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## Evaluation of Anticancer Activity of Nanoparticles of Erlotinib on A-549 Cell Line Induced Lung Cancer Model of Mice



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### ABSTRACT

Lung cancer is by far one of the malignant tumors with the highest incidence and mortality all over the world. It kills more people's yearly than any other neoplastic process. Mortality rates have changed little over the past several decades, despite improvements in surgical techniques, radiation therapy and chemotherapy. Lung cancer can be broadly classified into two categories small cell lung cancer and non-small cell lung cancer. A number of factors are responsible for lung cancer induction and include smoking, family history, exposure to asbestos, age etc. The aim of the present study was to develop a model which mimic the lung carcinogenesis and to evaluate the anticancer activity of nanoparticles of Erlotinib. Nanoparticles of Erlotinib were prepared by ion gelation method and their characterization was carried out by particle size determination, zeta potential measurement, TEM analysis and release study. Methodology includes the inoculation of A549 cell line into pleural cavity of mice. Erlotinib (50 mg/kg) was used as a standard. After 14<sup>th</sup> day, therapy was started with test drug nanoparticles and standard drug Erlotinib by giving them from oral route. Efficacy of prepared nanoparticles was then compared with standard therapy. Body weight changes, hematological parameters (like RBC, WBC and Hb), histopathology and immunoassays (TNF- $\alpha$ , VEGF and IL-6) were carried out to investigate the carcinogenesis.



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## INTRODUCTION:

Lung cancer is the uncontrolled growth of abnormal cells in one or both lungs. These abnormal cells do not carry out the functions of normal lung cells and do not develop into healthy lung tissue. Lung cancer is one of the leading causes of cancer mortality in developed and developing countries, and responsible for 25% of death due to cancer (1). Lung cancer can be broadly divided into small cell lung cancer and non-small cell lung cancer (NSCLC) with latter constitutes nearly 80% of lung cancer mortality. Presently, conventional treatment modalities such as surgical resection, radiotherapy and chemotherapy are standard treatment regimen for lung cancers. However, surgical removal of cancerous tissues is highly difficult in many cases; radiotherapy damages the normal healthy cells surrounding the cancer cells, and chemotherapy requiring high dose level of individual drugs to treat lung carcinoma (2). Most of the times, chemotherapeutic drugs act on the normal cells causing severe dose-limiting adverse side-effects and remains far from satisfactory (3). Therefore, a strong need to develop a therapeutic approach that can increase the efficiency and minimize the adverse effects continuously persists. To meet these needs, nanoparticle delivery systems present a novel approach for delivering cytotoxic drugs in the treatment of NSCLC, both with higher efficacy and lower toxicity (4).



In the present study, chitosan nanoparticles (CNP) of erlotinib were prepared by ion gelation method. Erlotinib is a reversible epidermal growth factor receptor (EGFR) - tyrosine kinase inhibitor, which has been available for the treatment of patients with NSCLC since 2004 and has been included in National Comprehensive Cancer Network (NCCN) guideline since 2010 as first line treatment option for advanced NSCLC patients who harbor EGFR mutation (5). This mechanism for stopping cancer cells from growing and multiplying is very different from the mechanisms of chemotherapy and hormonal therapy.

Present study aimed at treating lung cancer by administrating the nanoparticles of drug by oral route. Additionally, *in vivo* studies have been conducted in A-549 cancer cell bearing mice to systemically evaluate the antitumor efficacy. Body weight, hematological parameters, histopathology and immunoassays were carried out to investigate the carcinogenesis.

## **MATERIALS AND METHODS:**

### **MATERIALS:**

Low molecular weight chitosan derived from crab shell was purchased from TCI (China). Sodium tripolyphosphate (TPP, 85% pure) was procured from Sigma Aldrich. Erlotinib HCl was a kind gift from Cipla Pvt Ltd. Quantization Kit was purchased from Ray Biotech. All other chemicals were of reagent grade and used without any modifications.

