8 IN-VIVO STUDIES

8.1 INTRODUCTION

In cancer biology and responsiveness to treatment or formulation, preclinical research models on real animals are game changer. The target specificity and bioavailability of the drug molecule are demonstrated by animal models. Animal simulation breakthroughs in the area of genetic engineering are on the brink of understanding and treating a number of cancers and offer considerable power to potentiate the insightful function of in-vivo research.

In order to achieve targeted delivery to the lung, most studies of nanoparticulate systems have relied on the parenteral route of administration, but direct administration of PLHNCs into the airways has the benefit of circumventing systemic dilution and elimination by other tissues and organs (1). In order to compare preparations on an equal basis during animal experiments, it was important to ensure that 100% of the overall dosage was specifically administered to the lung in each event (2).

8.2 METHODS

Surgical procedures and protocol performed in the experiment were approved by the Institutional Animal Ethical Committee (IAEC), Faculty of Pharmacy, The M.S. University of Baroda wide protocol Id MSU/IAEC/2018-19/1827 with permission from Committee for the purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India.

8.2.1 Selection of animals

Healthy young adult animals, male or female Sprague Dawley rats, approx. weight of 300-400 gm with 8-12 weeks age were used for the study (3).

8.2.2 Housing and feeding conditions

The temperature in the animal room was 20-25°C. Artificial lighting with the sequence of 12 hr light and 12 hr dark was kept in animal housing. The animals were housed individually. For feeding, conventional rodent laboratory diets were used with ad libitum drinking water.

8.2.3 Preparation of animals

The animals were randomly selected, marked to permit individual identification, and acclimatized in their cages for at least 5 days prior to dosing for acclimatization to the laboratory conditions.

8.3 INTRATRACHEAL INSTILLATION

Animals were fasted for about 24 h with water ad libitum prior to the studies. After that, weighed animals were anesthetized using 100 mg/kg ketamine and xylazine cocktail. Once the

deep anaesthesia was induced, a small incised cut was made near the neck region. To make the trachea visible, surrounding muscle was torn step by step using forceps. Once a clear trachea was sighted, a small lateral puncture was made with help of a sterile syringe. Later, docetaxel solution or D-sh-PLHNCs at 10 mg/kg dose was injected to animals, followed by suturing. To prevent the spread of postoperative microbial infection, antibiotic Neosporin powder was applied on sutures. The operated rats were observed for any sign of toxicity for 10-15 min after the surgery. After that Amoxicillin at a concentration of 40 mg/kg and Tramadol were injected to rats to prevent infection as well as postoperative pain (4).

8.4 ACUTE TOXICITY STUDY AND MAXIMUM TOLERATED DOSE ESTIMATION

Acute toxicity studies were performed in two groups of animals (n=3); one with D-sh-PLHNCs (dose of 10 mg/kg Docetaxel & 40 ng shRNA plasmid equivalent DPI formulation) and another with Docetaxel solution (5). 6 rats of either sex were fasted overnight before dosing and as per the weight of the animal. Docetaxel solution and D-PLHNCs were administered to animal through intra-tracheal route at an initial dose of 50 mg/kg, which was 5 times higher than the therapeutic dose of Docetaxel solution. If the animal survived, then higher dose of Docetaxel was injected until 2 or more rats have been found dead in the same group. The dose at which an animal was found dead was considered as LD_{50} value of the respective formulation. The dose intensity was planned as 50, 60, 70 mg/kg and so on.

For MTD study Sprague Dawley rats were divided into five groups, each for D-PLHNCs, control saline solution, and Docetaxel solution (Three Dose of Docetaxel with two elevated doses as per the model guided estimation of MTD from literature (6)). 3 Rats in each group were anesthetized and given drug solution and PLHNC dispersion at 10, 20, and 30 mg/kg Docetaxel and 40 ng shRNA via an intra-tracheal administration (As per the results of P-gp inhibition study performed in chapter 7 dose of shRNA was fixed at 40 ng to determine that the applied dose of shRNA was tolerated by animal or not). After that rats were kept for 21 days under observation for estimation of MTD (Maximum tolerated dose). After the span of 21 days, animals were sacrificed and the left lung of the animal was stored in a 10 % solution of formalin and analysed for histological analysis. Very thin sections (4 mm) were sectioned and further observed under the inverted microscope for any sign of inflammation and congestion in muscle with help of eosin and hematoxylin staining. The separated right lung was weighed before drying and subjected to 60°C for drying of extracellular fluid. The former weight was considered as wet weight (WW) and after drying, weight was considered as dry

weight (DW). The oedema index was calculated by considering the ratio of the wet weight by dry weight using the following equation (7).

