

## Original Research Article

# Induction of apoptosis in response to improved gedunin by liposomal nano-encapsulation in human non-small-cell lung cancer (NCI-H292) cell line

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### Abstract

**Purpose:** To investigate the anti-proliferative activity of free and nanoencapsulated gedunin against human non-small-cell lung cancer (NCI-H292) cells.

**Methods:** Gedunin-loaded nanoliposomes (LG) were prepared using thin-film hydration method. Anti-proliferative effects of free and LD were evaluated by sulforhodamine B (SRB) assay. Apoptotic effects of gedunin-loaded liposomes were assessed by evaluating expressions of p53, Bax and survivin genes, caspase 3/7 activities, DNA fragmentation and morphological changes after staining with Hoechst 33342 and acridine orange/ethidium bromide (AO/EB).

**Results:** Cell proliferation data and microscopic visualization demonstrated a higher anti-proliferative activity for LG than the encapsulant (liposomes) alone. LG exhibited dose- and time-dependent 10-fold anti-proliferative activity compared to the free drug, while displaying tolerable belligerence towards normal human lung fibroblast (MRC-5) cells. Apoptosis detection assays and gene expression analysis revealed the transcriptional modulation of the apoptosis-related genes (p53, survivin and Bax), increased activity of caspase 3/7 and the condensation of nuclear chromatin, implying the induction of apoptosis by the nano-formulation in NCI-H292 cells.

**Conclusion:** LG may therefore be considered as a potential nano-formulation which can target non-small-cell lung cancer.

**Keywords:** Lung cancer, Gedunin, Liposome, Nanoencapsulation, Apoptosis, Anti-proliferative activity, Nuclear chromatin

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## INTRODUCTION

Lung cancer ranks as the most common cancer among men worldwide and every year approximately 1.35 million lung cancer cases are diagnosed. It ranks as the major cause of cancer deaths among Asian males [1]. Of the two major

types (small-cell and non-small-cell) of lung cancer, non-small-cell lung cancer (NSCLC) is the most common type [2]. Though chemotherapy, radiotherapy and surgeries are the only available treatment options for lung cancer patients, chemo and radiotherapy cause severe side effects. Thus, finding an effective

chemotherapeutic solution with fewer side effects will improve the prognosis of NSCLC patients.

Plant-derived anti-cancer compounds play a major role in the field of anti-cancer drug discovery and some natural compounds have been approved for clinical use [3]. Although many anti-cancer drugs have been isolated from natural sources, several challenges such as short half-lives, high metabolic instability, low solubility leading to poor absorption and systemic bioavailability are mainly associated with the development of anti-cancer drugs into a globally acceptable level. Further, drug resistance, rapid degradation of the drug, large dosage requirement, and undesirable toxicity are also existing challenges [4]. Research on nano-pharmaceuticals has helped overcome these challenges and a number of nano-material-based therapeutic approaches have been used for cancer treatment while some are under clinical development [5]. Lipid-based nano-particles (liposomes) have gained much attention as they possess some unique properties such as conjugation with targeting moieties like antibodies, ease of degradation under specific conditions, and drug-cargo-carrying capacity [6].

Gedunin is one of the major compounds present in the neem tree and it is an Hsp90 inhibitor [7]. Anti-proliferative effects of gedunin have also been reported in several cancer cells such as colon, prostate, ovarian, pancreatic, breast and cervical. However, anti-proliferative effects of gedunin in lung cancer have not been evaluated so far [7,8]. Since gedunin is poorly soluble (due to hydrophobic nature) in the aqueous extracellular environment which limits its bioavailability and pharmacokinetic profile, we hypothesize that nano-formulations of gedunin may enhance its anti-proliferative and apoptotic effects in lung cancer cells. Therefore, this study was planned to evaluate anti-proliferative and apoptotic effects of gedunin-loaded liposomal nano-particles in human non-small-cell lung cancer (NCI-H292) cell line.