### **METHODS:**

#### **PREPARATION OF CHITOSAN LOADED NANOPARTICLES:**

Chitosan nanoparticles (CNP) were synthesized via the ionotropic gelation (6-8) of chitosan with TPP anions. Chitosan was dissolved in acetic aqueous solution at various concentrations (1, 2, 3 mg/mL). The concentration of acetic acid in aqueous solution was 1.5% v/v (9). The TPP solution (1 mg/mL) was prepared by double-distilled water. Chitosan nanoparticles were spontaneously fabricated with the dropwise addition of 2 mL of TPP solution to 5 mL of the chitosan solution under magnetic stirring (1000 rpm, 1 hour) at room temperature. The opalescent suspension was formed under the same above mentioned conditions. The nanoparticles were separated by centrifugation at 20,000 g and 14°C for 30 minutes (Superspin R-V/Fm), freeze-dried and stored at 5±3°C. The weight of freeze-dried nanoparticles was calculated.

Drug loaded nanoparticles were formed by the addition of chitosan solution to 1 mg/mL erlotinib solution (erlotinib dissolved in 0.1% dimethyl sulfoxide and made up with water). Prepared erlotinib/chitosan solutions were flush-mixed with 2 mL of 1 mg/mL TPP. (10)

#### **CHARACTERISATION OF NANOPARTICLES:**

##### **Erlotinib encapsulation efficiency (EE) and loading capacity (LC)**

Encapsulation efficiency (EE) and loading capacity (LC) of erlotinib in the nanoparticles were measured using Spectrophotometric analysis of the filtrates obtained after centrifuging the nanoparticles in centrifugation tubes for 30 min. A double beam UV-VIS spectrophotometer connected to computer was employed with spectral bandwidth of 1 nm and wavelength accuracy of ±0.3 nm with a pair of 10 mm matched quartz cells. For

scanning, the wavelength range selected was 400 nm to 200 nm with medium scanning speed. All experiments were performed at room temperature ( $25\pm 1$ ) °C.

Erlotinib HCl stock solution was prepared by dissolving 25 mg of the drug in methanol in 25 mL volumetric flask (1000 µg/mL) and dilutions were made from the stock solution with 0.1N hydrochloric acid. The above solutions were scanned (200- 400 nm) against their reagent blank and the absorption spectra were recorded. (11)

Drug EE and LC were calculated from the following equations:

$$\%EE = (A-B)/A \times 100 \text{ and}$$

$$\%LC = (A-B)/C \times 100$$

Where:

A = total amount of erlotinib

B = free drug, and

C = weight of nanoparticles.



#### **Measurement of particle size, polydispersity and $\zeta$ potential:**

Prepared CNPs were analyzed for the particle size distribution and  $\zeta$  potential, by photon correlation spectroscopy (PCS; Zetasizer). Nanoparticles were dispersed in distilled deionized water (pH 7) for these measurements. The Z average and polydispersity index (PDI) were recorded in triplicate. For  $\zeta$ -potential measurement, samples were taken in a universal dip cell (Malvern Instruments) and the  $\zeta$  potential was recorded in triplicate.

#### **Transmission electron microscopy (TEM):**

The morphology of CNPs was identified through a high-resolution transmission electron microscopy (FEI Tecnai S Twin). Briefly, a liquid sample was placed on a carbon-coated copper grid and counterstained with phosphotungstic acid, followed by air drying for 2 hours.

### **Erlotinib nanoparticles release study:**

The release of erlotinib from CNPs was performed in pH 3 in acetate buffer and pH 7.4 in phosphate buffer. The freeze-dried CNPs encapsulating erlotinib were suspended in 0.5 mL of a buffered solution and transferred to a tubular cellulose dialysis membrane secured tightly at both ends.

The cellulose dialysis membrane was then incubated in 50 mL buffered reservoir at 37°C with gentle agitation of 100rpm on magnetic stirrer. The amount of erlotinib released was determined at selected time intervals and analyzed for percentage cumulative drug release using UV (10). Fresh buffer was used to replenish the receptor compartment at each time to maintain sink condition.

