MATERIALS AND METHODS
1.0 MATERIALS AND SUPPLIERS:

1.1 Ingredients:

Bleomycin hydrochloride  Nippon Kayaku Co., Ltd., Tokyo, Japan
Plumbagin  Sigma Chemical Co., Missouri, USA
Cholesterol  Sigma Chemical Co., Missouri, USA
Dicetyl phosphate  Sigma Chemical Co., Missouri, USA
Span20, Span40 and Span60  Sigma Chemical Co., Missouri, USA
Tween40 and Tween80  Sigma Chemical Co., Missouri, USA
Brij 35 and Brij 78  Sigma Chemical Co., Missouri, USA
Poloxamer F108  BASF Corporation, New Jersey, USA
Dipalmitoyl phosphatidyl choline (DPPC)  Sigma Chemical Co., Missouri, USA
Distearoyl phosphatidyl choline (DSPC)  Sigma Chemical Co., Missouri, USA
6-carboxy fluorescein  Sigma Chemical Co., Missouri, USA
Muramyl dipeptide  Sigma Chemical Co., Missouri, USA
Tuftsin  Sigma Chemical Co., Missouri, USA

1.2 Reagents:

Heparin  Gland Pharma, Pvt. Ltd., Hyderabad, India
Trichloro acetic acid  Riedel-Dehaen Ag Seelze-Hannover, Germany
Sodium hydroxide pellets  Ranbaxy Laboratories Ltd., Punjab, India
Acetic acid  Qualigens Fine Chemicals, Bombay, India
Chloroform (HPLC grade)  S.D. Fine Chemicals, Boisar, India
Acetonitrile (HPLC grade)  S.D. Fine Chemicals, Boisar, India
Methanol (HPLC grade)  S.D. Fine Chemicals, Boisar, India
Water (HPLC grade)  Qualigens Fine Chemicals, Bombay, India
1. Materials:

- Gluteraldehyde
- Acetone
- Anaesthetic ether
- Sodium chloride
- Sodium bicarbonate
- Potassium chloride
- Potassium dihydrogen phosphate

1.3 Materials:

- Para film
- Dialysis sac (cellulose)
- 1 ml insulin syringes
- 36 gauge needle
- 5 ml syringes

1.4 Equipments:

- Water bath, rotary evaporator and vacuum pump
- Centrifuge
- Vortex
- Water bath
- Ultra tome 5

S.D. Fine Chemicals, Boisar, India
E. Merck, Bombay, India
Industrial solvents and Chemicals Pvt. Ltd., Bombay, India
Astra IDL, Ltd., Bangalore, India
S.D. Fine Chemicals, Boisar, India
S.D. Fine Chemicals, Boisar, India
B.D.H. Laboratory Chemicals, Bombay, India
Sigma Chemical Co., Missouri, USA
Sigma Diagnostics Co., Missouri, USA
Hindustan Syringes Pvt. Ltd., India
Hindustan Syringes Pvt. Ltd., India
Hindustan Syringes Pvt. Ltd., India
Toshniwal, Madras, India
Remi, India
Remi, India
Julabo (PC 20B), Germany
LKB, Sweden
1.5 Instruments:

- pH meter
- UV spectrophotometer (UV-240 Graphicord)
- Spectrofluorimeter
- Gilson 305 HPLC gradient system
- Analytical column
  - Ultremex 3μ(ODS) 150x4.6 mm
- Guard column
  - Pellicular ODS (37-53 μm)
- Detector
  - (HM Holochrome)
- Loop injector (7125)
- Integrator CR-3A
- Sonicator
- Blood analyzer
- Electron microscope (TEM)
- Droplet and Particle size analyzer 2600

Control Dynamics, Bangalore, India
Shimadzu, Japan
Kontron, Germany
Gilson Medical Electronics, France
Phenomenex, California, USA
Whatmann Inc., Clifton, NJ, USA
Gilson Medical Electronics, France
Gilson Medical Electronics, France
Shimadzu, Japan
Sonics and materials Inc., Connecticut, USA
Technicon H1, Belgium
JEOL 100C, Japan
Philips (420 ST), Netherlands
Malvern, UK
2. OBJECTIVES OF THE PRESENT STUDY:

I

a. To formulate non ionic surfactant vesicular systems (niosomes and thermosensitive niosomes) as carriers for BLM.

b. To evaluate the size and shape of the vesicular carriers.

c. To determine in vitro the drug entrapment efficiency and vesicle stability.

d. Evaluate niosome encapsulated bleomycin for toxicity.

e. Monitor concentration of bleomycin at tumor site as an index of specific delivery.

f. To evaluate the antitumor efficacy of niosomal bleomycin in the presence and absence of hyperthermia.

g. To carry out pharmacokinetic studies of niosomal bleomycin.

i. To activate and exploit macrophages using niosomal encapsulated muramyl dipeptide and tuftsin for delivering niosomal bleomycin more quantitatively to tumor site.