$$Oedema\ index = \frac{Weight\ of\ wet\ lung\ (WW)}{Weight\ of\ dry\ lung\ (DW)}$$

8.5 PULMONARY PHARMACOKINETICS

Animals were divided into two experimental groups (3 rats per group) and intra-tracheal doses were given (1) Docetaxel solution (2) D-sh-PLHNCs (dose of 10 mg/kg Docetaxel and 40 ng shRNA). Bronchoalveolar lavage was performed on an anesthetized as well as re-cannulated rat using 12 mL of PBS, which was prewarmed at 37 °C. Lavage was performed using Hamilton syringe extended with PE50 tubing having 3-way stopcock, which was further extended with two 20 mL syringes. The tubing was inserted into the cannula in the trachea till it reaches tracheal bifurcation. After that, PBS with pH 7.4 was slowly injected into the trachea with one syringe and BAL (Broncho alveolar lavage) was ejaculated with another syringe. The yielded BAL fluid was centrifuged at 4000 g for 5 min. Supernatant was mixed with a 10 % solution of Triton X to burst the PLHNCs for Docetaxel release. Then, Docetaxel was extracted and analyzed by HPLC. After euthenization, the Lungs were excised along with the tracheal portion below the site of instillation and homogenized in 10 mL PBS having 1% Triton-X-100 to analyze diffused drug. Total concentration of drug in the lung was calculated and pulmonary and IV pharmacokinetic (By single IV bolus of lyophilized D-sh-PLHNCs suspension in rat tail vain) parameters were calculated (8).

The drug concentration in the lung is the drug estimated in Lung homogenate (LH) and BAL fluid and pulmonary and IV pharmacokinetic parameters were calculated using graph obtained by HPLC analysis.

- **Tmax:** The time point at which maximum drug concentration is attained in lung (i.e. Concentration of Docetaxel in LH and BAL was obtained as HPLC developed method in chapter 3. Further the time point at which highest amount of drug obtained in plasma was considered as Tmax for the particular sample treatment).
- **AUC_{total}**: The area under the curve of drug concentration in lung vs. time, over the period of study (12hrs).

(Plot of time Vs. Docetaxel concentration were constructed and Total area was calculated using given formula.)

$$AUC(0-\infty) = \int_0^\infty C(t) * dt$$

- **$t_{1/2}$** : Pulmonary half-life of drug is calculated by:
    a. Calculated the sum of the values of drug concentration (as obtained from each time point) in BAL and LH at individual sampling points.
    b. Regressing the calculated sum over the entire duration of study.
    c. Deriving the time point at which the sum of drug level is 50% compared to instilled quantity (i.e. deriving the median of the regression line).
- **C_{max}** : is the highest concentration of a drug in the blood, cerebrospinal fluid, or target organ after a dose is given.
  (From the graph of concentration of Docetaxel in LH and BAL vs. time, Cmax was calculated as maximum amount of Docetaxel found in LH and BAL.)
- **MRT (Mean residence time)** : is the average time that molecules of a dosed drug spend in the body.
  (Sum of the values of drug concentration (as obtained from each time point) in BAL and LH at individual sampling points were calcualted. After that mean of the reading were taken considering total time points to obtained Mean residence time.)

8.6 ENZYMATIC ACTIVITY ESTIMATION

Sprague Dawley rats were divided into two groups (One group for Doetaxel solution and another for D-sh-PLHNCs treatment), 3 rats/each group and 10 mg/kg dose was injected with intratracheal administration. One animal of saline control (negative control) and one with 0.1 µg/mL lipopolysaccharides (LPS) was considered as a positive control. Animals were euthanized with an overdose of phenobarbital at 75 mg/mL by intra-peritoneal route. BAL fluid was collected as per the above-mentioned procedure and supernatant from the BAL fluid was separated. Enzymatic activity of two enzymes called alkaline phosphatase and lactate dehydrogenase were evaluated using commercially available kits of Abcam, India. Fold increase in the enzyme activity was reported compared to the saline group. Lungs were removed surgically and the right lung was separated from the bronchus, heart, thymic tissue, trachea, and any other adherent clot or fibrinous exudates (9). An isolated piece of right lung was weighed on a calibrated weighing balance and lung weight to body weight ratio was calculated. Wights of lungs were reported as g/100g of rat body weight.

