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Folate Anchored Nanostructured Lipid Carrier for Lung A549 Adenocarcinoma Cell Targeting

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ABSTRACT

Background: Nanomedicine faces significant challenges in targeted drug delivery using nanocarriers. The background of this approach lies in addressing the challenges of conventional chemotherapy, such as systemic toxicity, poor bioavailability, and drug resistance. Nanostructured lipid carriers (NLCs) are advantageous due to their biocompatibility, ability to encapsulate both hydrophilic and lipophilic drugs, and controlled drug release properties. Folate conjugation further enhances the specificity of these carriers, making them a promising tool for lung cancer treatment.

Objective: A newly developed folate-anchored nanostructured lipid carrier (F-NLCs) with pegylated polymer shows potential in enhancing targeting efficiency toward cancer cells and minimizing drug-related side effects.

Method: This study evaluates the impact of F-NLCs on A549 lung adenocarcinoma cells using the SRB assay method. Paclitaxel, a widely-used anticancer agent, was incorporated into F-NLCs via the ethanol injection technique.

Result: The formulations were characterized for particle size and zeta potential, and their cancer targeting potential was assessed on A549 cells. F-NLCs displayed a particle size of 231.11±2.3 nm and a zeta potential of 10.2±0.3 mV. These carriers exhibited the highest cell growth inhibition rates compared to P-NLCs formulations, with a dose-dependent inhibition observed in A549 cells. Increased drug concentration in cancer cells was noted, likely due to receptor-specific targeting facilitated by folate conjugation.

Conclusion: F-NLCs represent a stable, secure, and potentially effective drug delivery system for lung cancer targeting, showcasing enhanced uptake and inhibition of cancer cells.

Keywords: *Lung cancer, Nanomedicine, A549, paclitaxel, nanostructured lipid carriers, adenocarcinoma cell*

1. INTRODUCTION

Lung cancer is the leading cause of death from malignant neoplastic diseases, accounting for 1.3 million deaths globally each year [1]. It is the most common in men and the second most common in women. The primary treatment options for lung cancer are surgery, chemotherapy, and radiation therapy [2]. Chemotherapy, which is typically administered intravenously for systemic circulation, is the first-line treatment for advanced stages of malignant lung disease. However, the overall survival rate over the past five years is only about 17%, highlighting the need for more effective and innovative treatment strategies [3].

Current chemotherapy treatments for lung cancer are not efficient as they also kill normal cells along with cancer cells, leading to severe side effects. Therefore, there is a need for site-specific and targeted delivery of anticancer drugs to improve bioavailability and reduce side effects. The use of a nanoparticle system for site-specific targeting of anticancer drugs is a promising approach in nanotechnology. This system enhances bioavailability and reduces the harmful side effects associated with anticancer drugs [4]. Recent research efforts have focused on using nanostructured lipid carriers (NLCs) as carriers to target lung cancer sites. These optimized nano-formulations offer several advantages over conventional drug formulations, including improved drug solubility, facilitated drug delivery across lipid membranes, selective targeting, and reduced drug-related side effects. NLCs, which are prepared from lipids that remain solid at room temperature, have shown better stability as the solid lipid core prevents the particle from losing its properties [5]. Nanostructured lipid carriers (NLCs) have shown promise as a delivery system for lipophilic drugs. Their advantages include improved drug encapsulation, increased stability, and enhanced absorption by cells, leading to more effective drug delivery to targeted tissues and cells [6].

Paclitaxel (PTX), a compound that inhibits cell division, is commonly used in the management of various cancers such as those of the lung, ovary, breast, head, neck, and advanced Kaposi's sarcoma [7]**.** Paclitaxel promotes the formation of tubulins and lends stability to microtubules against depolymerization [8]. Active targeting can enable nanoparticles anchored with ligands to specifically recognize and deliver drugs to the intended cells, thereby minimizing drug distribution to normal cells and reducing side effects [9]. Folate receptors (FR) are found in abundance on the surface of many cancer cells, up to 100 times more than on healthy cells and tissues [10]. They are rarely present on the surface of normal cells or are located on the apical surfaces of polarized epithelial cells. Certain cancers, including those of the pancreas, testicles, bladder, prostate, and liver, reportedly have low levels of folate receptors [11]. The folate receptor, a glycosylphosphatidyl-inositol-anchored cell surface receptor, is an ideal target due to its low expression in healthy tissues and high expression in cancer cells [12]. It facilitates receptor-mediated endocytosis through a carrier protein known as the reduced folate carrier. The combination of pegylation and folate could enhance the effectiveness of drug delivery by promoting the uptake of nanoparticles by tumor cells that express folate receptors [13]. This can also positively alter the surface hydrophobicity of particles, stabilize them in a sterile state, and prevent the binding of serum proteins (e.g., apoproteins) and other opsonic factors.

