Section: Research Paper
ISSN 2063-5346

Development and assessment of long-circulating betulinic acid-loaded dammar gum nanoparticles for enhanced anti-tumor effectiveness

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Abstract
Betulinic Acid, a triterpene found in nature, has potent anticancer effects. However, it has limited solubility in the aqueous phase as a result of its poor bioavailability. Damar Gum nanoformulations are a practical method to give Betulinic Acid better solubility and bioavailability. Betulinic Acid Damar Gum nanoparticles (BDNs) were made in the current study, and their physical and chemical properties were examined and modified. BDNs demonstrated the highest qualities for usage in drug delivery applications with a particle size range of 48-135 nm, a zeta potential of -25.1 mV, and a percent entrapment effectiveness of 68.7%. The cytotoxic efficacy of the BDNs against the A-549, MCF-7, and Hela cell lines revealed its capacity to prevent cancer cell proliferation. The current study project demonstrated how BDNs could be employed as promising nanoformulations for cancer treatment.

Keywords: Cancer treatment, Damar Gum, Betulinic Acid, and Damar Gum nanoformulations.

Introduction

Chemotherapy is only successful if the anticancer drugs reach their molecular targets. The anticancer drug's random and untargeted circulation inside the biological system reduces its therapeutic efficacy. It also raises the possibility of toxicity and negative side effects. A fundamental tactic for site-specific drug administration and to reduce side effects is to entrap an anticancer agent in a particular carrier. For carrier-mediated drug delivery to cancer spots, however, the particle's surface must be coated with the proper agent and its biocompatibility must be ensured [1].

Cancer diagnosis and treatment may change as a result of nanotechnology [2]. Numerous nanotechnologies have been created and are currently being used to treat cancer more efficiently and safely due to the inherent disadvantages of conventional chemotherapy. Patients have already received more than 40 nanotherapeutics, including chemotherapeutic and imaging drugs. Nanomaterials enable the integration of many therapeutic effects onto a single platform [3]. For this reason, they might be targeted at certain tissues. For successful systemic delivery of nanotherapeutics to solid tumors, a deeper understanding of the biological features and physicochemical properties of nanotherapeutics is also required [4].

Well-known drug carriers that are non-immunogenic, biocompatible, and biodegradable are called Damar Gum [5]. The improved bioavailability of the bioactive element as it passes through the digestive system and enters the bloodstream is another advantage of Damar Gum

bioactive delivery. During formulation, hydrophilic substances may disintegrate into the watery center of Damar Gums while hydrophobic substances may be bonded to the bilayer. Therefore, Damar Gums could contain both hydrophilic and hydrophobic bioactive molecules. Thanks to the discovery and development of phyto nanoformulations, advanced nano-herbal products can now be created using nanotechnology [6]. Several herbal medications are used to treat CVS issues, respiratory disorders, diabetes, cancer, etc. Cancer death rates and the severity of the disease have both decreased as a result of better cancer treatment made feasible by nanoformulations. Betulinic Acid, a triterpenoid molecule, has been demonstrated to have anti-inflammatory [7] and hepatoprotective [8] properties. Additionally, two examples of Betulinic Acid triterpenoids are the aglycone of saponins and free carboxylic acid) [8]. It affects the glucocorticoid receptor and decreases Bcl2 levels in human breast cancer cell lines (Michigan Cancer Foundation-7) [9, 10]. Because of its low level of toxicity and natural origin, Betulinic Acid is frequently used in pharmaceutical formulations that can be administered locally and taken orally [11, 12]. The Betulinic Acid-loaded novel Damar Gum nanoformulations, which also provide a potential way for effectively administering Betulinic Acid, could be able to get over these limitations. A formulation with increased solubility, bioavailability, and therapeutic potential might be provided by Betulinic Acid Damar Gum. In this study, Betulinic Acid Damar Gum was created, characterized, and tested for anti-cancer activity against cancer cell lines.

Material and Methods

Sigma Aldrich in India was the source of the Betulinic Acid. The cholesterol came from Molychem in Mumbai. The cell lines A-549 (Human lung adenocarcinoma epithelial cells), MCF-7 (Human breast adenocarcinoma cells), and Hela (Human cervical carcinoma cell line) were provided by the National Center for Cell Science (NCCS), Pune, while lecithin, minimum essential eagle medium, fetal bovine serum (FBS), penicillin, and streptomycin were purchased from HI media, Mumbai. All study projects utilized chemicals of the analytical reagent grade.