8.7 IN-VITRO METABOLISM ASSAY

A clear understanding of Docetaxel metabolism after pulmonary administration was necessary as the development of D-PLHNCs may alter the Docetaxel metabolism pattern compared to Docetaxel solution. Liver and lung of the Sprague Dawley rats were collected and

microsomes were separated using the method described by Samanth et al (10). Thereafter, Docetaxel solution, D-PLHNCs, and FA-D-PLHNCs at dose of 10mg/kg Docetaxel were incubated with obtained liver or lung microsomes at a concentration of 0.2 or 0.5 mg/mL with 5 mM $MgCl_2$ along with 1 mM of NADPH (nicotinamide adenine dinucleotide phosphate) with a final volume make up to 200 µL using 0.1 M PBS. Once the treatment was done, the further activity of microsomes was terminated using 400 µL of methyl t-butyl ether (ice cold). The non-metabolized Docetaxel was separated using centrifugation at 20,000 rpm for 1 h. The amount of Docetaxel in supernatant was estimated using the HPLC method described in chapter 3. Folic acid conjugated PLHNCs were used to determine the effect of folate conjugation of Docetaxel metabolism.

8.8 HEMOLYTIC STUDY

8.8.1 Hemolytic study by UV method

In a heparin-lined tube, fresh blood from Sprague-Dawley rats was obtained for Retro-orbital plexus. At 2500 rpm for 15 min, erythrocytes were separated from the heparinized human blood by centrifugation. The supernatant was discarded and the settled pellet of erythrocytes was resuspended in phosphate buffer saline pH 7.4. The fluid was centrifuged again and red cell precipitates were rinsed over and over again until the liquid's upper fraction became clear. To achieve 2 percent v/v of the RBC suspension, the filtered erythrocytes were then re-suspended in standard saline. After that, 1.8 ml of the erythrocyte suspension was incubated in an incubator shaker with 0.2 ml of Docetaxel solution, Blank PLHNCs, D-PLHNCs, D-sh-PLHNCs and SLS (Sodium lauryl sulphate)at 37 ° C for 30 min and then centrifuged for 5 min at 2500 rpm. Both tests used control samples of 0 percent lysis (in saline) and 100 percent lysis (in SLS containing double purified water). The percent hemolysis of the supernatant at the wavelength of 545 nm was measured by UV-Vis spectroscopic analysis. The mean value of three measurements using different samples was recorded. The percent hemolysis was calculated using equation below

$$\% \text{ Hemolysis} = \frac{(ABS - ABS_0)}{(ABS_{100} - ABS_0)} \text{ X } 100$$

Here, ABS- Absorbance of test sample, ABS_0 – Absorbance of saline, ABS_{100} Absorbance of SLS (Positive control).

8.8.2 Hemolytic study by Optical method

Fresh blood from rats was collected in a heparin-coated tube. Erythrocytes were isolated from the heparinized human blood by centrifugation at 2500 rpm for 15 min. The supernatant was discarded and the settled erythrocytes were re-suspended in phosphate buffer saline pH 7.4. The mixture was centrifuged again, and pellet red cells were rinsed again and again until the upper fraction of the liquid became transparent. The purified erythrocytes were then re-suspended in normal saline to obtain 2% v/v of the RBC suspension. Then it was subjected to optical microscope and Shape of RBC was observed.

8.9 RESULTS AND DISCUSSION

8.9.1 Estimation of the maximum tolerated dose and LD_{50}

Estimation of the LD_{50} was accomplished by observing the signs of mortality as well as morbidity on Sprague-Dawley rats after intratracheal administration of the escalated dose of 50, 60, 70, and so on mg/kg. Mortality study suggest that (As shown in Table 8-1, Docetaxel solution showed mortality at 100 mg/kg dose with intra-tracheal administration, while at the same concentration, PLHNCs could not show any mortality, which suggests the PLHNCs as a safe delivery vector for controlled release of Docetaxel in the lung tissue. The most appropriate reason for mortality of Docetaxel solution is instantaneous exposure of large dose of drug directly to lung where in PLHNCs, drug is released over prolonged period, which prevents the sudden rise of the Docetaxel in the pulmonary region.

Table 8-1 Results of sighting study for acute toxicity determination

Treatment	Dose	Observation	
		Toxicological signs	Mortality
Saline	-	NOAEL	No mortality
Blank PLHNCs	-	NOAEL	No mortality
Docetaxel solution	50 mg/kg	NOAEL	No mortality
	60 mg/kg	NOAEL	No mortality
	70 mg/kg	NOAEL	No mortality
	80 mg/kg	Stastically significant weight loss	No mortality
	90 mg/kg	Stastically significant weight loss	1 animal showed mortality

	90 mg/kg	Stastically significant weight loss	1 animal showed mortality
D-sh-PLHNCs	50 mg/kg	NOAEL	No mortality
	60 mg/kg	NOAEL	No mortality
	70 mg/kg	NOAEL	No mortality
	80 mg/kg	NOAEL	No mortality
	90 mg/kg	NOAEL	No mortality
	100 mg/kg	NOAEL	No mortality

*****No Observed Adverse Effect Level.

Experiment was performed in triplicate.