II

a. To formulate non ionic surfactant vesicular system for plumbagin.

b. To evaluate niosome encapsulated plumbagin for acute toxicity and antitumor efficacy.
STUDY DESIGN

Bleomycin niosomes:

1. Preparation of niosomes
   a. Lipid layer hydration method.
   b. Reverse phase evaporation.

2. Size distribution pattern studies of the prepared niosomes.

3. Determination of vesicular drug entrapment efficiency.

4. Study of drug leakage from the vesicles.

5. Determination of vesicle stability.


7. Toxicity studies following treatment with free and niosomal bleomycin.
   a. Haematological toxicity.
   b. Gastrointestinal toxicity.
   c. Pulmonary toxicity.

8. Comparative pharmacokinetics and tissue distribution profiles of free and niosomal bleomycin.

9. Antitumor efficacy of niosomal bleomycin subsequent to activation of macrophage by niosomal MDP and tuftsin.
Thermo-sensitive bleomycin niosomes:

1. Preparation of thermo-sensitive niosomes by Lipid layer hydration method.

2. Size distribution pattern studies of the prepared niosomes.

3. Determination of vesicular drug entrapment efficiency.

4. Study of drug leakage from the vesicles.

5. Determination of vesicle stability.


7. Comparative pharmacokinetics and tissue distribution profiles of free and thermo-sensitive bleomycin niosomes in the presence and absence of hyperemia (43°C, 30 min.).

8. Antitumor efficacy of thermo-sensitive bleomycin niosomes subsequent to activation of macrophages by thermo-sensitive niosomal MDP and tufts.

Plumbagin niosomes:

1. Preparation of niosomes by Lipid layer hydration method.

2. Determination of vesicular drug entrapment efficiency.

3. Acute toxicity studies following treatment with free and niosomal plumbagin.

2. METHODS

2.1.1 Determination of calibration curve for bleomycin hydrochloride (BLM) in phosphate buffered saline by UV spectrophotometry:

Pure BLM (15 mg) was dissolved in phosphate buffered saline. Serial dilutions were carried out to get final concentrations of 0.375, 0.750, 1.5, 3, 7.5, 15, 22.5 and 30 μg/ml. The absorbance was measured at 254 nm and plotted against corresponding concentrations (Table-1 and Figure-1).

2.1.2 Determination of calibration curve for plumbagin (PLM) in phosphate buffered saline by UV spectrophotometry:

Pure PLM (1 mg) was dissolved in minimum quantity of alcohol (1 ml) and made up the volume with buffered saline. Serial dilutions were carried out to get final concentrations of 1, 2, 4, 6, 8, 10 and 20 μg/ml. The absorbance was measured at 422 nm and plotted against corresponding concentrations (Table-2 and Figure-2).

2.1.3 Determination of calibration curve for bleomycin in plasma of mice transplanted with S-180 tumor by HPLC with UV detection:

Sample preparation:

Reported high performance liquid chromatographic (HPLC) methods where unsuitable for routine quantitative analysis. The method adopted in the present study was developed by Shiu et al, 1979. Standard curves for BLM were determined by adding varying quantities of BLM (equivalent to 0.375, 0.750, 1.5, 3, 7.5 and 10 μg) to 1 ml of pooled S-180 bearing mice plasma.
### Table - 1

**Calibration curve data for bleomycin in saline by UV spectrophotometry**

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>Average absorbance ± S.D. (n=3)</th>
<th>Percentage coefficient of variation (% C.V.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.375</td>
<td>0.007 ± 0.000</td>
<td>0.0</td>
</tr>
<tr>
<td>0.750</td>
<td>0.012 ± 0.001</td>
<td>8.3</td>
</tr>
<tr>
<td>1.500</td>
<td>0.018 ± 0.001</td>
<td>5.5</td>
</tr>
<tr>
<td>3.000</td>
<td>0.028 ± 0.002</td>
<td>7.1</td>
</tr>
<tr>
<td>7.500</td>
<td>0.063 ± 0.002</td>
<td>3.1</td>
</tr>
<tr>
<td>15.000</td>
<td>0.118 ± 0.006</td>
<td>5.1</td>
</tr>
<tr>
<td>22.500</td>
<td>0.178 ± 0.004</td>
<td>2.2</td>
</tr>
<tr>
<td>30.000</td>
<td>0.231 ± 0.004</td>
<td>1.7</td>
</tr>
</tbody>
</table>