Folate-anchored nanostructured lipid carriers (F-NLCs) demonstrate potential for targeted delivery, particularly to carcinoma cells, and exhibit good biocompatibility, making them an attractive choice as a carrier and targeting vehicle [14-16]. In this research, an *ex-vivo* evaluation of the cytotoxicity of Folate-anchored Nanostructured Lipid Carriers (F-NLCs) was performed on a Lung Adenocarcinoma A549 cell line. This evaluation was conducted using an SRB assay with cell uptake study. This comprehensive approach allowed for a thorough investigation of the potential applications of F-NLCs in targeted cancer therapy. This paper presents the findings of the cell uptake and *ex-vivo* study conducted on A549 cell line using SRB assay. The schematic representation was shown in Figure 1.

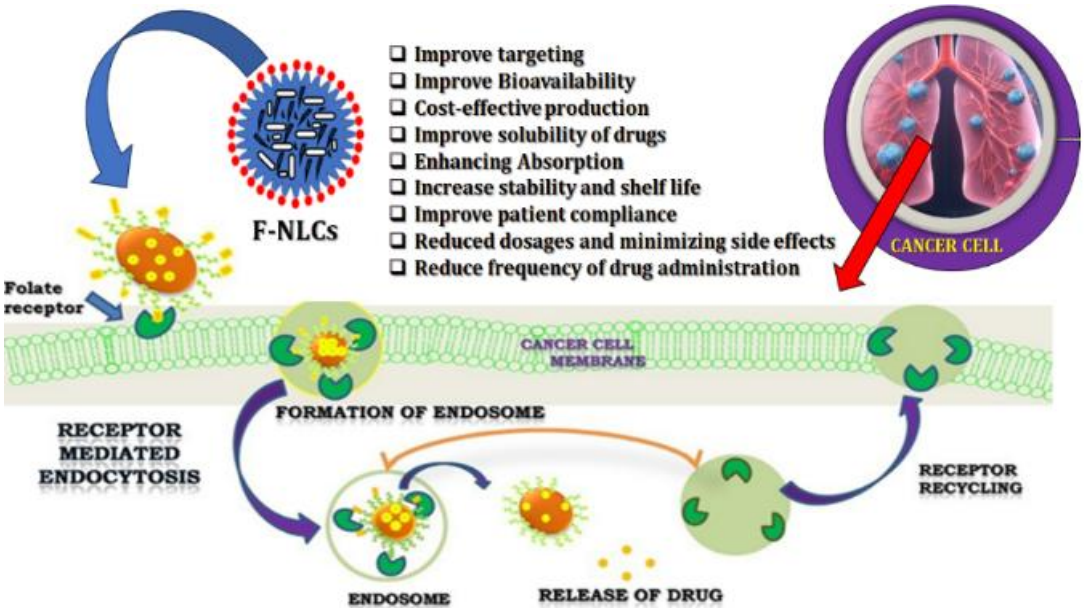


Figure 1: Schematic representation of F-NLCs for receptor mediated targeting to cancer cells

2. MATERIALS AND METHODS

The drug Paclitaxel was gift sample from Neon Laboratory Industries Ltd, Maharashtra (Mumbai), India. FA-PEG-SA was procured as gift sample from lipoid Germany. Glyceryl monostearate (GM)and oleic acid was procured from Hi-media Laboratories Pvt. Ltd, Mumbai. Polaxamer-188 and ethanol were procured from Central drug house Pvt. Ltd. Mumbai**.**

Preparation folate anchored Nanostructured Lipid Carriers (F-NLCs)

The Nanostructured lipid carriers (F-NLCs) were formulated using hot homogenization, utilising ethanol injection method with little modifications [16, 17]. Briefly, Glyceryl monostearate (GM), Oleic acid and FA-PEG-SA were dissolved in ethanol: acetone (7:3 v/v) at a concentration of 10 mg/ml, after this paclitaxel (10 mg) was added and shake for 10 min. for complete homogenization and injected into 20 ml of aqueous phase contain 0.5% w/v of Polaxamer-188 as surfactant solution and stirrer at 3000 rpm for 45 min. Both phases were pre warmed to and kept at 60˚C during the mixing. The liquid suspension was prepared, then further sonicate for 5 min using a probe sonicator to form F-NLCs. The F-NLCs were then concentrated by centrifugation at 5000 rpm for 10 minutes and re-suspended in phosphate buffer saline (pH 7.4). The P-NLCs prepare by using PEG-SA polymer.