BDNs preparation

Thin film hydration was utilized to produce BDNs Tween 20 was the major excipients used. Tween 20 (20 ml), and 200 mg of Betulinic Acid were dissolved in a solution of 35 ml ethanol and 65 ml carbon tetra chloride. To create nanoformulations centrifuge at 8000 rpm. The particles were then hydrated with 90 ml of deionized water containing 2 ml of 0.035% Tween-80 solution for an hour at 70 degrees Celsius.

Characterization of BDNs

Dynamic light scattering was used to determine the average particle size of the Damar Gum and the degree of heterogeneity (polydispersity index) of the size-optimized Damar Gum. The electrokinetic potential in a colloidal dispersion of vesicles was calculated using the Zetasizer Nano ZS-90 (Malvern Instruments, Malvern, UK) to see if the Damar Gum nanoformulations is stable at 25 °C. The amount of unbound medication in the supernatant was assessed after centrifugation at 10,000 rpm, 4 °C for 30 min, and the % encapsulation efficiency was calculated. The examination of the enhanced batch's morphology was done using a transmission electron microscope (TEM-Hitachi-H-7501SSP/N-817-0520, Japan). One drop of optimized BDNs was first placed onto a copper grid, dried by air, and then

Section: Research Paper
ISSN 2063-5346

scanned at 60,000 magnification factor and 80,000 V accelerating voltage in order to obtain a TEM micrograph. The morphology of BDNs was analyzed prior to extrusion using a fluorescent photomicroscope (LEICA DM 2500 M) magnified 100X. The KBr pellet of powdered samples of Betulinic Acid, lecithin, cholesterol, and BDNs was evaluated in the 4500–500 cm-1 range using the FTIR spectrophotometer Affinity-1 (Shimadzu, Japan). To determine the physical properties of the medication and liposomes, BDNs and dummy nanoparticles were subjected to a differential scanning calorimetry-thermogravimetric analysis (DSC-TGA) evaluation using a TGA/DSC 3+ Stare System, Mettler Toledo AG, Analytical, Switzerland. Alumina pan was used to scan samples (5 mg) between 30 and 500 C at a heat flow rate of 15 C/min.

In Vitro Drug Release from BDNs Release

The release profile was examined using the dialysis sac technique. BDNs (10 mg) were placed in a dialysis sac and kept there while being continuously stirred at 90 rpm at a constant temperature of 37 °C in a solution of ethanol (25%) and phosphate buffer (0.1 M) saline with a pH of 7.4. One ml samples were taken and collected at predetermined intervals of 1, 2, 3, 6, 12, and 24 hours. After that, they were put through an HPLC analysis (Agilent 1200 Infinity Series) on a ZORBAX SB C-18 column (5 m, 150 x 4.6 mm), acetonitrile: water (75:25 v/v), 218 nm, and 9.01 minutes.

Anti-Oxidant Activity

As a measure of antioxidants' capacity to scavenge DPPH, the antioxidant activity was measured. Pure Betulinic Acid and BDNs were exposed to 3.9 mg/100 ml of the free radical 1, 1-diphenyl-2-picrylhydrazyl (DPPH), which was dissolved in methanol, for 30 minutes in the dark. A UV spectrophotometer was used to gauge the absorption at 517 nm. Utilizing Betulinic Acid (positive control), Blank Damar Gum (negative control), and BDNs, the inhibition of DPPH was evaluated. The equation below was used to compute the percentage of DPPH inhibition by pure Betulinic Acid and BDNs.