## EXPERIMENTAL

### Materials

Cell culture medium and reagents were purchased from the American Type Culture Collection (ATCC), Rockville, MD, USA. Caspase-Glo 3 and 7 assay kit was a product of Promega. Egg yolk phosphatidylcholine (approx. 60 %, TLC) and cholesterol ( $\geq 99.0$  %), dialysis tubing (12 000 MWCO), ethanol, methanol and acetonitrile (HPLC grade) were purchased from Sigma-Aldrich. Gedunin was procured from

Santa Cruz Biotechnology, Texas (sc-203967). Deionized water was filtered through a 0.45  $\mu\text{m}$  cellulose nitrate filters (Sartorius Stedim Biotech GmbH 37070, Goettingen, Germany). All other chemicals and reagents used in this study were of analytical grade and purchased from Sigma Aldrich Chemical (St. Louis, MO, USA) unless otherwise indicated.

### Cell culture

NCI-H292 (non-small cell lung cancer) and MRC-5 (normal human lung Fibroblast) cell lines were purchased from American Type Culture Collection (ATCC), Rockville, MD, USA. Cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10 % fetal bovine serum (FBS), and a 0.1 % antibiotic cocktail (penicillin/streptomycin). Cells were seeded in T-25 flasks, incubated in a humidified atmosphere of 5 %  $\text{CO}_2$  at 37 °C to 70 – 80 % confluence and sub-cultured as a mono-layer.

### Preparation of liposomal nano-formulation

LG were prepared using the thin-film hydration method originally described [9]. Phosphatidylcholine (100 mg), cholesterol (20 mg) and gedunin (1 mg) in 5 mL ethanol were dissolved in 25 mL chloroform. Then, a thin film of lipids was prepared after evaporating the organic solvents using a rotary evaporator. The lipid film was allowed to dry overnight at room temperature in a vacuum oven and hydrated with 10 mL phosphate buffered saline (PBS, pH 7.4) with continuous stirring to incorporate the gedunin. The liposomal suspension was kept overnight at 4 – 8 °C, to complete hydration. Then, the liposomal suspension was sonicated for 5 min at 4 °C in a sonicating bath. It was then dialyzed against PBS (pH 7.4) for 3 days at 4 °C to purify LG. The liposome nano-particles were sterilized by autoclaving at 121 °C for ~ 15 min, followed by slow cooling for ~ 1 h [10].

### Characterization of LG

LG (500  $\mu\text{L}$ ) was dissolved in 10 mL ethanol and the solution was analyzed by HPLC in a reversed phase C18 column. Deionized water and acetonitrile were used for gradient elution in which the percentage of acetonitrile was increased from 30 to 100 % gradually over a period of 20 min. Next, 100 % acetonitrile was eluted for 5 min. The flow rate used throughout the analysis was 1 mL/min. The wavelength used for the detection of gedunin was 224 nm and the retention time was 13.7 min. A series of concentrations of ethanol gedunin solutions was

used for the construction of the standard curve. These data were used for calculating encapsulation efficiency (EE) and loading capacity (LC). Particle sizes of liposomes were determined using dynamic light scattering technique (zetasizer NanoZS, Malvern Instruments, UK) fitted with a red laser of 633 nm. Liposome suspensions were diluted in PBS and the samples were equilibrated at 25 °C. Then, scattering intensity was measured at an angle of 173° relative to the incident radiation. Particle size was obtained from size distribution by intensity. Zeta-potential of the same liposomal suspensions described above were measured using Laser Doppler Electrophoresis technique. The values reported are the mean zeta-potential of the liposomal formulations.

### Anti-proliferative assays

NCI-H292 and MRC-5 ( $5 \times 10^3$  cells per well) cells were seeded in 96-well tissue culture plates (BD Biosciences, Cowley, UK). The cells were then incubated for 24 h temperature before treatment with test compounds. After incubation, six serially diluted gedunin concentrations (1.56 – 50 µg/mL) and media containing LG (at six serial concentrations ranging from 0.50 to 15 µg/mL) were tested for anti-proliferative activity by sulphorhodamine B (SRB) assay [11]. Three time intervals (24, 48 and 72 h) were used and the standard anticancer drug paclitaxel (at the same doses with LG) was used as the positive control. Prior to the SRB assay, wells were washed with PBS (three times) and fixed in ice-cold 50 % trichloroacetic acid (TCA) at 4 °C for 1 h. The cells were then stained with 0.4 % SRB dye (prepared in 1 % acetic acid) for 15 min at room temperature and washed off with 1 % acetic acid, and subsequently solubilized with 10 mM Tris-base in a plate shaker for 1 h at room temperature. Absorbance was measured at a wavelength of 540 nm using a microplate reader (Synergy HT, USA). Half-maximal inhibitory concentration ( $IC_{50}$ ) values were determined for tested treatments.