The highest dose tolerated by the animal without any unfavourable adverse effect is considered as MTD (11). To estimate MTD, histopathological images and alteration of the oedema index, as well as the severity of damage to pulmonary tissues, were considered. Pulmonary oedema index of five different groups are shown in Table 8-2. It can be concluded that the oedema index increases proportionally with the concentration of drug solution. At the same concentration, oedema index of the Docetaxel PLHNCs was found to be comparatively lower than the same dose of Docetaxel solution. The results showed concordance with images of the Hematoxylin-Eosin staining of the lung. In Figure 8-1 (A and B), saline and blank PLHNCs, a regular arrangement of bronchi epithelial cells along with mild inflammation and infiltration of cells were found. When Docetaxel solution was introduced at 10 mg/kg, there was a mild sign of cell infiltration but bronchial epithelial cells were found undisturbed with no sign of hemorrhage. When the concentration increased to 20 mg/kg, some of the stiffing was shown in bronchial cells and alveolar destruction was noticed. At the dose of 30 mg/kg, complete destruction of the bronchial cell was observed along with oedema development and hemorrhage. In D-sh-PLHNCs groups, which showed that the integrity of the broncho-epithelial cells were well maintained with negligible evidence of any cell infiltration and oedema. Hence, it was confirmed that Docetaxel solution was found safe up to 10mg/kg. At the same time, there was no trace of drug present in BAL and LH when an intravenous dose of Docetaxel solution was injected, although intravenous dose of D-sh-PLHNCs have some concentration of Docetaxel in lung tissue after a span of 2 h owing to long circulation of the PLHNCs in the bloodstream which could distribute Docetaxel into different blood compartment. The mass balance of total Docetaxel absorbed and Docetaxel entrapped was found near 95 %.

Table 8-2 Estimation of oedema index

Dose(mg/kg)	Docetaxel solution	D-sh-PLHNCs
10	1.12 ± 0.15	1.02 ± 0.09
20	1.52 ± 0.19	1.05 ± 0.12
30	2.01 ± 0.21	1.16 ± 0.11
Saline solution	1.07	
Blank PLHNCs	1.05	

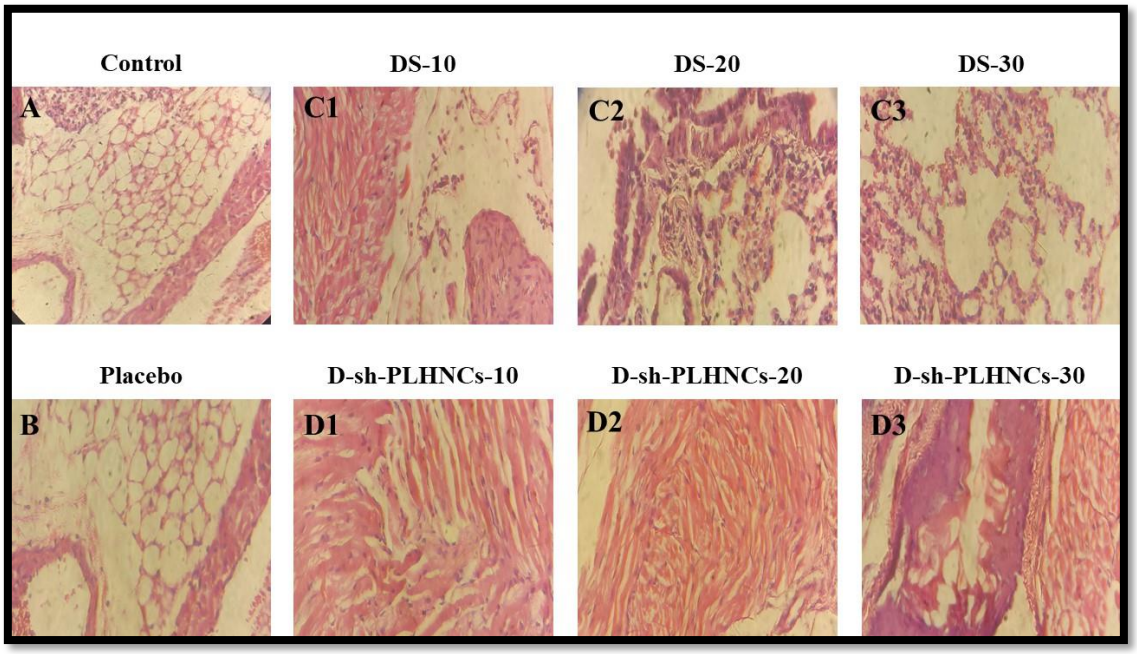

Figure 8-1 Histogram images of Sprague Dawley rat lungs after intratracheal instillation