Antioxidant activity percentage equals (Control Abs.) - (Sample Abs.) X 100 A control absence

Cytotoxicity Activity

The in vitro cytotoxicity activity of BDNs solutions was evaluated using the MTT assay on the 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 tests are used to determine whether a therapeutic bioactive agent or dangerous chemicals have cytotoxic or cytostatic effects. Experiments are often conducted in a dark setting since the MTT reagent is typically photosensitive [11]. The cultivation of cell lines—both healthy and cancerous—in media supplemented with 10% inactivated FBS, 100 l/ml streptomycin, and 100 l/ml penicillin was followed by an incubator humidifier incubation at 370C and 5% CO2. After achieving 70% confluence, the cells were subcultured in a sterile solution containing 0.25% trypsin. Compound and standard stock solutions were created in DMSO (M/ml), and then 96-well plates were used to create dilutions of 1 M, 10 M, 20 M, 50 M, and 100 M per ml in the medium. The density of each cell line was determined using the characteristics of its proliferation. After an initial 8-hour incubation, three days of treatment with various LNL concentrations (0.1-1000 g/ml) and Betulinic Acid were applied to

triplicate wells. 3 l of MTT solution (5 mg/ml) was added to the media three days later. The results were based on the mitochondrial conversion of 3-(4, 5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT), and the total relative percent of metabolically active cells was compared to Formazan crystals and untreated controls. The incubation duration was 180 minutes. The absorbance at 570 nm was measured using a microplate reader (BIORAD) after formazan crystals had been dissolved in DMSO. The effectiveness of synthesized chemicals as anticancer agents was assessed using Betulinic Acid as a reference drug.

The Findings and Discussion

Betulinic Acid Nanoliposome (BDNs) Synthesis

The thin film hydration procedure was used to make Betulinic Acid Damar Gum. Since Damar Gum contains both lipid and aqueous phases, it can be utilized to trap, distribute, and release both lipid and water-soluble compounds. The hydrophobic drug Betulinic Acid's pharmacological profile in the current study generally improved once it was absorbed by the liposomes.

BDNs Characterization

Analysis of particle size and Zeta potential

Particle size is a crucial characteristic of Damar Gum nanocarriers. Cellular uptake, drug release profile, encapsulation efficiency, and biodistribution are all impacted [12,13]. Damar Gum of a particular size (150 nm) can exit or enter the microenvironment of cancer cells. The cancer cells are bigger and more vascular [14]. Vascular mediators will subsequently accumulate at the locations of the tumor. The leaky vasculature of cancer cells, however, is what enables the enhanced permeability & and retention (EPR) impact of high molecular weight medicines. Drugs included in liposomes as small as 400 nm can passively target cancer cell sites, but the endothelium wall prevents them from reaching healthy tissues [15–20]. In the present study, the particle size of BDNs was found to be 211 nm, indicating its high vasculature and concentration. Zeta potential calculations were done to assess the relationship between surface charge and stability [21–22]. Zeta potential can help control the fusion, precipitation, and aggregation of nano Damar Gum formulations. A more stable preparation and greater cellular absorption are indicated by a larger negative value. Zeta potential measurements of BDNs were found to be -42.5 mV, indicating higher stability.

% EE, or percent encapsulation efficiency

The encapsulation efficiency of the BDNs formulation was estimated using the free drug concentration in the dispersion medium. After the suspension was centrifuged for 30 minutes at 10,000 rpm (4 C), the amount of free Betulinic Acid in the supernatant was determined by HPLC (Fig. 1). Encapsulation efficiency is calculated using the following equation:

EE(%) = 100 * (Cinitial - Cfinal)/Cinitial

where C initial refers to the first medication concentration and after centrifugation, the Cfinal-free medication was quantified in the supernatant. Betulinic Acid was discovered to have a 65.2% encapsulation rate.

Morphological investigations employing TEM and photomicroscopy

Multilamellar vesicles containing Betulinic Acid were found to be stable [23]. The multilamellar vesicles were observed under a photomicroscope at a 100X magnification prior to extrusion. The transmission electron microscope (TEM) is an analytical tool used to

investigate minute morphological aspects of Damar Gum vesicles at the nanometric scale. The release, solubility, and dissolution rates of molecules and drugs are influenced by the size of nanoparticles [24–26]. Damar Gum can pass through a variety of human organs depending on their shape and size [27, 28]. The BDNs nano formulation was seen to be distinct and consistently spherical (Fig. 2). The size of the prepared BDNs, according to TEM, ranged from 60 to 150 nm.