### Light microscopy

Microscopic images of untreated and treated cells were captured using an inverted phase-contrast microscope (Olympus CKX41, Japan) to evaluate the changes in cell morphology following treatments.

### Fluorescence microscopy

Cells cultured on ( $2 \times 10^4$  cells/well) coverslips in 24-well tissue culture plate, were treated with liposomal gedunin (1, 2 and 4 µg/mL) for 24 h.

Following incubation, the wells were washed with PBS and fixed with 4 % formaldehyde (pH 7.4) at room temperature for 15 min. The cells were washed again with 1 mL PBS and triple-stained in the dark with three dyes: acridine orange/ethidium bromide and Bis-benzimide (Hoechst 33258) and observed under a fluorescence microscope (Olympus, BX51TRF, Japan).

### Caspase 3/7 activity assay

Caspase3/7 activity in liposomal gedunin treated NCI-H292 cells was measured using Caspase-Glo 3/7 assay kit (Promega) according to manufacturer's protocol.

### DNA fragmentation analysis

NCI-H292 cells ( $1 \times 10^6$  cells) were treated with LG at 1, 2 and 4 µg/mL and paclitaxel (4 µg/mL) for 48 h. Cells were collected by trypsinization and centrifugation. Resulting pellets were washed with ice-cold PBS to remove cell debris present. Freshly prepared lysis buffer (5 mL), containing Tris-HCl (5 mM, pH 8.0), EDTA (5 mM, pH 8.0), 0.5% SDS, 1 M NaCl, proteinase K (0.1 mg/mL), RNase (0.03 mg/mL) and autoclaved distilled water was added to each sample (300 µL) and incubated for 1 h in a 55 °C water-bath. DNA was extracted by phase separation method [11]. The DNA pellet was then re-dissolved in 20 µL autoclaved distilled water and equal amount of DNA (1 µg) was loaded onto the 2% agarose gel containing ethidium bromide (0.5 µg/mL) and run along with 100 bp ladder at 55 V for 3 h. The banding pattern was visualized by UV illumination using a Gel-Doc system (Quantum-ST4 1100/20M).

### Real-time quantitative PCR of apoptosis-related genes

The NCI-H292 cells were sub-cultured ( $2 \times 10^5$  cells/mL) in T-25 tissue culture flasks and maintained for 24 h. They were treated with liposomal gedunin (0.5 and 1 µg/mL) and incubated for another 24 h along with an untreated control. TRIzol reagent was used to extract RNA. Extracted RNA (2 µg) was mixed with 50 ng of random primers and 13.5 µL of ultrapure PCR water to make a total volume of 25 µL and incubated for 5 min at 70°C. cDNA was then synthesized by adding 5 µL 10 mM dNTP mix, 5 µL MMLV 5X reaction buffer, 25 units of RNasin and MMLV RT enzyme (200 units), and the reaction mixture (25 µL) was incubated at 37 °C for 60 min in a thermal cycler. Stratagene Mx3000P real-time PCR system (Agilent Technologies, CA, USA) was used to

perform quantitative real-time PCR (qPCR) with the help of MESA Green qPCR MasterMix Plus for SYBR<sup>®</sup> Assay (Eurogentec, Seraing, Liège, Belgium) with primers previously described [12]. The reaction mixture (25 µL) contained 0.5 µL of respective forward and reverse primers, 12.5 µL SYBR Green MasterMix and water. GAPDH (glyceraldehyde 3-phosphate dehydrogenase) was used as the internal control. The thermal cycle programme was as follows: denaturation at 95 °C for 10 min, 40 cycles of denaturation at 95 °C for 30 s, annealing at 56 °C for 1 min, and extension at 72 °C for 30 s. The relative quantification of each gene expression profile was computed by the  $2^{-\Delta\Delta Ct}$  method [13].