### **LUNG CANCER MODEL OF MICE:**

Swiss albino mice of about 8 weeks of age with an average body weight of 35-40 g were used for the experiment. Four groups of 10 mice each were grouped, housed in polyacrylic cages and maintained under standard laboratory conditions (temperature- $25 \pm 2^{\circ}\text{C}$  and relative humidity 55.60% with dark/light cycle i.e. 12/12) and were allowed free access to standard dry pellet diet and water *ad libitum*. The animals were acclimatized to laboratory conditions for 1 week before initiation of the experimental studies. All the described experimental procedures were reviewed and approved by the Institutional Animal Ethics committee.

A total of 30 mice were intraperitoneally injected with pentobarbital sodium (2mg/kg i.p.) to induce anesthesia and fixed in the right lateral decubitus position after anesthesia (12). Then 100  $\mu\text{l}$  A549 single cell suspension ( $1.12 \times 10^5$  ml) prepared with the 1ml injector was percutaneously inoculated into the upper margin of the sixth intercostal rib on the right anterior axillary line to a depth of about 5mm rapidly and after that, the needle was promptly pulled out. Mice were maintained in the right lateral decubitus position after injection and observed until complete recovery (12). All the animals were regularly observed for their general behavior.

The drug therapy was started after 14 days. Group-A received normal saline while Group-B received blank nanoparticles. Group C and D were provided with the drug erlotinib (50mg/kg, oral) in standard dosage form and nanoparticle form. Blood hematology was performed on each group to check the red blood cell (RBC) count, white blood cell (WBC)

count and hemoglobin (Hb) count. After dissection lung tissue samples were given for histopathological examination. Immunoassay was also performed to detect the presence of tumor necrosis factor (TNF- $\alpha$ ), vascular endothelial growth factor (VEGF) and interleukin (IL-6), which usually rises in case of lung cancer.

**Statistical analysis:** The results are expressed as mean  $\pm$  SD. Statistical evaluation was performed using one-way analysis of variance (ANOVA) using Graph pad prism 5.0. P-values (\*p<0.05, \*\*p<0.01, \*\*\*p<0.001) are considered statistically significant.

## RESULTS:

### CHARACTERISATION OF NANOPARTICLES:

#### EE and LC measurement:

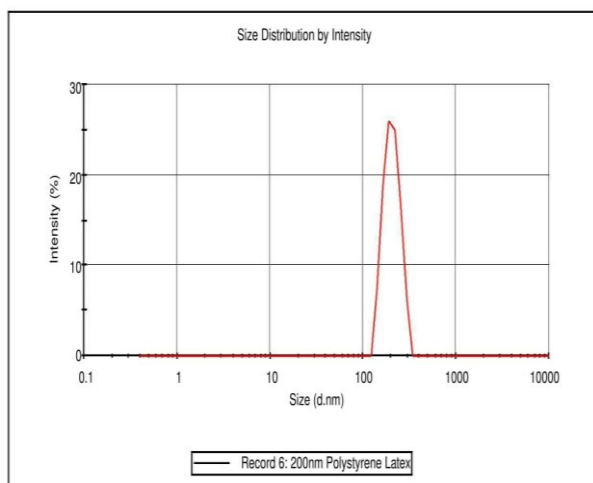
EE of erlotinib was found to be 74% when the concentration of chitosan used was 1mg/ml. A similar trend was observed in the LC as well. The LCs of the nanoparticles was 41%. The percentage yield was calculated to be 62%

#### Measurement of particle size, polydispersity and $\zeta$ potential:

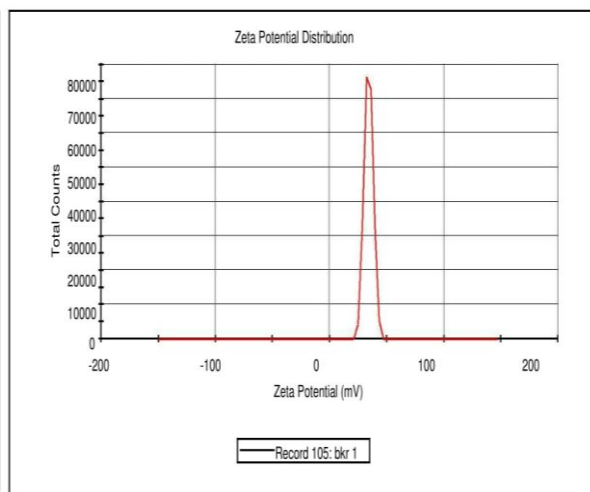
The average size, measured by Zetasizer, of erlotinib, loaded chitosan nanoparticles were approximately 308.47 nm (Fig. 1). The PDI value was 0.359 (Table 1), thus indicating a narrow and favorable particle size distribution (PDI < 0.5). Zeta potential was 35.567 mV, which shows nanoparticles are positively charged (Fig. 2).