Characterization

Particle size, Zeta-potential, and Size distribution

The polydispersity index (PDI) and particle size of P-NLCs and F-NLCs were measured using the NanoPlus AT zeta analyzer, after dispersing the nanocarriers in phosphate-buffered saline (pH 7.4). Additionally, the zeta potential of F-NLCs was evaluated by dispersing them in deionized sterile water at 25°C [16, 18]. All measurements were recorded in triplicate (n=3).

Transmission Electron Microscopy

P-NLCs and F-NLCs were examined using a transmission electron microscope (FEI Tecnai G2 F-20 S-Twin, Netherlands). A drop of the formulation was placed onto a carbon-coated copper grid, forming a thin film, and was negatively stained with 1% phosphotungstic acid (PTA) before drying. The grid was then air-dried at room temperature, after which the formulations were observed under the microscope, and photomicrographs were captured at appropriate magnifications [16, 19].

Percent entrapment efficiency

The paclitaxel content in P-NLCs and F-NLCs was quantified using UV-Visible spectrophotometric methods [16, 20] (Shimadzu 1900, Kyoto, Japan). Both formulations were centrifuged at 1000×g and 4°C for 30 minutes using a HITACHI Ultracentrifuge. The supernatant was then filtered through a membrane filter with a 0.45 µm pore size and subsequently analysed using a UV instrument to determine the entrapment efficiency of paclitaxel. The percentage entrapment efficiency was calculated using the following equation

$$\textbf{Percent Entrapment Efficiency} = \frac{\textbf{Concentration of total drug} - \textbf{Concentration of free drug}}{\textbf{Concentration of total drug}} \times 100$$

Drug Loading

The drug entrapment efficiency of paclitaxel in P-NLCs and F-NLCs was assessed using a Sephadex mini column [16, 21]. To prepare the Sephadex mini column, 1 g of Sephadex G-50 was first allowed to swell in 0.9% NaCl aqueous solution for 8 hours. The hydrated gel was then loaded into the barrel of a 2 ml disposable syringe plugged with a filter pad. The barrel was centrifuged at 2000 rpm for 2 minutes to remove excess saline solution, forming the Sephadex separating column. To isolate the free drug from the NLC nanocarrier, 5 ml of NLC dispersion was carefully applied dropwise onto the top of the Sephadex column and centrifuged again at 2000 rpm for 2 minutes, allowing the void volume containing NLCs to be expelled into centrifuge tubes [22]. The eluted NLC dispersion was collected and lysed by disruption with 5% Triton-X100. The amount of entrapped drug was then analyzed using a UV-Visible spectrophotometric method [31].

***In-vitro* drug release**

The in-vitro release of paclitaxel from P-NLCs and F-NLCs was evaluated in four different pH environments (PBS pH 4.0, 6.8, 7.4, and 8.0) using the dialysis bag method [23]. The NLC formulation was first purified by separating it from the free drug using a Sephadex column, followed by centrifugation. The isolated NLC formulation (5 ml) was then transferred into a dialysis tube (molecular weight cutoff: 12,000 Da, Hi Media, India) and immersed in a beaker containing 50 ml of PBS (pH 7.4) [16]. The beaker was placed on a magnetic stirrer, maintaining a temperature of 37±2°C throughout the procedure. At specific time intervals, samples were withdrawn and replaced with an equal volume of phosphate buffer [24, 30]. The drug content was analyzed spectrophotometrically by measuring absorbance at 237.0 nm against a blank using a UV-Visible spectrophotometer (Shimadzu 1900, n=3). The same protocol was followed for three other PBS media (pH 4.0, 6.8, and 8.0).

Stability studies

"Stability testing plays a crucial role in the development of pharmaceutical dosage forms and is widely acknowledged within the pharmaceutical industry [16]. Predicting stability and ensuring the therapeutic efficacy of a dosage form are essential for moral, legal, competitive, and public health reasons. The degradation of active ingredients in pharmaceutical formulations can occur through various mechanisms, including hydrolysis, oxidation, reduction, racemization, cyclic cleavage, decarboxylation, and photolysis [25]. "Stability was assessed based on turbidity, color changes, crystallization, and other visual alterations. Drug leakage was evaluated for P-NLCs and F-NLCs under varying temperature conditions (0–4°C, room temperature 20–30°C, and 60°C) after storage for five weeks in both dark conditions (amber-colored glass vials) and light exposure (colorless vials) [26].