of (A) saline solution, (B) Blank PLHNCs, (C1) drug solution 10mg/kg Docetaxel, (C2) drug solution 20mg/kg Docetaxel, (C3) drug solution 30mg/kg Docetaxel, (D1) D-sh-PLHNCs 10 mg/kg Docetaxel, (D2) D-sh-PLHNCs 20 mg/kg Docetaxel, (D3) D-sh-PLHNCs 30 mg/kg Docetaxel

8.9.2 Estimation of Docetaxel in lung homogenate (LH) and broncho-alveolar lavage (BAL fluid) after intra-tracheal and intravenous administration

An in-vivo study was performed by estimating Docetaxel in BAL fluid and Lung homogenate after the administration of the Docetaxel solution and D-sh-PLHNCs (Figure 8-2). The equivalent dose of 10mg/kg was instilled by the intra-tracheal route. The amount of drug that remain in lung homogenate was considered as absorbed Docetaxel by the lung tissues and it was readily available for cytological action. While amount present in BAL fluid was

considered as reservoir drug that could be gradually absorbed by lung tissues. Hence, from the Figure 8-2, it was inferred that D-sh-PLHNCs have a higher concentration in the LH as well as BAL when delivered by intra-tracheal route, while Docetaxel solution had initially high BAL concentration, which falls very fast due to rapid clearance of the drug by lung macrophage. At the same time, there was no trace of Docetaxel present in BAL and LH when an intravenous dose of Docetaxel solution was injected although intravenous dose of D-sh-PLHNCs have some concentration of Docetaxel in lung tissue after a span of 2 h which shows the long circulation of the PLHNCs in the bloodstream.

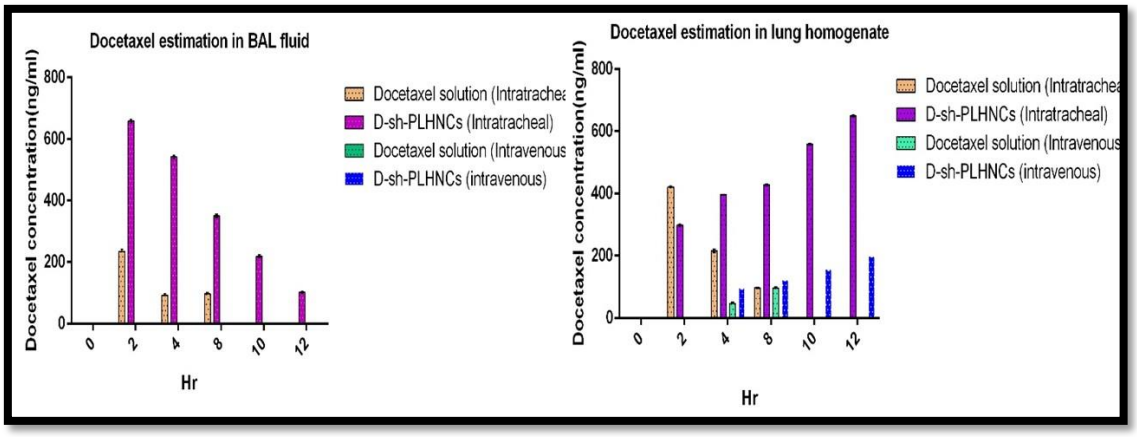

Figure 8-2 Concentration of Docetaxel in BAL and LH.