### Statistical analysis

All data in this study are expressed as mean ± standard deviation of three independent experiments. GraphPad Prism 6.0.1 software (GraphPad Software Inc., San Diego, CA, USA) was used for statistical analysis and two-way analysis of variance (ANOVA) with Tukey's multiple comparisons test was used to determine significant differences between experimental groups. The differences were considered statistically significant at  $p < 0.05$ .

## RESULTS

### Characteristics of liposomal gedunin formulation

The prepared nanoparticles showed polydispersity index of 0.709 and exhibited characteristics of successful encapsulation, as determined by HPLC. Encapsulation efficiency (EE), loading capacity (LC), particle size

distribution and zeta-potential of nanoparticles are presented in Table 1.

### Anti-proliferative activity

The IC<sub>50</sub> values of the free gedunin, liposomal gedunin, and the standard anticancer drug, paclitaxel for NCI-H292 and MRC-5 cells, as well as their safety factors, are displayed in Table 2. Free gedunin showed significant ( $p < 0.05$ ) dose-dependent and a time-dependent cytotoxic effect in NCI-H292 cells and it was less cytotoxic to MRC-5 cells. Liposomal gedunin also exerted significant ( $p < 0.05$ ) dose-dependent and time-dependent cytotoxic effects in NCI-H292 cell. However, liposomal gedunin showed higher cytotoxic effects in NCI-H292 cells than to pure gedunin and paclitaxel. Paclitaxel demonstrated higher cytotoxicity in MRC-5 cells than to prepared liposomal gedunin nano-particles.

### Cytomorphological changes

Phase-contrast microscopic images obtained in the presence and absence of treatments, as shown in Figure 1, depict dose-dependent as well as time-dependent characteristic cytomorphological changes: cell shrinkage, cytoplasmic granulation, surface blebbing and blistering and cell lysis were the major visible hallmarks of apoptosis.

### Caspases 3 and 7 activities

According to the results obtained, all three doses (0.0125, 0.0625 and 1 µg/mL) of liposomal gedunin increased the activity of caspase 3 and 7 in a dose-dependent manner, which was only significant ( $p = 0.03$ ) for the highest dose (Fig 2).

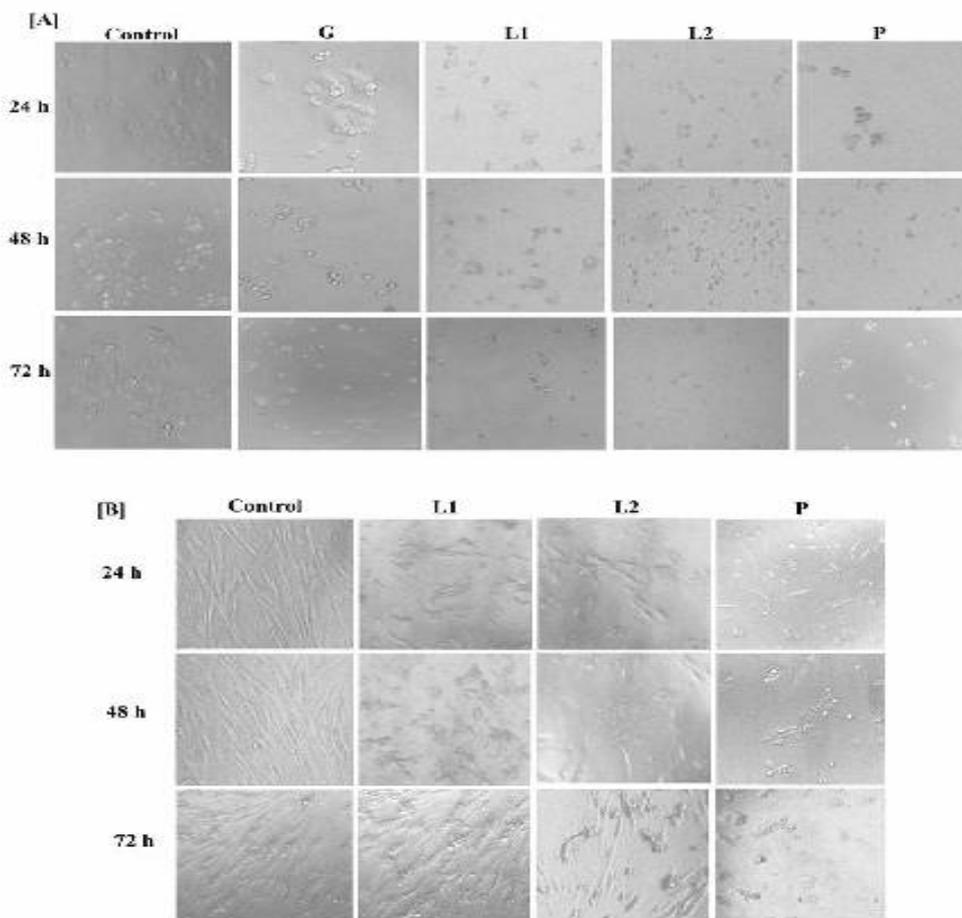
**Table 1:** EE, LC, size and zeta potential of liposome nano-particles