FTIR analysis

FTIR can distinguish a wide range of functional groups due to the significant interaction between medications and excipients. It can also provide data in the form of peaks or spectra that show structural information and is sensitive to changes in molecular arrangement. Betulinic Acid-specific FTIR signal for the -OH stretch may be found at 3447 cm-1. The -CH2 group vibrations' FTIR peak is located at 3030 cm-1. The FTIR signal at 1742 cm-1 implies the presence of the C=O functional group. The stretch-OH group is related to the 1318 cm-1 FTIR peak. Fig. 3A shows the stretch or C-O group peaks at 1043 cm-1 and 1421 cm-1, respectively. The hydroxyl group at 3512 cm-1 is the FTIR signal for cholesterol. As shown in Fig. In figure 3B, the ester stretch, the carboxylic acid C=O group, and the aromatic stretch of CH=CH are all responsible for the peaks at 2930 cm-1, 1565 cm-1, and 988 cm-1, respectively. The FTIR peak that distinguishes lecithin from other substances is the peak of the amide group at 3546 cm-1. A vibration for the -OH carboxylic stretch is present at 2989cm-1, a P=O stretch vibration is present at 1011cm-1, and a P-O-C stretch vibration is present at 1320cm-1 (Fig. 3C).

The FTIR spectrum of BDNs shows the characteristic peaks for Betulinic Acid, cholesterol, and lecithin. Peaks for the cholesterol ester stretch are at 909 cm-1, the carboxylic acid C=O group is at 1501 cm-1, the hydroxyl group is at 3445 cm-1, an aromatic stretch of CH=CH is at 3023 cm-1, and the hydroxyl group is at 3445 cm-1 (Fig. 3D).

Amide has a peak at 3021 cm-1, the -OH carboxylic stretch vibration at 2897 cm-1, the P=O stretch vibration at 1365 cm-1, and the P-O-C stretch vibration at 1102 cm-1. Betulinic Acid is indicated by the FTIR peaks. The peak for the -OH group (Stretch) is at 1390 cm-1, while the FTIR signal for the vibrations of the -CH2 group is at 3012 cm-1. At 965 cm-1 and 1300 cm-1, respectively, stretch or the C-O group peaked. P=O and P-O-C both change significantly as a result of the interaction between Betulinic Acid and lecithin, moving from 1338 cm-1 to 1387 cm-1 and 988 cm-1 to 999 cm-1, respectively.

Analytical research using differential scanning calorimetry and thermogravimetry

The free Betulinic Acid DSC thermogram revealed two endothermic peaks. The low intensity of the first endothermic peak at 286 oC confirmed the presence of Betulinic Acid, while the high intensity of the second peak demonstrated the substance's crystalline shape (29–30). The thermogram for BDNs revealed an endothermic peak at 375 oC and the beginning of disintegration at 370 oC (Fig. 4A). The amorphous character of BDNs was reinforced by the peak's low intensity. In blank Damar Gum, two endothermic peaks were observed: a low intensity peak at 170 oC and a second peak at 365 oC that disintegrates above 365 oC (Fig. 4B). The observed peaks were not sharp but had a recognizable shape. TGA was used to measure the weight loss in response to temperature. Maximum weight loss in phony Damar Gum was discovered at 340 °C, but maximum weight loss in BDNs happened at 350 °C

following a slight melting temperature adjustment, indicating a significant difference in the existence of the co-amorphous phase.

HPLC-based in vitro drug release profile for BDNs

The nanoparticle matrix's regulated drug release guards against rapid metabolism and degradation. 85.6% of the pure form of Betulinic Acid was released in 3 hours, based on results from in-vitro drug release experiments. Only 38.9% of Betulinic Acid was, however, sustainably released from the BDNs after 3 hours. In under 24 hours, 71.6% of Betulinic Acid was released from BDNs. The drug release profile of BDNs as a whole shows a continuous release of Betulinic Acid throughout time because Betulinic Acid is hydrophobic (nonpolar). Additionally, Damar Gum nanoformulations encased the Betulinic Acid particles in lipid bilayers thick and firmly walled dense matrix, guaranteeing their protracted release (Fig. 5).