Table 8-3 Pulmonary and IV Pharmacokinetic Parameters

Formulation	**AUC_{total} (ng.h/mL)**	**C_{max} (ng/mL)**	**T_{max} (h)**	**$t_{1/2}$ (h)**	**MRT (h)**
Docetaxel solution **(intra tracheal)**	4215±125.5	522±5.5	2.1 ±0.85	1.98±0.54	4.64±1.58
D-sh-PLHNCs **(intra tracheal)**	12345±212.5	1105±22	14.2 ± 1.8	6.05±1.23	12.42±2.45
Docetaxel solution **(intravenous)**	12431±188	1296±21.5	1.8 ± 1.2	1.24±1.12	4.10±2.15
D-sh-PLHNCs **(intravenous)**	7209±121.5	954±7.0	6.4 ± 1.54	2.52±1.24	6.90±1.98

The pulmonary as well as IV pharmacokinetic parameters are represented in Table 8-3. The t½ of 6.05 h was recorded for D-sh-PLHNCs compared to 1.98 h for Docetaxel solution. A significant improvement was observed in the AUC of the D-sh-PLHNCs compared to the Docetaxel solution in pulmonary pharmacokinetics. Two-three times higher AUC was achieved with PLHNCs compared to Docetaxel solution in pulmonary pharmacokinetics. The

residence time of the D-sh-PLHNCs was also high compared to the Docetaxel solution. Hence, from the above trend, it can be inferred that Docetaxel was quickly absorbed by the lungs entered the systemic circulation and was further metabolised when administered as a solution form, whereas PLHNCs formulations provided higher residence time of Docetaxel in lungs and slowed its metabolism.

8.9.3 Enzymatic activity estimation

As depicted in Figure 8-3, lowest L/B ratio of D-sh-PLHNCs suggested the lowest adverse effect of the Docetaxel due to encapsulation in the PLHNCs. The weight of the lungs were rationalized considered 100 g of body weight. For the saline-treated animals, L/B ratio was obtained as 0.51, which suggested a normalized condition, while the L/B ratio of 0.92 for lipopolysaccharides suggested the accumulation of the extracellular fluids, which indicated lung cell injury. L/B ratio of 0.54 for D-sh-PLHNCs had suggested that the formulation is as safe as saline in terms of the cellular damage to lung linings. Even a higher L/B ratio of the Docetaxel solution provided the esteem for the development of safer formulations. From Figure 8-3, it can be observed that enzymatic activities of lactose dehydrogenase (LDH) and alkaline phosphatase (ALP) were noted highest in the Lipopolysachharides (Positive control) , while D-sh-PLHNCs showed a similar activity as the saline solution indicates a high safety of the developed formulation.