Formulation	Encapsulation efficiency (%)	Loading capacity (%)	Diameter (nm)	Zetapotential (mV)
Gedunin-loaded liposomes	86.82	$7.2 \times 10^{-3}$	394.1 ± 89.4	-54.1 ± 5.91

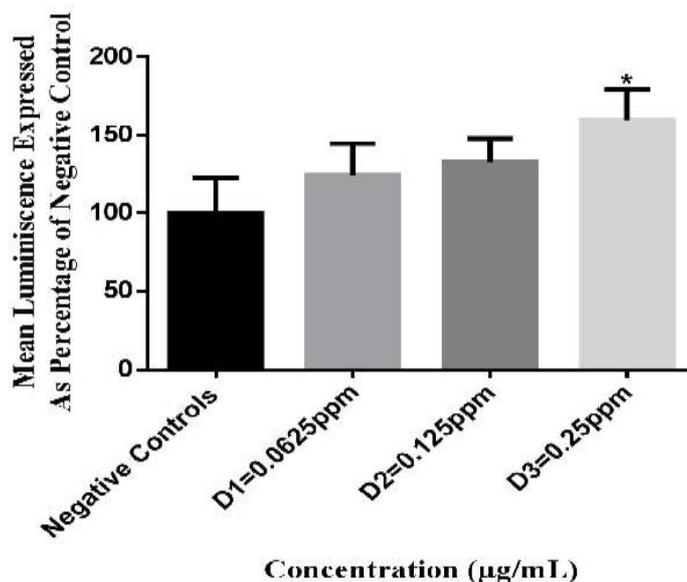
**Table 2:** IC<sub>50</sub> (µg/mL) and safety factors for the test compounds and the standard anticancer drug, Paclitaxel on cancerous and normal lung cell lines at different experimental timelines

Formulation	NCI-H292			MRC-5		
	24 h	48 h	72 h	24 h	48 h	72 h
Gedunin	26.430	24.640	21.600	>50	>50	>50
Gedunin liposomes	3.354	1.900	1.795	5.315 <b>(1.58)*</b>	2.937 <b>(1.55)*</b>	4.902 <b>(2.73)*</b>
Paclitaxel	8.372	3.286	1.596	1.518 <b>(0.18)*</b>	0.964 <b>(0.29)*</b>	0.351 <b>(0.22)*</b>

\*Bold data in brackets represent the safety factor, i.e. evaluation of the biotoxicity of test compounds on cancerous (NCI-H292) cells relative to normal (MRC-5) cells, determined as the ratio of IC<sub>50</sub> value for MRC-5 cells to that for NCI-H292 cells – the higher the value, the safer the drug. An IC<sub>50</sub> value for liposome (without drug) was not detected within the highest dose tested (100 ppm)