**Table 1: Measurement of particle size, polydispersity and  $\zeta$  potential**

| Sr. No.       | Particle Size (nm) | Polydispersity Index (PDI) | Zeta Potential (mV) |
|---------------|--------------------|----------------------------|---------------------|
| 1             | 308.4              | 0.353                      | 34.3                |
| 2             | 310.7              | 0.376                      | 37.8                |
| 3             | 306.3              | 0.347                      | 34.6                |
| Mean $\pm$ SD | 308.47 $\pm$ 2.201 | 0.359 $\pm$ 0.0153         | 35.567 $\pm$ 1.939  |



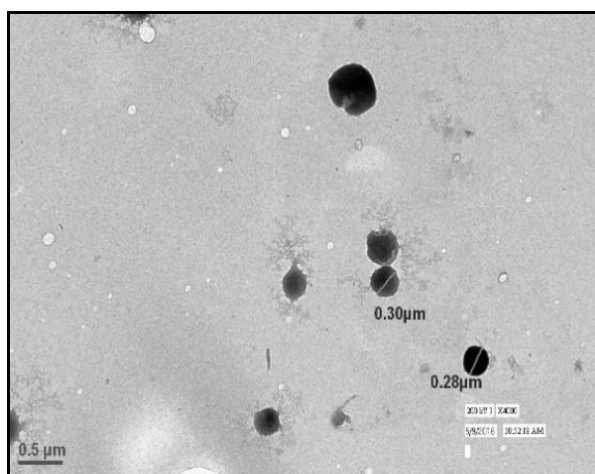
**Fig. 1 Particle size of nanoparticles**



**Fig. 2 Zeta potential of nanoparticles**

### Transmission electron microscopy (TEM)

In the present study, TEM images have shown the morphological properties and surface appearance of nanoparticles. The nanoparticles have nearly spherical shape, smooth surface and size range of about 280-300 nm (Fig. 3).



**Fig. 3 TEM image of nanoparticles**

### Erlotinib nanoparticles release study:

Our observations showed that about 61% of the loaded erlotinib was released within 24 hours of incubation in acetate buffer at pH 3. The release profile of erlotinib loaded nanoparticles exhibits an initial burst release of about 51% in the first 8 hours followed by a slow release of 10% for the subsequent 16 hours (Fig. 4).

The release study was also conducted using phosphate buffer (pH-7.4) for nanoparticles. But there was no release of erlotinib, in first 8 hours of the study.

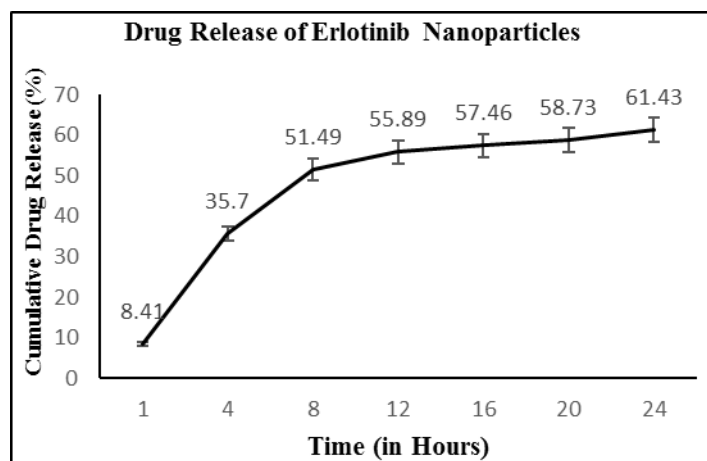


Fig. 4 Release study of nanoparticles

**ANIMAL STUDY:**

**Body weight changes:**

Mean body weight of mice continually declined following intrathoracic inoculation of tumor cells (Fig. 5). At around two weeks following inoculation of tumor cells, tumor bearing mice developed similar cachectic symptoms as observed in clinical patients with tumors and symptoms of labored mouth breathing could be observed in dying mice. (13)