***Ex-vivo* studies**

Ex-vivo studies involve experimentation on living tissue in an artificial environment outside the organism. The developed P-NLCs and F-NLCs formulations were assessed for their storage stability and demonstrated sufficient stability with a desirable drug release profile, making them suitable for further evaluation in an ex-vivo cell line study [16]. "The cell line study was conducted at ACTREC, Tata Cancer Centre, Mumbai, India (Protocol No.: R-811696, 18/11/2024). An in-vitro evaluation of the formulations was performed to assess their targeting capability, cytotoxicity, and cellular absorption against

A549 lung cancer cell lines [27].

***In-vitro* cytotoxicity studies**

In-vitro cytotoxicity studies were conducted using the A549 cell line, a well-established model for non-small cell lung cancer [28], to evaluate the efficiency of paclitaxel based NLCs formulation against lung cancer. The studies were conducted to evaluate the effectiveness of P-NLCs and F-NLCs compared to the standard drug in inhibiting cell growth [29-30]. Analysis was conducted using two different samples, P-NLCs and F-NLCs, among those tested. A positive control, ADR, was included to validate the significance of the cell line for the study. The samples were appropriately diluted to achieve concentrations of 10, 20, 40, and 80 µg/ml [31]. The prepared solutions were introduced into cultured cell lines to evaluate their anti-cancer activity. Cell uptake images of the tested samples were captured using a Ti-S Inverted Research Microscope (Nikon) at ×20 magnification, utilizing Eclipse Image Processing Software (NIS-Elements) at ACTREC, Tata Cancer Centre, Mumbai, India.

Statistical analysis

Statistical analysis was conducted using Graph Pad Instant Software (Version 3.0, Graph Pad Software, California, USA). Either an unpaired t-test or one-way ANOVA, followed by the Tukey–Kramer multiple comparison test, was applied as appropriate. A p-value of less than 0.05 (where applicable) was considered highly significant [32].

3. RESULTS

Particle size, Zeta-potential, and Size distribution

The zeta potential, polydispersity index (PDI), and particle size of P-NLCs and F-NLCs are summarized in Table 1. The particle sizes of P-NLCs and F-NLCs were measured as 190.1±2.3 nm and 231.3±3.7 nm, respectively. The PDI values for F-NLCs and P-NLCs were found to be 0.335 and 0.292, respectively [16]. The data representing particle size, zeta potential, and PDI for the NLC formulations are detailed in Table 1.

Table 1: Particle Size, Zeta-Potential and PDI for NLCs Formulation

S. No.	Formulation Code	Particle Size (nm)	PDI	Zeta Potential (mV)	Percent Drug Loading	Percent Entrapment Efficiency
1.	P-NLCs	190.1±2.3	0.292±0.09	13.9±0.5	15.3.±1.1%	65.14 ±1.4
2.	F-NLCs	231.3±3.7	0.335±0.05	10.2±0.3	14.2±1.3%	59.42± 1.2

Mean±SD, n=3

Transmission Electron Microscopy

TEM studies validated that the developed nanocarrier systems were within the nanometric size range. P-NLCs and F-NLCs were examined under a transmission electron microscope, with photomicrographs captured at suitable magnifications [31]. The images are presented in Figure 2a and 2b [16].