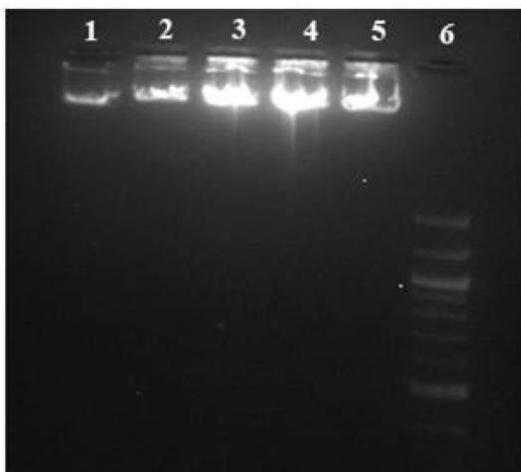
**Figure 1:** Cytomorphological changes in [A] NCI-H292 and [B] MRC-5 cells following 24, 48 and 72 h drug treatments, as observed under a phase-contrast light microscope: Across rows: Untreated control; G = Gedunin (25  $\mu\text{g}/\text{mL}$ ); L1 = Liposomal gedunin (3.75  $\mu\text{g}/\text{mL}$ ); L2 = Liposomal gedunin (15  $\mu\text{g}/\text{mL}$ ); P = Paclitaxel (3.75  $\mu\text{g}/\text{mL}$ )



**Figure 2:** Dose-dependent activation of effector caspases 3 and 7 in untreated and liposomal gedunin--treated NCI-H292 cells. Error bars indicate the  $\pm$  standard deviation of three replicates.  $p = 0.03^*$ ;  $p$  value represents the significance of the difference between the control and the treated groups (two-way ANOVA).  $P < 0.05$  indicates significant difference among treatments

## DNA fragmentation

Typical DNA fragmentation was not observed in liposomal gedunin and paclitaxel treated cells. However, a small smearing of DNA was observed at 3 and 4  $\mu\text{g}/\text{mL}$  treatments compared to the untreated control (Figure 3).

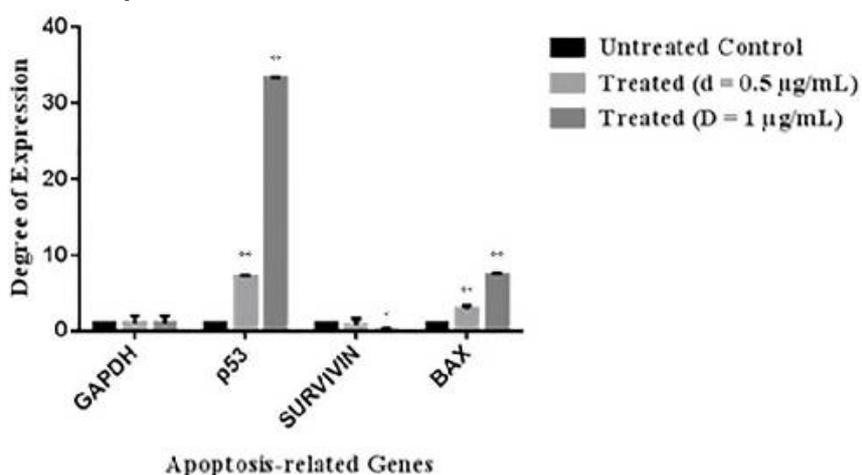


**Figure 3:** Electropherogram showing induction of high-molecular-weight DNA fragmentation in response to drug exposure: 1 – Untreated control; 2, 3, 4 – Liposomal gedunin (1, 2, and 4  $\mu\text{g}/\text{mL}$ ); 5 – Paclitaxel (4  $\mu\text{g}/\text{mL}$ ); 6 – 100bp DNA ladder.

## Gene expressions of *p53*, *Bax* and *Survivin*

The data presented in Figure 4 indicate that liposomal gedunin significantly up-regulated the expression of the genes *p53* and *Bax* ( $p < 0.0001$ ). However, a slight down regulation of the expression of the anti-apoptotic gene, *survivin* was observed in liposomal gedunin treated cells.