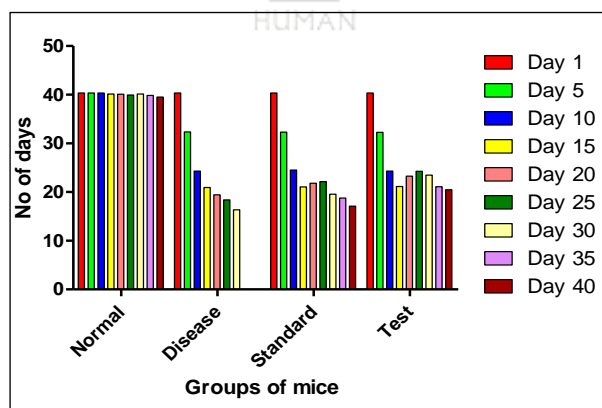


Fig. 5 Body weight changes in mice (Values are mean ± SD, n=8)

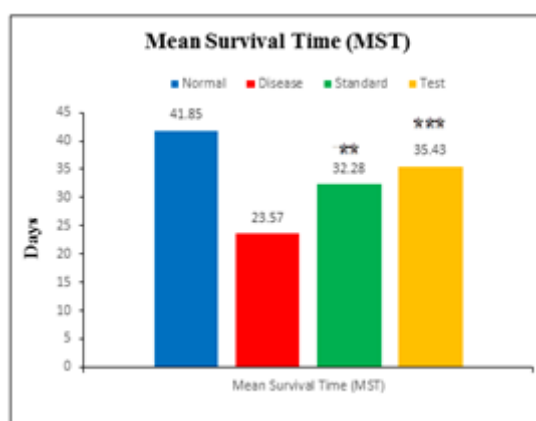
**Mean survival time of mice:**

The mean survival time was calculated for all the four groups of mice and then percentage increase in life span was calculated for standard and test treated group. The MST was significantly increased in case of standard ( $p < 0.01$ ) and nanoparticles ( $p < 0.001$ ) treated group when compared with diseased group (Fig.6). The percentage increase in life span was higher in test group (41.83%) as compared to standard group (36.95%) (Table 2).



**Table 2: Mean survival time of mice (Values are mean ± SD, n=8,\*p<0.05, \*\*p<0.01, \*\*\*p<0.001.)**

| Group    | Mean Survival Time (MST) | Percentage Increase in Life Span (%ILS) |
|----------|--------------------------|---|
| Normal   | 41.85 ± 3.02             | -                                       |
| Disease  | 23.57 ± 3.8              | -                                       |
| Standard | 32.28 ± 4.84**           | 36.95%                                  |
| Test     | 35.43 ± 4.54***          | 41.83%                                  |



**Fig. 6 Mean survival time of mice**

### BLOOD HEMATOLOGY

The RBC and Hb count in disease control group was decreased as compared to normal group. Treatment with standard drug showed increase in RBC and Hb count when compared with the disease group. Treatment with test nanoparticles shown significant ( $p<0.001$ ) increase in RBC count when compared with the standard group (Table 3).

The WBC count of diseased group shows significant ( $p<0.05$ ) rise in their count which is indicative of cancer, while the standard treatment group shows significant ( $p<0.05$ ) decrease in WBC count. Treatment with test nanoparticles also shows significant ( $p<0.001$ ) decrease in WBC count as comparable to normal when compared with standard treated group (Table 3).