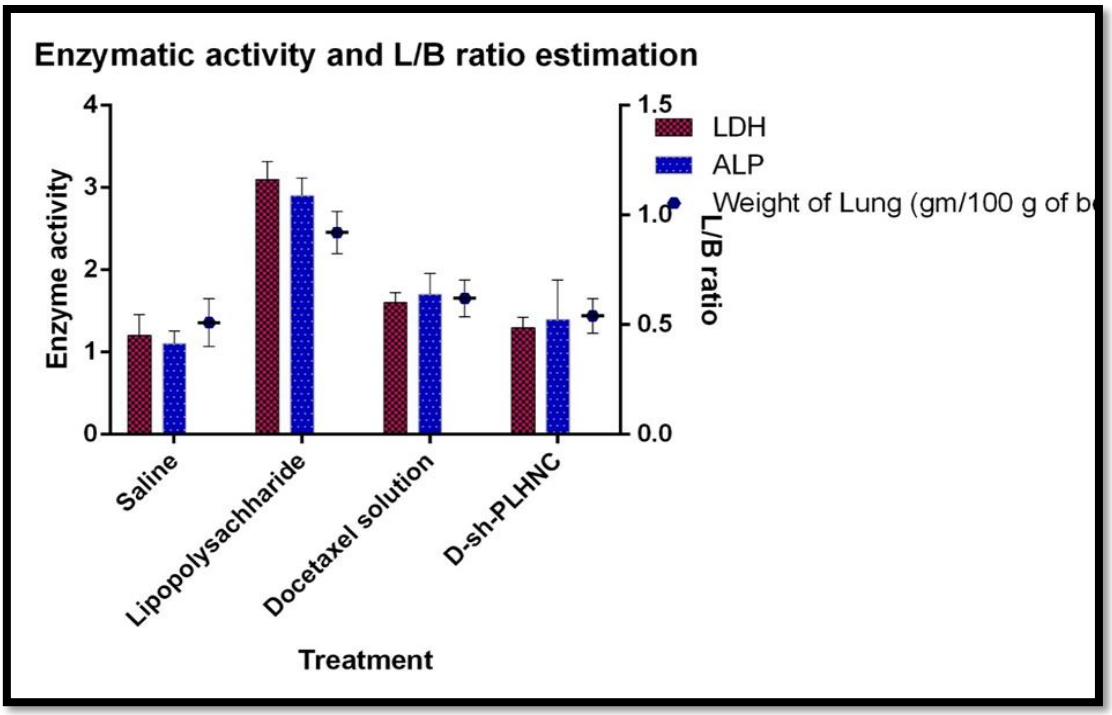

Figure 8-3 Enzyme activity and L/B ratio

8.9.4 Metabolism of Docetaxel by rat liver and lung microsomes

Lungs play a prominent role in the absorption after pulmonary instillation, while the liver plays a crucial role in its metabolism (12). Figure 8-4 (A and B) shows that the metabolism of Docetaxel was reduced, in the lung as well as liver microsomes when it is formulated in PLHNCs. Moreover, the highest amount of drug remained intact during a time span of 90 min with lung microsomes which suggested the advantage of pulmonary delivery. Docetaxel solution with liver microsomes was metabolized very fast. The mean half time of the FA-D-PLHNCs (folic acid conjugated and drug loaded PLHNCs) was higher compared to the Docetaxel solution suggest low Docetaxel clearance with FA-D-PLHNCs. In lung microsomes, low clearance was observed despite high concentration because metabolic function of the lung was incomparable in the context of the liver due to less enzymatic activity of the lungs. Hence, higher $t_{1/2}$ was achieved by pulmonary administration, which accounts for higher pulmonary activity of Docetaxel. Hence, metabolic capacity of FA-D-PLHNCs was decreased compared to the Docetaxel solution and resulted in the longer $t_{1/2}$ value that could produce a more sustainable effect of the chemotherapeutic agent. The folic acid conjugated PLHNCs were used for metabolism study to verify role of folate conjugation to drug metabolism.

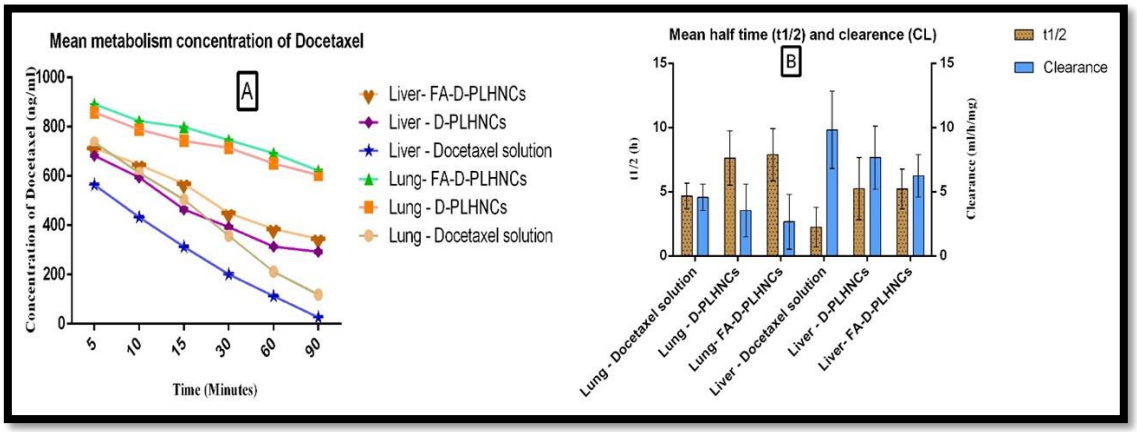

Figure 8-4 Metabolism of Docetaxel solution, D-PLHNCs, and FA-D-PLHNCs in Sprague Dawley lung and liver microsomes

(A) mean metabolism concentration of Docetaxel at 0.2 mg/mL microsomes (B) Mean half time (t1/2) and clearance (CL) according to metabolism concentration-time curve. Data shown here are mean ± SD (n = 3).

8.9.5 Hemolytic study by UV spectroscopy

From the graph shown in Figure 8-5, It can be clearly seen that Docetaxel PLHNCs formulation are much safer than docetaxel solution. Blank PLHNCs, D-PLHNCs and D-sh-PLHNCs was showed less hemolytic property than Docetaxel solution and positive control.

