was a slight transition in melting temperature and the maximum weight loss occurred at 350 ºC, which suggested a noteworthy difference in existence of co-amorphous phase.

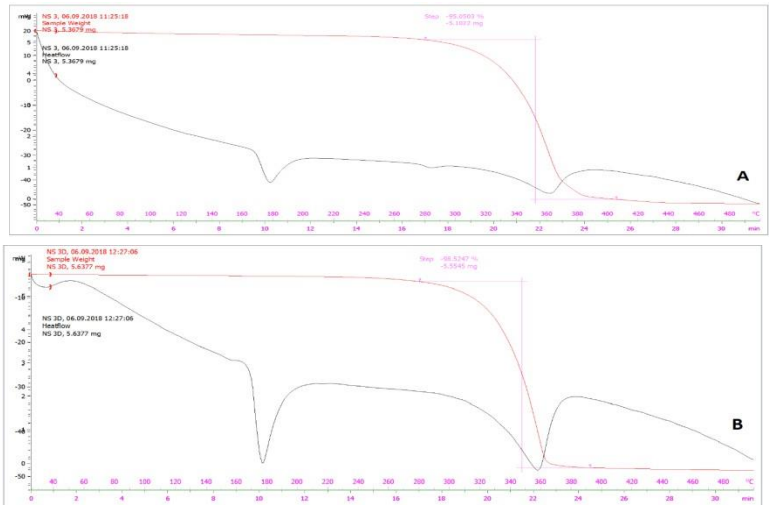


Figure 4. DSC of Betulinic acid loaded liposomal nanoformulation (A) and Blank liposomal nanoformulation (B).

In vitro drug release profile for BNLs using HPLC

The rate controlled the discharge of drug from nanoparticles matrix shields it from rapid metabolism and degradation. The *in-vitro* drug release data suggested that 85.6% of the pure form of BA was released within 3 h. But the sustained release was attained from the BNLs after 3 h, only 38.9% BA was released. In 24 h, 71.6 % of BA was released from BNLs. Overall the drug release profile of BNLs shows a sustained release of BA with the passage of time, which is due to the hydrophobic (nonpolar) nature of BA. Liposomal nanoformulation also formed a lipid bilayer thick and strong walled dense matrix around the BA particles, which assures its sustained release (Fig 5).

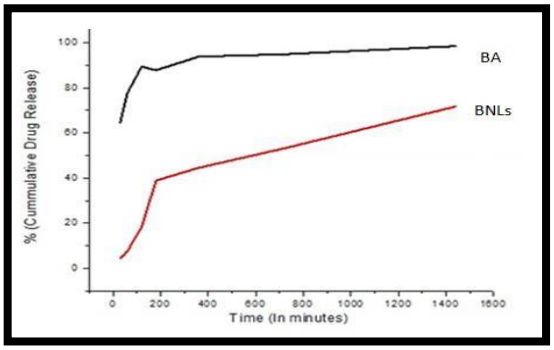


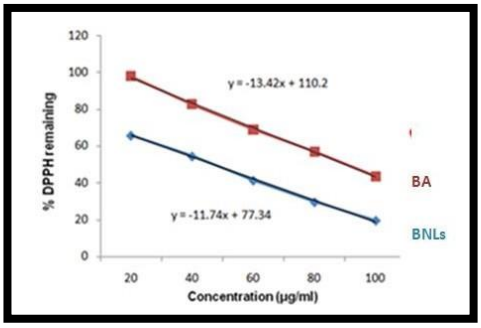
Figure 5. *In vitro* drug release of Betulinic acid nanoliposomes

Antioxidant activity

The DPPH analysis is frequently used to find out the antioxidant activity of encapsulated molecules [31-32]. 1,1-diphenyl-2-picrylhydrazyl is a stable free radical having spare electrons delocalized over the whole molecule, giving a deep violet coloration. It shows an absorption band near 517 nm [33-34]. The disappearance of violet color is observed when a DPPH solution is uniformly mixed with a molecule that can donate (oxidizing nature) a hydrogen atom. The BA is a well known antioxidant molecule. The solution of BA was incubated with DPPH (a hydrogen atom donor) and change of color i.e. from violet to pale yellow was seen. Hence, absorbance band was reduced. BNLs exhibited higher percent DPPH inhibition when compared to the unencapsulated analog of free Betulinic acid, nanoencapsulation of BA by lipid bilayer leads to size reduction

at nanometer scale with more exposed surface which results in enhanced antioxidant activity as compared to the pure drug (Fig 6).