## Morphological changes observed under fluorescence microscope

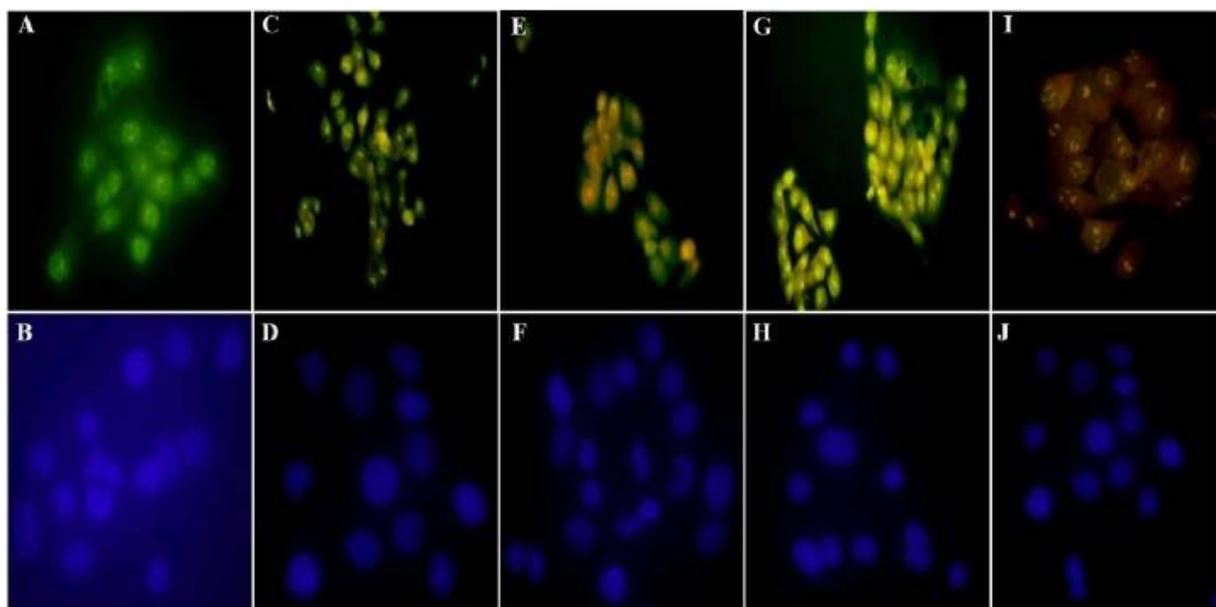


**Figure 4:** Liposomal gedunin-induced changes in expression of apoptosis-related genes (*p53* and *Bax*;  $**p < 0.0001$ ); *Survivin* ( $*p > 0.05$ )] after basal normalization with a reference gene (*GAPDH*); an arbitrary figure of one (1) was allotted to all control samples as a calibrator;  $p < 0.05$  indicates significant difference among treatments

Morphological changes of the cells treated with liposomal gedunin were observed with the help of a fluorescence microscope after staining with Hoechst 33342 and acridine orange/ethidium bromide (AO/EB) (Figure 5). Viable control cells containing normal nuclei reflected bright green fluorescence, while apoptotic cells that have lost their membrane integrity exhibited different chromophorous (orange to red staining) characteristics (cells stained with AO/EB). Granulations in the nucleus were observed in cells treated with liposomal gedunin, whereas no granulations were observed in untreated controls.

## DISCUSSION

In this study, we have demonstrated the enhanced *in vitro* anti-cancer activity of improved gedunin by liposomal nano-encapsulation in non-small-cell lung cancer (NCI-H292) cell line. When considering anti-proliferative effects of liposomal gedunin and free gedunin, liposomal gedunin showed greater anti-proliferative effects in NCI-H292 cells than to free gedunin. Moreover, liposomal gedunin is less cytotoxic to normal lung fibroblast cells (MRC-5) than the positive control (paclitaxel). Drugs with  $\text{IC}_{50}$  values as low as 4 or 10  $\mu\text{M}$  have been identified as highly potent cytotoxic agents [14-16]. Thus liposome-encapsulated gedunin fits into this class of anticancer drug leads and may therefore, be considered a potent formulation. Furthermore, liposomal gedunin with comparatively less cytotoxicity to the MRC-5 cells than the positive control, paclitaxel, highlights the possible use of liposomal gedunin as a more potent drug lead for the treatment of lung cancer.