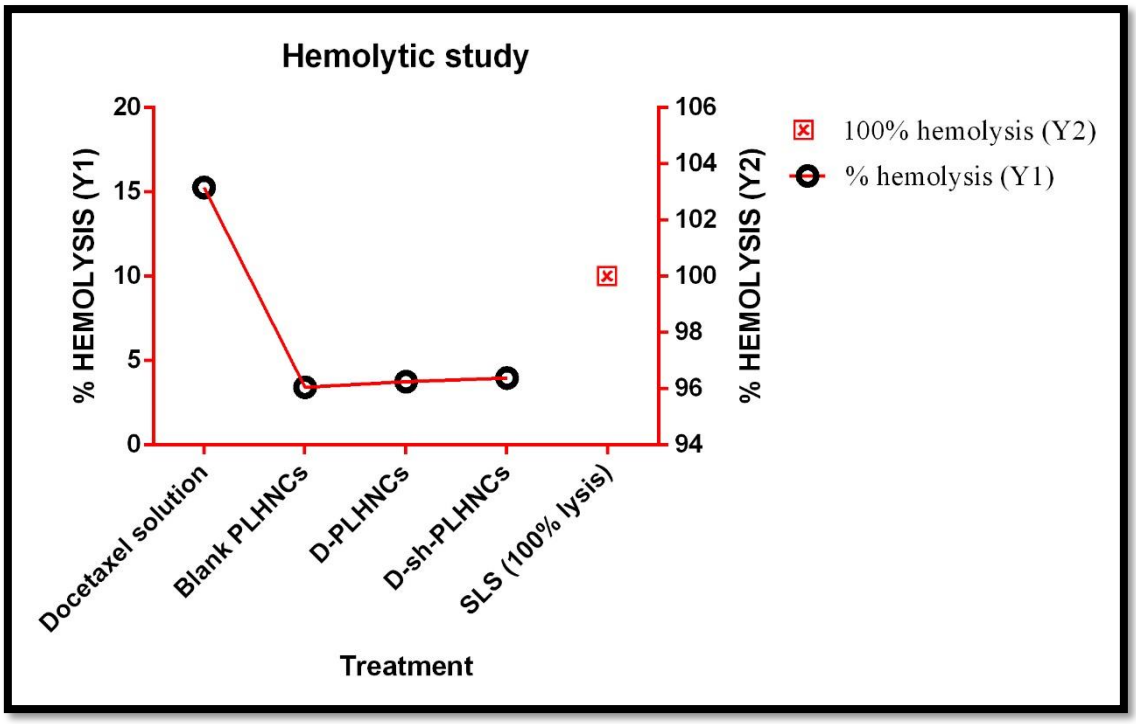

Figure 8-5 Haemolysis by UV spectroscopy

8.9.6 Hemolytic study by optical method

From the Figure 8-6, it was clearly determined that triton X has shown lysis of all the RBC while negligible disruption of RBC was seen with treatment of saline. Blank PLHNCs as well drug and shRNA pDNA loaded PLHNCs (D-sh-PLHNCs) shows disruption of RBC as similar as phosphate buffer saline which suggested that developed formulation is as safe as saline.

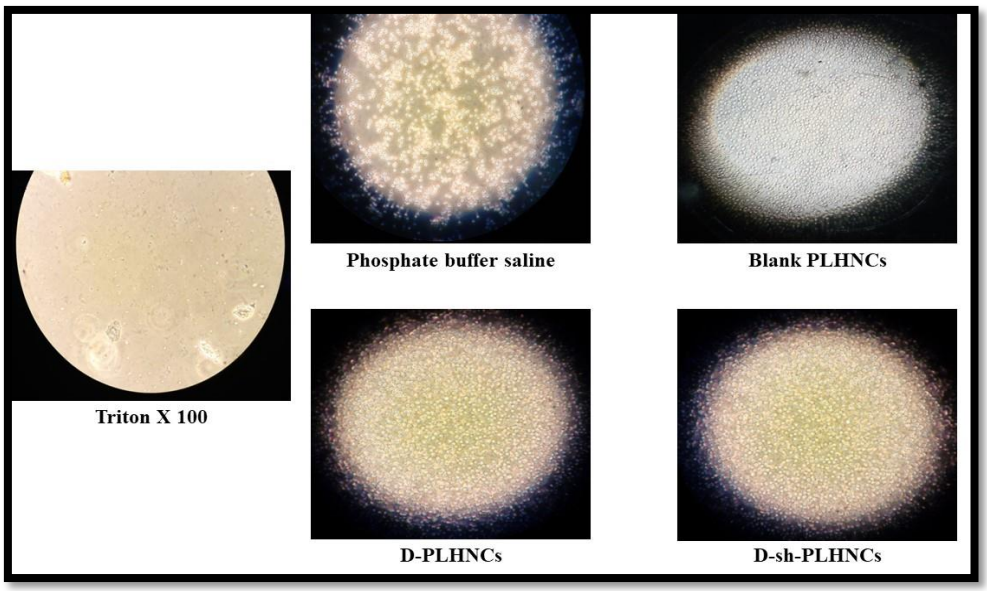

Figure 8-6 Hemolysis by Optical method

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