Anti-Oxidant Function

The DPPH technique is frequently used to assess the antioxidant activity of substances that are encapsulated [31, 32]. Because spare electrons are dispersed across the entire molecule of the stable free radical 1,1-diphenyl-2-picrylhydrazyl, the molecule exhibits a deep violet hue. It contains an absorption band at around 517 nm [33, 34]. Violet hue vanishes when a DPPH solution is uniformly mixed with a molecule that has the ability to donate (have an oxidizing character) one atom of hydrogen. Betulinic Acid, an antioxidant substance, is frequently employed. The Betulinic Acid solution's hue changed from violet to pale yellow when DPPH (a hydrogen atom donor) was added. BDNs demonstrated a higher level of DPPH inhibition compared to the unencapsulated analog of free Betulinic Acid, which caused the absorption band to narrow. Increased antioxidant activity is the result of lipid bilayer nanoencapsulation of Betulinic Acid, which results in size decrease at the nanometer scale with more exposed surface (Fig. 6).

Anti-Cancer Properties

A-549, MCF-7, and Hela cell lines were employed to assess the Betulinic Acid and BDNs for anticancer activities using the MTT assay. The outcomes are displayed in Table 1. The enormous surface area of Damar Gum is primarily responsible for the cytotoxic efficacy of BDNs. The deepest levels of the tumor can be more successfully penetrated by smaller bilayer vesicles [35–37]. Betulinic Acid's anti-cancerous capabilities have already been mentioned in the literature [38]. Mitochondrial dehydrogenase is present in healthy cells. In order to produce dark purple formazan crystals that are impermeable to cell membranes and accumulate inside of the cells [40–42], it destroys the tetrazolium ring structure of the pale yellow MTT dye [39]. Through inducing apoptosis, a Damar Gum nanoplatform containing oleanolic acid, an isomer of Betulinic Acid, demonstrated strong cytotoxic effects on many cancer cell types. Oleanolic acid was discovered to be more effectively delivered by NPs loaded via mPEG-PLA/PLGA, which were also proven to be more cytotoxic to cancer cells [43]. With IC50 values of 5.8 g/ml, 5.2 g/ml, and 4.9 g/ml against A-549 cells, MCF-7 cell lines, and Hela cell lines, respectively, the results demonstrated that BDNs display a significant anticancer impact. Compared to pure BA particles, which had IC50 values of 26.66 g/ml, 25.89 g/ml, and 31, this effect was more pronounced.

Section: Research Paper
ISSN 2063-5346

Conclusions

Phytochemicals have been employed successfully for a range of therapeutic purposes because of their beneficial effects and excellent therapeutic-to-toxicity ratios. The findings of this study demonstrated the creation, characterization, optimization, and in vitro antioxidant and anticancer capabilities of BDNs. Betulinic Acid's size was lowered as a result of the formation of lipid bilayer vesicles, which boosted its bioavailability and extended-release. The lipid-based nanoformulations enable the therapeutic potential of Betulinic Acid at low doses along with increased drug residence time. Damar Gum (lipid bilayers) are compatible chemically with plasma membranes and can diffuse bioactive compounds straight inside of cells. Our results specifically show that BDNs outperformed their structural sibling in the in vitro fight against cancer. Thus, BDNs may be helpful in the battle against cancer.

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Section: Research Paper
ISSN 2063-5346