**Table 3 RBC, Hb and WBC profile of mice (Values are mean ± SD, n=8,\*p<0.05, \*\*p<0.01, \*\*\*p<0.001.)**

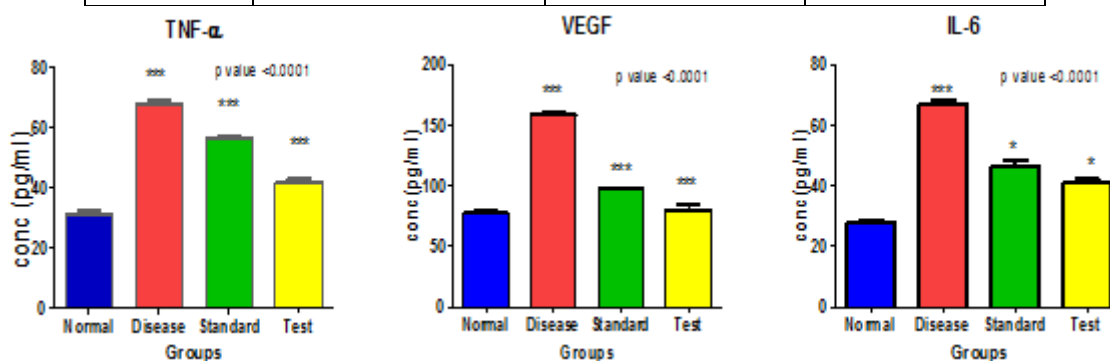
| Groups   | RBC x 10 <sup>6</sup> /mm <sup>3</sup> | WBC x 10 <sup>3</sup> /mm <sup>3</sup> | Hb (g/dl)     |
|----------|--|--|---------------|
| Normal   | 11.58 ± 0.19                           | 10.73 ± 0.24                           | 14.46 ± 0.26  |
| Disease  | 5.17 ± 0.06                            | 18.65 ± 0.28                           | 6.05 ± 0.07   |
| Standard | 6.5 ± 0.05                             | 5.45 ± 0.07*                           | 8.1 ± 0.28**  |
| Test     | 8.61 ± 0.028***                        | 7.6 ± 0.42***                          | 9.4 ± 0.42*** |

### IMMUNOASSAYS

The TNF- $\alpha$ , VEGF and IL-6 levels in Disease Control group were significantly higher (p<0.001) compared to Normal (Table-4), whereas the Standard and Nanoparticles treated mice showed mean TNF- $\alpha$ , VEGF and IL-6 levels lower than Disease Control but remain higher than Normal (\*\*\*p<0.0001) (Fig.7).

**Table 4 Effect of standard and its Nanoparticles on TNF- $\alpha$ , VEGF and IL-6 levels evaluated by ELISA. Each value represents Mean± S. D. (\*\*\*p<0.0001)**