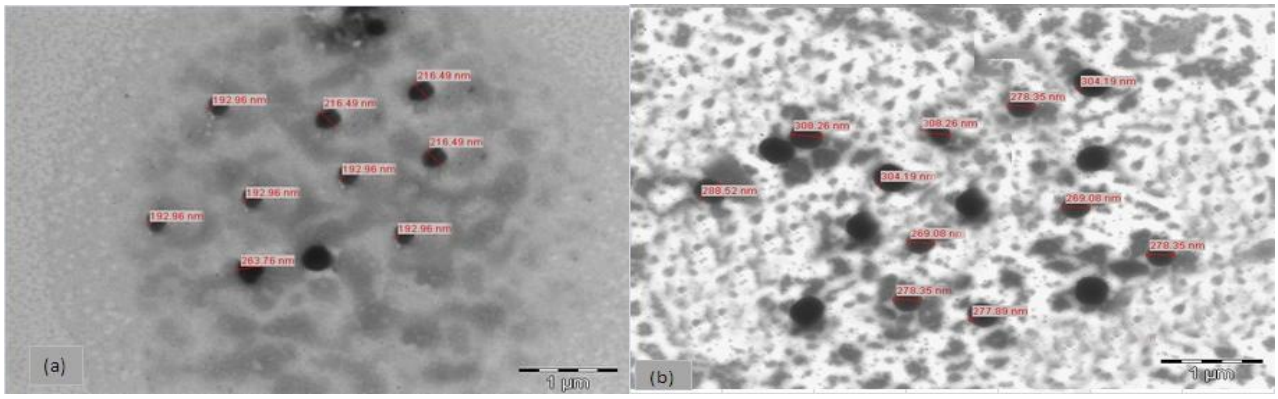


Fig. 2: TEM image of (a) P-NLCs (b) F-NLCs (16)

Scanning Electron Microscopy (SEM)

SEM studies demonstrated that P-NLCs and F-NLCs exhibited a smooth surface and a nearly spherical shape, as illustrated in Figure 3 [16].

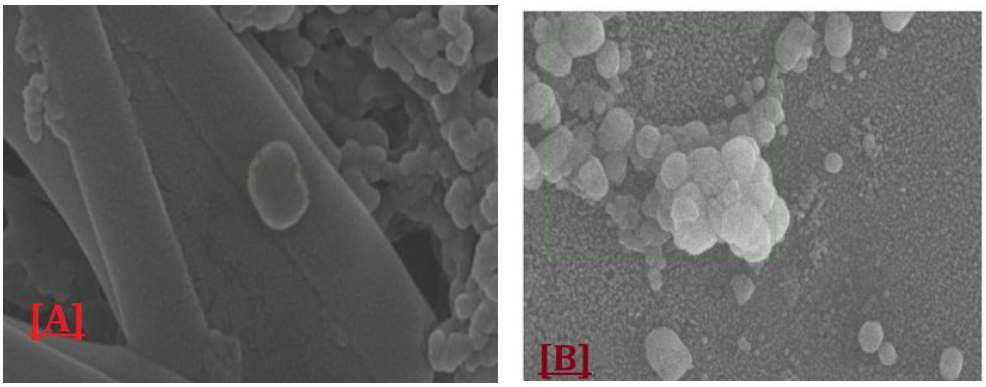


Fig. 3: SEM image of (a) P-NLCs (b) F-NLCs [16]

Percent entrapment efficiency

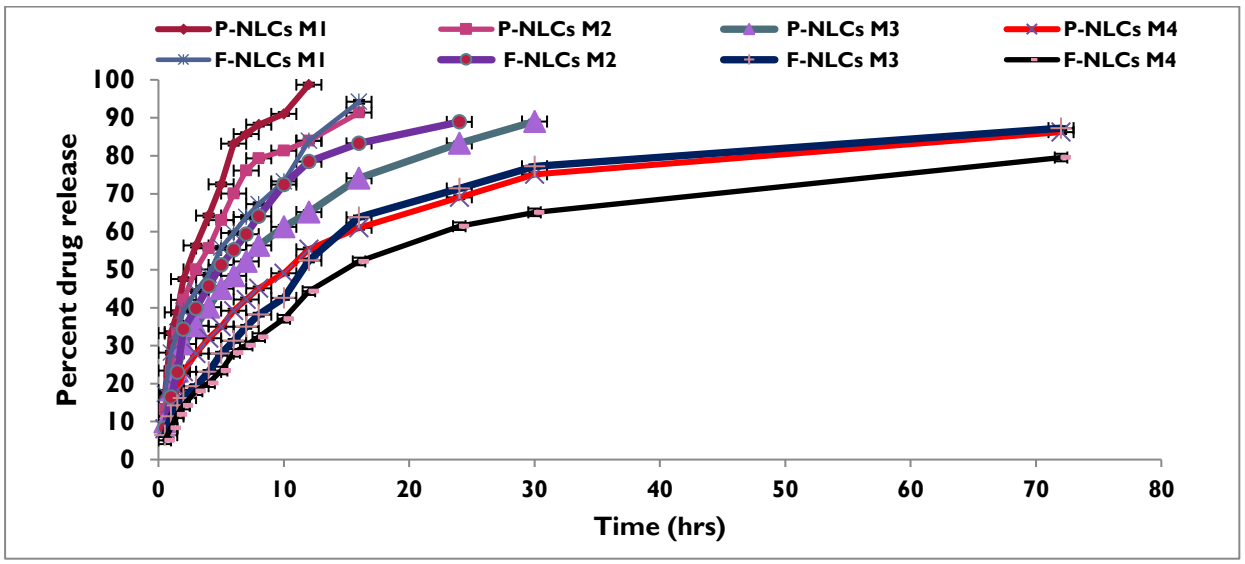
The entrapment efficiency of P-NLCs and F-NLCs was determined to be 65.14±1.4% and 59.42±1.2%, respectively, as summarized in Table 1.