Figure 1: Zeta potential of BDNs

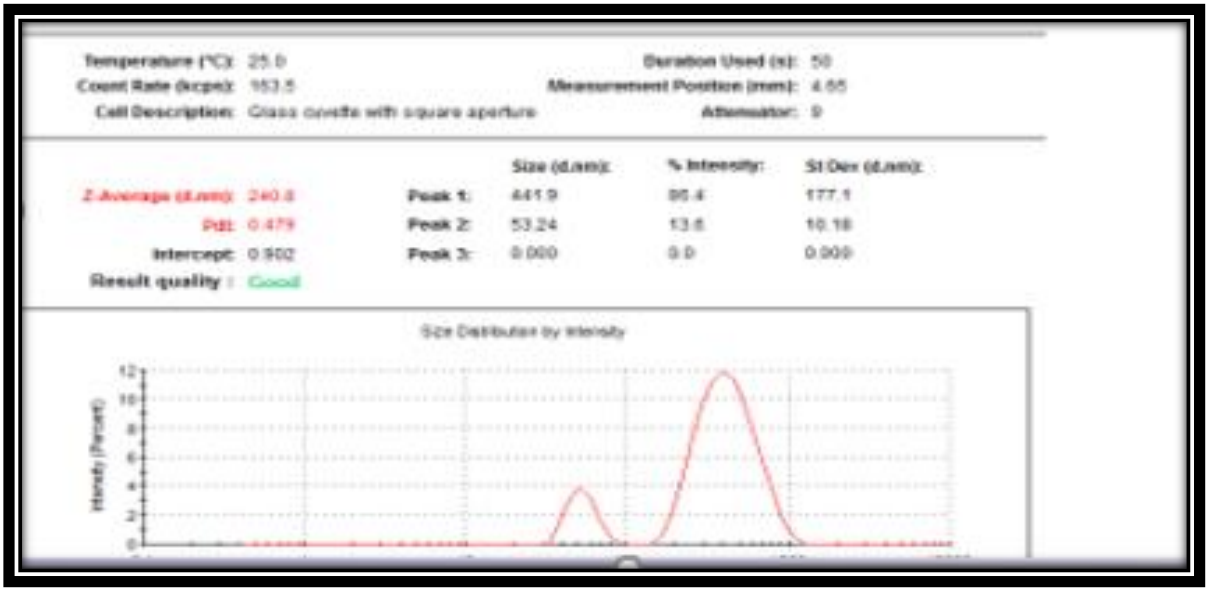
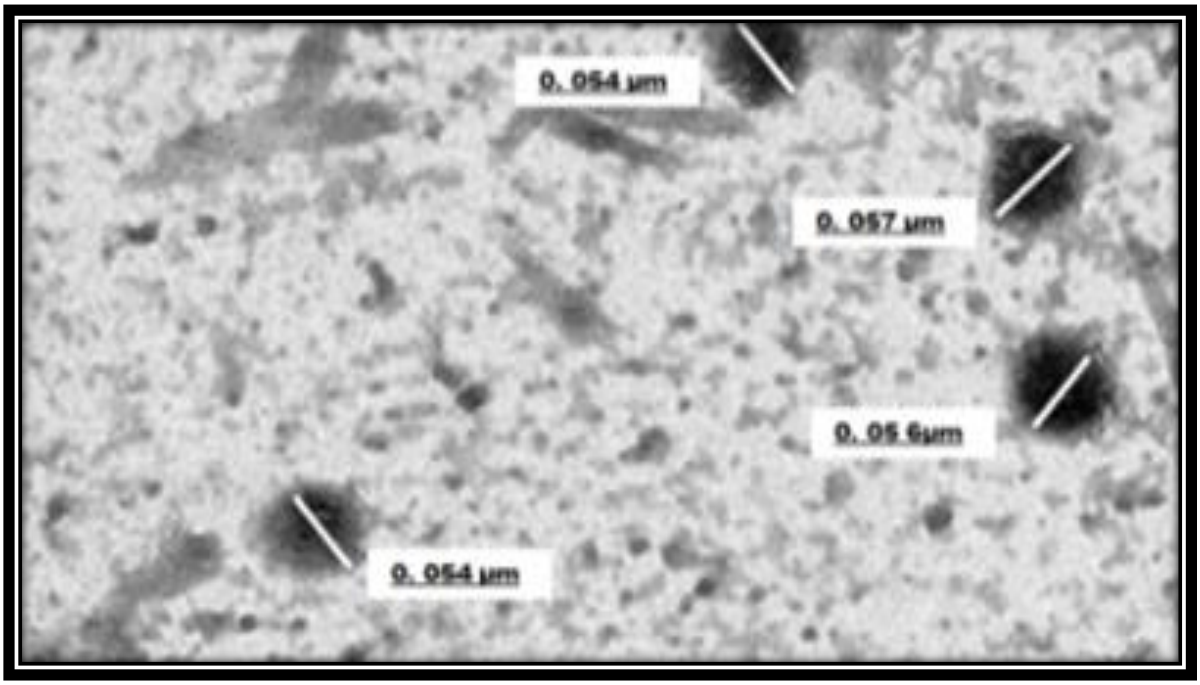