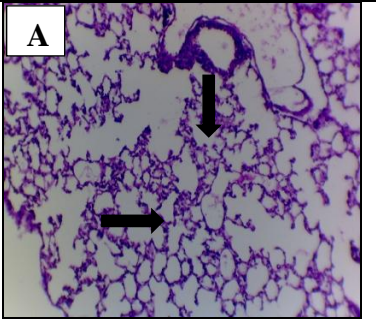
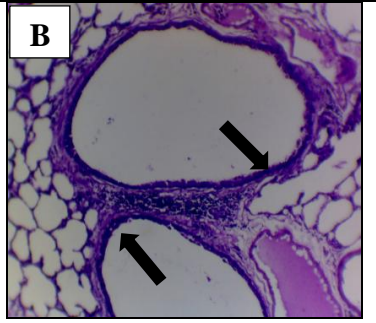
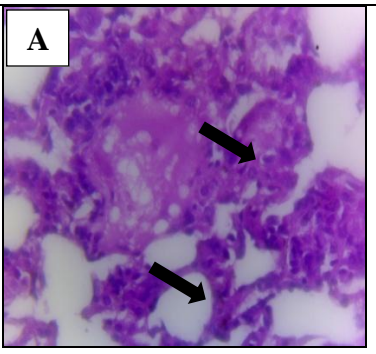
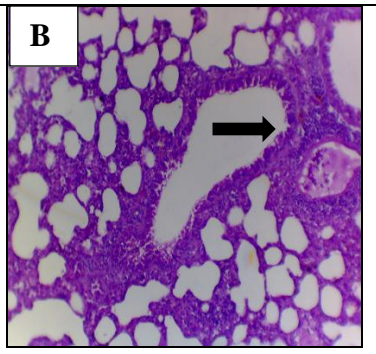
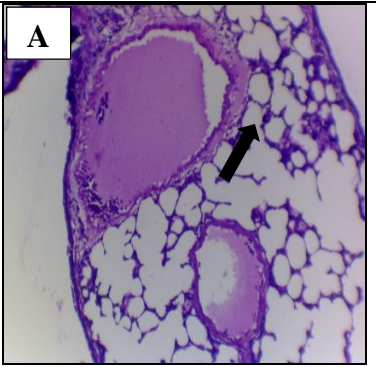
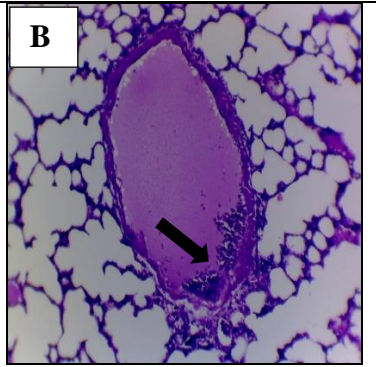
| Group    | TNF- $\alpha$    | VEGF             | IL-6            |
|----------|------------------|------------------|-----------------|
| Normal   | 31.16 ± 1.827    | 77.69 ± 2.63     | 27.86 ± 1.28    |
| Disease  | 67.78 ± 2.578*** | 158.34 ± 4.01*** | 67.17 ± 1.97*** |
| Standard | 46.61 ± 0.987*** | 97.65 ± 0.99***  | 46.55 ± 2.04*   |
| Test     | 43.13 ± 0.334*** | 90.95 ± 2.03***  | 41.69 ± 1.11*   |

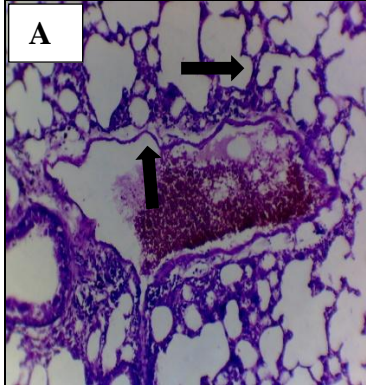
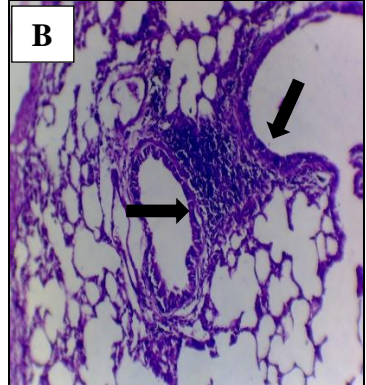


**Fig. 7 Effect of standard and its Nanoparticles on TNF- $\alpha$ , VEGF and IL-6 levels evaluated by ELISA. Each value represents Mean±S.D. (\*\*\*p<0.0001)**

**HISTOPATHOLOGY**

Mice were sacrificed at the end of the therapy and the lungs were removed, fixed with 10% buffered formalin and embedded in paraffin. The cut sections were stained with hematoxylin and eosin (H and E) respectively, for microscopic examination. The results of histopathological findings are described in Fig.8.

|   |   |  |   |
|---|---|--|---|
| <p>Group-A<br/>Normal Group</p>           | <p>A-Normal Alveoli and pulmonary veins</p> <p>B-Normal Respiratory bronchioles</p>                                     |    |    |
| <p>Group-B<br/>Diseased Group</p>         | <p>A-Damaged alveoli with exudation of fibrin.</p> <p>B-Damaged bronchiole due to proliferation of tumor cells.</p>     |   |   |
| <p>Group-C<br/>Standard treated group</p> | <p>A-Normal alveoli and alveolar duct</p> <p>B-Dilated veins containing transudate and RBCs adjacent to bronchiole.</p> |  |  |

|   |  |  |   |
|---|--|--|---|
| <p>Group-D<br/>Nanoparticles<br/>(Test) Treated<br/>group</p> | <p>A- Dilated veins and<br/>normal alveolar ducts<br/>and alveoli</p> <p>B-Normal alveoli and<br/>Lymphocytes<br/>infiltration adjacent to<br/>bronchiole.</p> |  |  |
|---|--|--|---|