Drug Loading

The drug loading capacity of P-NLCs and F-NLCs was determined to be 15.3±1.1% and 14.2±1.3%, respectively, as presented in Table 1

***In-vitro* drug release**

In-vitro drug release studies were conducted at pH 4 and 6.4, considering the acidic nature of the tumor microenvironment. The rapid drug release behavior of NLC formulations at pH 4 supports the hypothesis that the formulation will release the drug in higher concentrations upon reaching the target site, where the pH is generally below 7. The F-NLC formulation exhibited distinct release patterns across various pH conditions: at pH 4.0, it demonstrated 94.21% drug release within 16 hours; at pH 6.4, 88.92% release within 16 hours; at pH 7.4, 87.28% release within 30 hours; and at pH 8.0, 79.54% release within 72 hours. Additionally, at pH 7.4 and 8.0, the F-NLC formulation showed sustained release patterns, with approximately 65.78% and 52.18% drug release within 16 hours, respectively [16]. The release patterns in different pH media for the NLC formulations are depicted in Figure 4.



M1: Phosphate Buffer Saline pH 4.0; M2: Phosphate Buffer Saline pH 6.4; M3: Phosphate Buffer Saline pH 7.4 and M4: Phosphate Buffer Saline pH 8.0. Values represent mean ± SD (n=3): P-NLCs: Paclitaxel loaded Nano-lipid Carrier; F-NLCs Paclitaxel loaded folate conjugated Nano-lipid Carrier0

Fig. 4. Drug release of NLCs formulation on different pH Environment (pH 4, 6.4, 7.4 and 8.0). [16]

Stability study

Stability was assessed based on turbidity, color change, crystallization, and other visual alterations. Drug leakage was measured for P-NLCs and F-NLCs under various temperature conditions (0–4°C, room temperature 20–30°C, and 60°C) after storage for five weeks in both dark conditions (amber-colored glass vials) and light exposure (colorless vials). Leakage was negligible at 0°C. After five weeks, P-NLCs exhibited the highest drug leakage at 60°C, with values of 13.8% in darkness and 35.8% in light, compared to 3.5% and 3.4% (in dark) and 5.5% and 4.9% (in light) at 0–4°C and 20–30°C, respectively. Similarly, F-NLCs showed the highest drug leakage at 60°C, with values of 8.4% in darkness and 20.7% in light, compared to 2.1% and 1.0% (in dark) and 4.0% and 2.4% (in light) at 0–4°C and 20–30°C, respectively. Based on these results, folate-conjugated NLCs formulation exhibited greater stability in dark conditions at room temperature compared to storage at 0–4°C and 60°C

Cancer Cell Inhibition Assay

A549 human adenocarcinoma cells were selected for analysis due to their extensive use in previous studies. The inhibition of cell growth was observed to be higher with F-NLCs compared to P-NLCs (Table 2, Figure 5). Among the tested formulations, F-NLCs exhibited the highest percentage of cell growth inhibition, surpassing Paclitaxel solution (PS). A549 cells were treated with four equivalent doses ranging from 10 to 80 µg/ml. The cellular inhibition test demonstrated significant differences (Table 2), with F-NLC formulations showing the greatest inhibitory effect. F-NLCs exhibited a higher percentage of cell growth inhibition compared to P-NLCs (Figure 5), with all formulations displaying dose-dependent inhibition of A549 carcinoma cells. The increased uptake observed in F-NLCs was likely due to receptor-specific targeting enabled by folic acid surface conjugation. These findings indicate that F-NLCs exhibited a higher level of cell inhibition compared to P-NLCs, closely resembling the positive control (Adriamycin). At 80 µg/ml, P-NLCs showed 29.4% cell growth inhibition, while F-NLCs demonstrated 23.7% inhibition at the same concentration. Notably, Adriamycin exhibited a comparable growth inhibition effect to F-NLCs at 80 µg/ml