Figure 2: TEM and Photo microscopic images of BDNs



Section: Research Paper
ISSN 2063-5346

Figure 3. FTIR spectra of BDNs and Betulinic acid

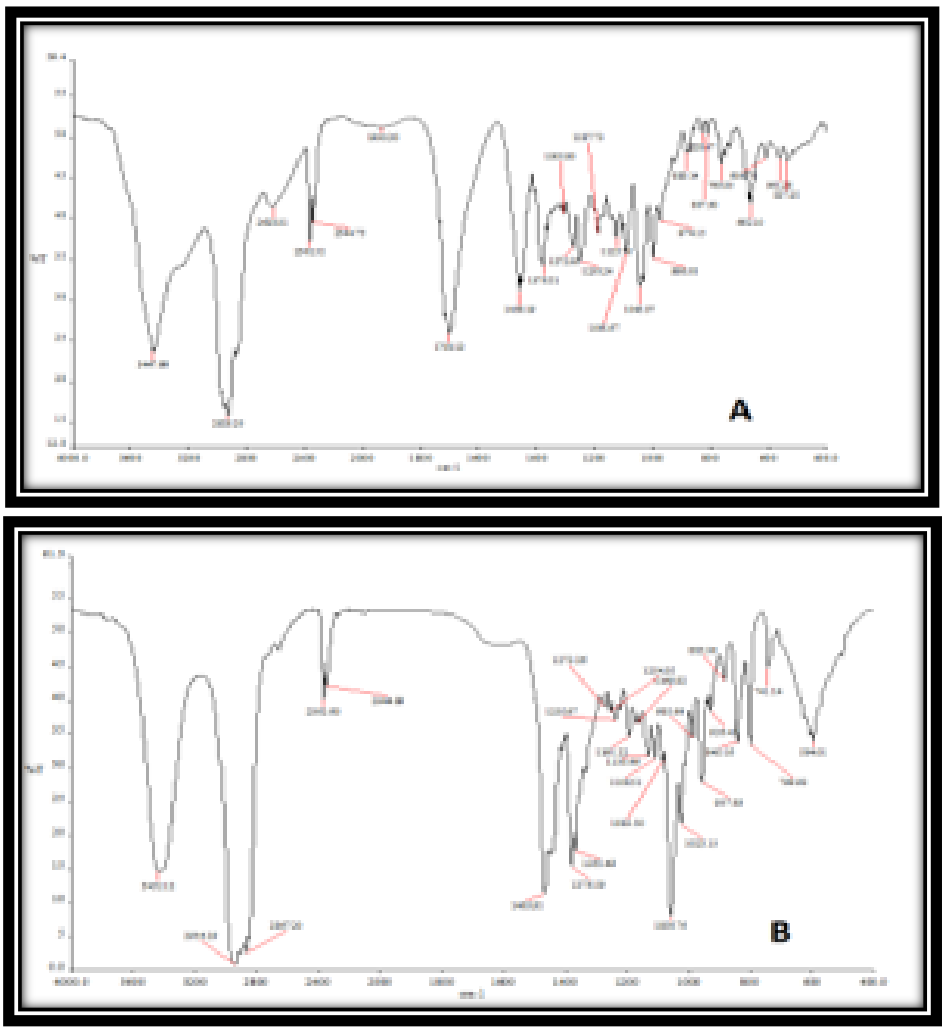
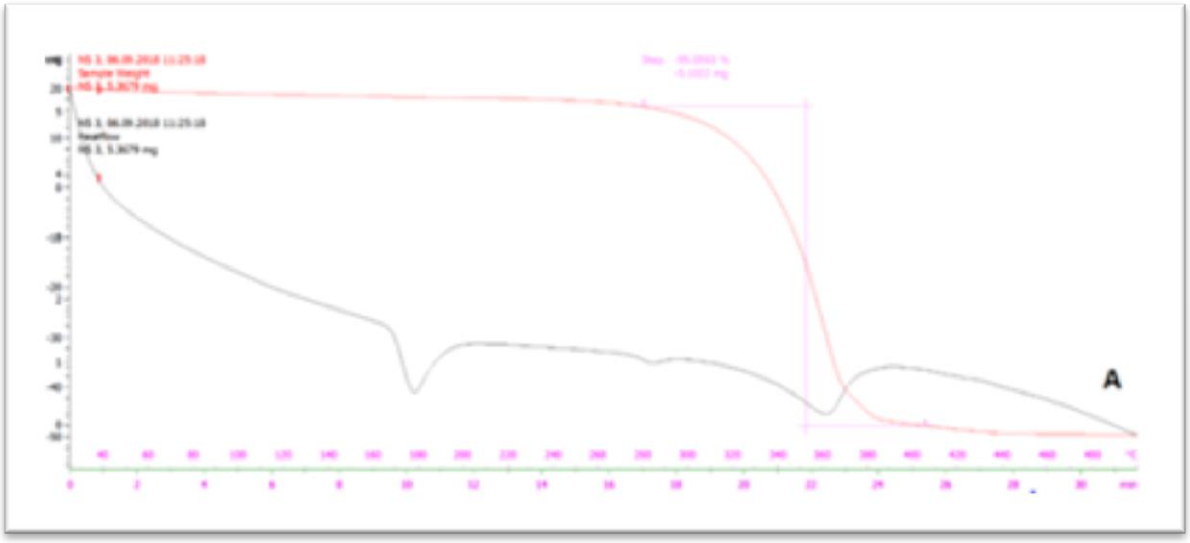