**Fig. 8 Histopathological findings**

### DISCUSSION:

In the recent years, oral delivery of anticancer drugs encapsulated in polymeric nanoparticles has gained much attention. This is mainly due to the potential of nanoparticles to protect the entrapped drug molecules from the harsh conditions (chemical and enzymatic degradation) of gastrointestinal tract. (14, 15)

In the present study, we aimed to investigate the effects of chitosan loaded nanoparticles (CNP) of erlotinib in the treatment of non-small cell lung carcinoma. CNPs were spontaneously formed following the addition of TPP because of the ionic cross-linking between the TPP and chitosan solutions (16). The *in vitro* release profile of erlotinib loaded chitosan nanoparticles was performed at two different pH i.e. pH 3 in acetate buffer and pH 7.4 in phosphate buffer (10) which showed two distinct phases. The initial burst release phase (for about 4 hour) might be due to the dissolution and diffusion of the drug that was poorly entrapped in the polymer matrix. On the other hand, the slower and continuous release after this initial burst can be attributed to the diffusion of the drug localized in the core of the nanoparticles (17).

In our study, we developed a rapid and reproducible model of orthotopic lung cancer in which human lung adenocarcinoma cell line A549 was engrafted throughout the pulmonary parenchyma of mice and the tumor forming rate of modeling was 90% (18-20). Body weight changes in tumor bearing mice reflected a declining trend of mean body weight for about 10 days after inoculation of tumor cells. After 14 days of model development, treatment was started with standard drug as well as nanoparticle formulation of drug. Treatment was carried for 30 days by giving the drug by oral route. During the therapy, body weight measurement

and blood profile monitoring were done in all the four groups of mice. At the end of the therapy, MST and % ILS was calculated for standard and nanoparticle treated groups.

Lung cancer bearing mice show decrease in Hb% and RBC count which is an indication of anemia. The complication of anemia result from hypoxia of virtually all organs and tumor hypoxia is frequently considered as a potential therapeutic problem. Increase in WBC count has been suggested to be the hallmark of carcinogenesis. In our study, lung cancer animals showed marked increase in WBC count. The standard as well as nanoparticle treated group show decrease in WBC count which again proves the potential of the treatment in lung cancer. Immunoassays performed on plasma sample of different groups of mice also suggest the efficacy of treatment in controlling the lung cancer progression. The level of TNF- $\alpha$ , VEGF, and IL-6 was found to be lower in standard and nanoparticle treated group as compared with diseased control group. There is an increase in % ILS of mice when treated with the drug.

At the end of the study, mice were sacrificed and lung tissue was collected and stored in 10% formalin for histopathological testing. Histopathological findings reveals the disease progression and its correction after giving the therapy.

## CONCLUSION



In conclusion, the results of the present study demonstrated that Chitosan loaded nanoparticles of erlotinib were developed successfully, to target EGFR overexpressing lung cancers, by ion gelation method. Orthotopic lung cancer model was developed successfully within 14 days through transplantation of A549 cells into the lung of mice. The administration of nanoparticles by oral route increases its therapeutic efficacy, which can be high lightened with hematological profile of standard and test drug treated mice.

Further, there appears to decrease in toxicity of the drug when it is given in nanoparticles form, as evidenced by, increase in body weight, MST and % ILS of nanoparticles treated group. The histopathological findings and Immunoassays performed by using ELISA kits also revealed that the drug given in nanoparticles form showed better results as compared to standard treated group.

The results of the study clearly indicated that there is a great potential for nanoparticulate delivery of Erlotinib-HCl in minimizing drug-induced toxicity. However, extensive studies in

terms of chronic toxicity, pharmacokinetic and attachment of more specific targeting molecule are needed before establishing nanoparticle-mediated delivery of erlotinib.

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