Figure 6. Antioxidant activity of Betulinic acid nanoliposomes



Anticancer activity

The BA and BNLs were screened for anticancer activity against A-549, MCF-7, and Hela cells lines using MTT assay and the results are depicted in Table 1. The large surface area of nanoliposomes is primarily responsible for the cytotoxic efficacy of BNLs. Small-sized bilayer vesicles can enter deep inside the tumor more efficiently [35-37]. The anti-cancerous activity of BA is already reported in the literature [38]. Mitochondrial dehydrogenase is present in viable cells. It cleaves tetrazolium ring structure of MTT dye (Pale yellow in color) [39] forming dark purple crystals of formazan (impermeable to cell membranes) that accumulate inside the cells [40-42]. Oleanolic acid, an isomer of Betulinic acid loaded liposomal nanoplatform delivered critical cytotoxic impacts through apoptosis on cancer cell lines. As a rule, the NPs loaded via mPEG-PLA/PLGA were found more cytotoxic to malignancy cells and were in this way viewed as a more proficient strategy for Oleanolic delivery [43]. The results demonstrated that UENPs exhibit potent anticancer effect with IC_{50} of 5.8 µg/ml, 5.2 µg/ml and 4.9 µg/ml against A-549 cells, MCF-7 and Hela cell lines, respectively and which was more pronounced than pure BA particles anticancer effect with IC_{50} of 26.66 µg/ml, 25.89 µg/ml and 31.12 µg/ml against A-549 cells, MCF-7 and Hela cell lines, respectively (Fig 7).

Sample code	**A-549**		**MCF-7**		**Hela**	
	IC50	**pIC50**	**IC50**	**pIC50**	**IC50**	**pIC50**
Ursolic acid	26.66	-1.443732	25.89	-1.4403	31.12	-1.48187
UNLs	5.8	-0.76343	5.2	-0.7160	4.9	-0.6902

Table 1: IC50 values of BNPs along with pure drug Betulinic acid

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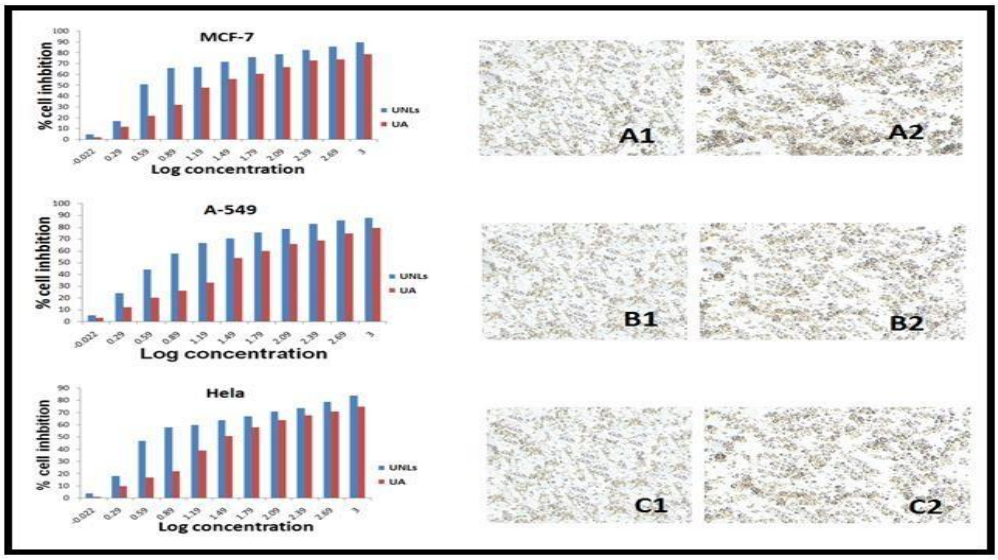

Conclusions

Phytochemicals have been utilized effectively for a wide range of therapeutic applications along with their beneficial effects and appreciative therapeutic to toxicity index. The data presented in this paper demonstrated the preparation, characterization, optimization, *in vitro* release rate, *in vitro* antioxidant activity & anticancer activity of BNLs. The reduction in the size of BA due to vesicle formation of lipid bilayer yield enhanced bioavailability as well as the sustained release of BA. The lipid based nanoformulation open new arena for the therapeutic potential of Betulinic acid at low dose along with enhanced drug residence time. Nanoliposomes (lipid bilayer) have chemical compatibility with plasma membrane and deliver bioactive directly inside the cells by diffusion. Prominently, our results indicated superior *in vitro* antioxidant activity and anticancer efficiency of BNLs, when compared to their structural sibling. Thus, BNLs can be used as promising candidates in treatment of cancer.

Conflict of Interest

There is no conflict of interest whatever.

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