Figure 4. DSC of BDNs



Section: Research Paper
ISSN 2063-5346

Figure 5. *In vitro* drug release of BDNs

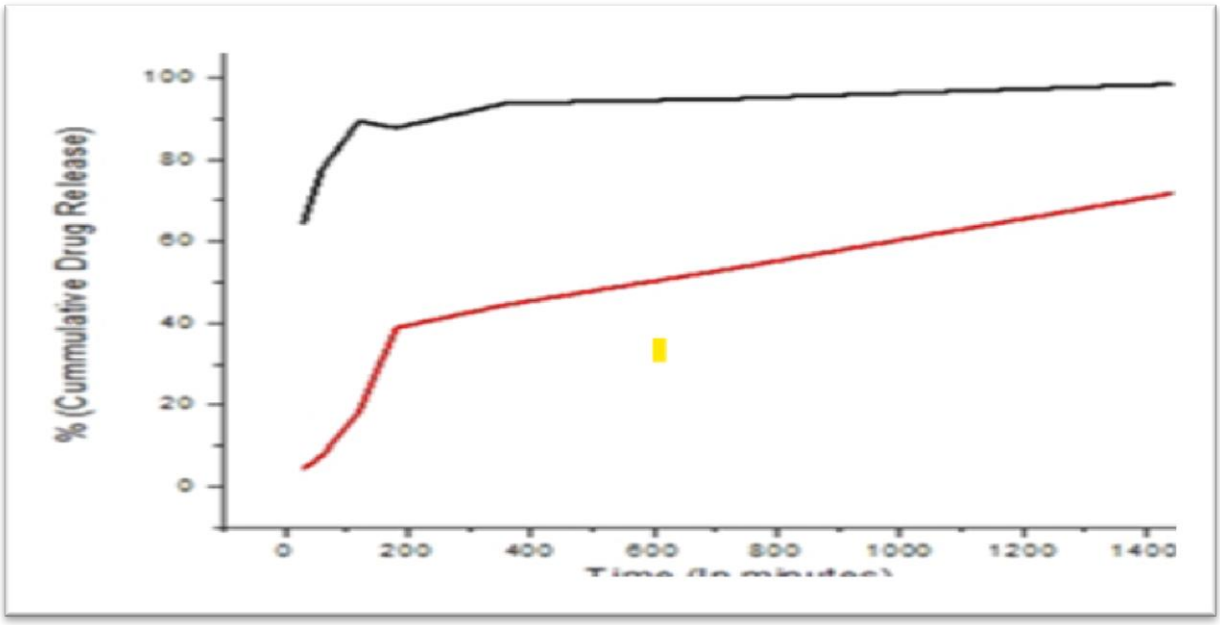


Figure 6. Percentage cell inhibition of BDNs on MCF-7, A-549 and MCF-7 Cell lines after 24 h.