**Figure 5:** Apoptotic changes in NCI-H292 cells following 24 h drug treatments, as observed under a fluorescence microscope: A = Control 1 (AO/EB); B = Control 2 (H); C = Gedunin (12.5 µg/mL, AO/EB); D = Gedunin (12.5 µg/mL, H); E = Gedunin (25 µg/mL, AO/EB); F = Gedunin (25 µg/mL, H); G = Liposomal gedunin (1 µg/mL, AO/EB); H = Liposome (1 µg/mL, H); I = Liposomal gedunin (4 µg/mL, AO/EB); J = Liposomal gedunin (4 µg/mL, H) (Note: AO/EB- stained with acridine orange/ethidium bromide and H-stained with Hoechst 33342)

Apoptosis is important in homeostasis of organs and tissues. Deregulation of apoptosis is one of the hallmark features of cancer [17]. Apoptosis involves activation of proteases, known as caspases (via extrinsic or intrinsic pathway), shrinkage of cells, fragmentation of DNA, chromatin condensation and formation of apoptotic bodies, etc. Genes *Bax* and *p53* play an important role in apoptosis. *Bax* and *p53* are highly expressed in apoptotic cells [17]. Moreover, *p53*, regulates the anti-apoptotic gene, survivin.

In the present study, apoptosis detection assays and apoptotic related gene expression analysis indicate induction of apoptosis in liposomal gedunin treated NCI-H292 cells. The fluorescence microscopic images after 24 h treatment further indicated that liposomal gedunin caused death of NCI-H292 cells mainly by apoptosis. The significant increase of *Bax* and caspase 3 and 7 activity in liposomal gedunin treated cells suggest the involvement of the intrinsic mitochondrial pathway of apoptosis. It has been reported that *Bax* induces the release of complex proteins such as cytochrome C, and calcium ions ( $\text{Ca}^{2+}$ ), from the mitochondria into the cytosol, activating the intrinsic pathway of *p53*-mediated apoptosis [18,19]. Significant up-regulation (33-fold) of *p53* could be linked to an alternative pathway of apoptosis induction or other DNA damage pathways [20].

Induction of apoptosis was not sufficiently

reflected by DNA fragmentation pattern obtained in the present study. Certain cells have also been reported to undergo apoptosis without degradation of the nuclear DNA implicitly due to the activities of inhibitor of caspase-activated DNase [21]. DNA fragmentation is a hallmark of late apoptosis which is highly dependent on the inducing agent, endonuclease class and tissue-specificity [21].

The physico-chemical characteristics of nanoparticles hold crucial implications for their capacity as drug delivery vehicles, their fate and toxicity [5-6]. As shown in Table 1 the encapsulation efficiency of liposomes (86.82%) was as high as those produced by other researchers [22], but the ones used in the present study were of larger size distribution (394 nm), and had an anionic zeta potential (-54) in contrast to their cationic liposomes. Unlike anionic liposomes, cationic liposomes cause induction of cellular stress and change in gene expression [22]. Most of the phosphatidylcholines usually used for liposomal drug delivery have been proven as non-toxic [5,6]. Particle size is instrumental to enhanced permeation and retention (EPR) effect, cellular internalization, sub-cellular trafficking, degradation and clearance. A size range of  $\leq 100$  nm is rapidly and efficiently internalized [23]. Although liposomal nano particles prepared in the present study were not in the standard nanoparticle size range, other researchers have been reported an effective chitosan nanoparticles within a size

range of 150 – 350 nm which is almost similar to the size of the nano particles used in the present study [24,25]. This observation underscores the need for implementing metrology standards related to nanoparticle suspensions in order to ensure uniformity and reproducibility. Collectively, the enhanced cytotoxicity of the liposomal formulation is a function of successful gedunin encapsulation and intensified uptake into the cells after treatment, thus validating the quality of the nano-formulation and the preparation method for physical entrapment of drugs.

## CONCLUSION

Enhanced anti-cancer activity of an improved gedunin is achieved by liposomal nanoencapsulation. This is the first documented evidence to demonstrate that nano-encapsulated (liposomal) gedunin enhances anti-proliferative effects against human non-small-cell lung cancer (NCI-H292) cells through p53-initiated, Bax-associated, caspase-dependent activation of apoptosis.

## DECLARATIONS

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### Conflict of Interest

No conflict of interest associated with this work.

### Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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