Table 2: *In-vitro* cytotoxicity of Paclitaxel, Adriamycin, P-NLCs and F-NLCsinA549cell line

Concentration (µg/ml)	Percent Cell Growth Inhibition (SRB Assay)		
	P-NLCs	Adriamycin	F-NLCs
10	20.0	7.6	9.2
20	20.8	8.0	11.2
40	21.8	11.9	14.5
80	29.4	23.7	23.7

Values represent mean ± SD (n=3); P-NLCs: Paclitaxel loaded nanostructured lipid carriers; F-NLCs- Folate anchored nanostructured lipid carriers

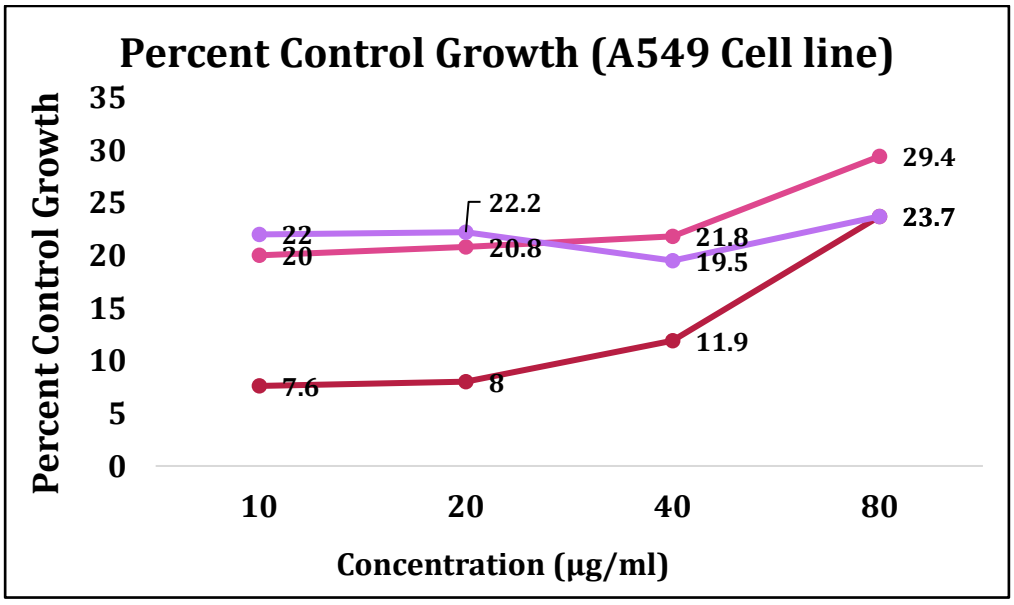


Figure 5: Cell growth inhibition assay in A549 cells treated with Paclitaxel, P-NLCs and F-NLCs

Cell Uptake Assay

A cell uptake study was conducted to evaluate the ability of different Paclitaxel-loaded formulations to target A549 carcinoma cells (Figures 6c and 6d). The higher uptake of F-NLCs was likely due to the presence of folate residues on the surface of the NLCs (Figure 6d), compared to P-NLCs (Figure 6c). Figure 6a represents A549 control cells, while Figure 6b illustrates A549 cells treated with Adriamycin as a positive control. The results further support the strategy that ligand-assisted delivery of anti-cancer drugs enhances drug uptake in A549 cancer cells.

Additionally, a fluorescence-based cell uptake study was performed to assess the targeting efficiency of P-NLCs, F-NLCs, and Adriamycin as a positive control (Figures 6a–6d). The fluorescence study demonstrated a higher uptake of F-NLCs (Figure 6d) compared to the control group (Figure 6a). The increased uptake of F-NLCs, relative to P-NLCs (Figure 6c) and A549 control cells (Figure 6a), was likely due to receptor-specific targeting enabled by folic acid conjugation. Notably, F-NLCs (Figure 6d) exhibited results similar to those of A549 cells treated with Adriamycin (Figure 6b). Among the tested formulations, F-NLCs displayed superior uptake efficiency compared to P-NLCs (Figure 6c), further supporting its potential for targeted cancer cell delivery.

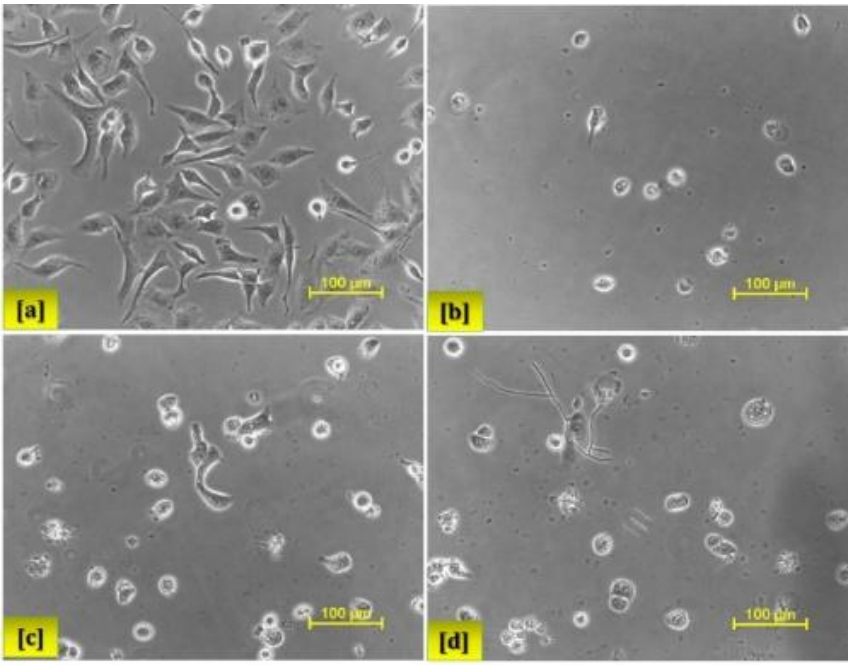


Fig. 6: Cell uptake images of [a] A549 control (20X) [b] Positive control adriamycin (20X)[c] P-NLCs (20X) [d] F-NLCs (20X) after 48 hrs.

4. DISCUSSION

The Nanostructured lipid carriers (F-NLCs and P-NLCs) were formulated using hot homogenization, utilising ethanol injection method. The average P-NLCs size recorded for different formulation was in a range of 182 to 200 nm. The average F-NLCs size recorded for different formulation was in a range of 220 to 240 nm [16]. It was evident from TEM studies that the developed nanocarrier systems were of nanometric size range. In-vitro drug release behaviours indicate that F-NLCs perform better at lower pH ranges (4 and 6.4), which are more acidic environments. This result supports the hypothesis that the F-NLC formulation releases the drug in higher concentrations in a shorter time when it reaches tumour sites, where the pH is always below 7. This sustained release is attributed to the surface engineering of folic acid, which led to more sealing at the nanoparticle periphery and hydrophobic interactions that delayed drug release. Stability data indicates that temperature-dependent drug leakage has occurred in almost every formulation. Overall, all formulations were found to be more stable at ambient temperature in darkness. In general, drug leakage was higher in light than in darkness, which can be attributed to temperature and light-induced structural cleavage. The smaller amount of drug was leached out form NLCs formulation at room temperature as compared at 0-4ºC and 60±2ºC. At 0ºC shrinkage of the polymer architecture may be the possible reason leading to the reduction of the cavity enclosing the drug molecules and thus a higher leakage. However, the greatest leak at 60ºC may be due to the higher kinetics of the solution. The reason for the lesser drug leakage from F-NLCs formulations might be ascribed to surface conjugation of folate which imparts rigidity to the NLCs. Cell uptake study suggested that administration of anti-cancer drugs via ligand can increase drug uptake in A549cancer cells. The results again support the strategy that F-NLCs entered into the cancer cell by receptor-mediated endocytosis and ligand-mediated NLCs can provide higher uptake of the drug to the A549carcinoma cell line.

5. CONCLUSION

Paclitaxel-loaded folate-anchored NLCs (F-NLCs) were successfully developed, leveraging folate receptors as a unique platform for targeted drug delivery through multiple ligand attachments. This system has the potential to enhance therapeutic efficacy by enabling dose reduction and improving biocompatibility [31]. Drug delivery via F-NLCs demonstrated a 1.25-fold increase in lung carcinoma treatment efficacy compared to a plain drug solution. These findings highlight F-NLCs as promising carriers for lung-targeted drug delivery, laying the foundation for optimizing anticancer therapy.

CONFLICT OF INTEREST

The authors report no declaration of interest.

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LIST OF ABBREVIATION

F-NLCs- folate-anchored nanostructured lipid carrier

P-NLCs- paclitaxel loaded nanostructured lipid carrier

PTX- Paclitaxel

NLCs- Nanostructured lipid carriers

FR- Folate receptors

GM-Glyceryl monostearate

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