Linear regression coefficient = 0.9998
Slope = 7.55 x 10^{-3}
Y axis intercept = -5.75 x 10^{-3}
Standard graph of BLM in saline
(UV Spectrophotometry)

---

Series 1

Figure - 1
### Table - 2

**Calibration curve data for plumbagin in saline by UV spectrophotometry**

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>Average absorbance ± S.D. (n=3)</th>
<th>Percentage coefficient of variation (% C.V.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>0.008 ± 0.000</td>
<td>0.0</td>
</tr>
<tr>
<td>1.0</td>
<td>0.017 ± 0.001</td>
<td>5.9</td>
</tr>
<tr>
<td>2.0</td>
<td>0.038 ± 0.002</td>
<td>5.3</td>
</tr>
<tr>
<td>4.0</td>
<td>0.076 ± 0.004</td>
<td>5.3</td>
</tr>
<tr>
<td>8.0</td>
<td>0.157 ± 0.002</td>
<td>1.3</td>
</tr>
<tr>
<td>10.0</td>
<td>0.194 ± 0.006</td>
<td>3.1</td>
</tr>
<tr>
<td>20.0</td>
<td>0.388 ± 0.004</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Linear regression coefficient = 0.99996

Slope = 0.0195

Y axis intercept = $-1.42 \times 10^{-3}$
Standard graph of PLM in saline
(UV Spectrophotometry)
To one ml of plasma in a 10 ml glass-stoppered conical centrifuge tube was added 200 µl of a 20% trichloroacetic acid solution. The samples were gently vortexed and then centrifuged at 3000 rpm for 10 minutes. A 50 µl aliquot of the clear supernate was then injected onto the column and peak heights were determined. The HPLC analysis was carried out as follows:

**HPLC instrument conditions and assay of BLM:**

A Gilson 305 programmable instrument equipped with the fixed wave length (254 nm) UV detector.

A Shimadzu CR-3A integrator was connected to the detector.

The C₁₈ guard column and a C₁₈ phenomenex 3 µ analytical column were connected to a Rheodyne injector.

**Chromatographic conditions:**

The mobile phase consisted of methanol-acetonitrile - 0.0085 M heptane sulfonic acid - acetic acid (30:10:59:1). A flow rate of 2 ml/min. was established (2.3 Kpsi). A minimum of 3 injections from each sample of bleomycin were made, and the mean of the peak height was used for determination of the calibration curve, depicted in Table-3, Figure-3. The procedure was repeated for 3 times for each data point to statistically validate the calibration curve.

2.2 Evaluation of antitumor efficacy:

The antitumor efficacy was assessed using two tumor models viz.

1. Sarcoma-180 (solid tumor).
2. Ehrlich ascites.
**Table - 3**

*Standard graph of bleomycin in plasma of S-180 bearing mice*  
*(HPLC with UV detection)*

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>Mean peak height in cms ± S.D. (n=3)</th>
<th>Percentage coefficient of variation (% C.V.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.375</td>
<td>0.4 ± 0.00</td>
<td>0.0</td>
</tr>
<tr>
<td>0.750</td>
<td>0.7 ± 0.00</td>
<td>0.0</td>
</tr>
<tr>
<td>1.500</td>
<td>2.0 ± 0.10</td>
<td>5.0</td>
</tr>
<tr>
<td>3.000</td>
<td>4.0 ± 0.10</td>
<td>2.5</td>
</tr>
<tr>
<td>7.500</td>
<td>9.4 ± 0.20</td>
<td>2.1</td>
</tr>
<tr>
<td>10.000</td>
<td>12.5 ± 0.20</td>
<td>1.6</td>
</tr>
</tbody>
</table>

Linear regression coefficient = 0.999462  
Slope = 1.26  
Y axis intercept = 0.011
Standard graph of BLM in plasma by HPLC

<table>
<thead>
<tr>
<th>Concentration (μg/ml)</th>
<th>Peak height (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.375</td>
<td></td>
</tr>
<tr>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
</tr>
<tr>
<td>7.5</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td></td>
</tr>
</tbody>
</table>

* Peak height

Figure - 3
**Animals:**

The mice were of BALB/c strain, obtained from the Cancer Research Institute, Bombay, India and reared by brother sister mating. Mice were housed in polypropylene cages containing sterile paddy husk as bedding and maintained under controlled conditions of temperature (23 ± 2°C), humidity (50 ± 5%) and light (10:14h of light and dark respectively). The animals were fed balanced diet water ad libitum.

**Tumors:**

The tumor sarcoma-180 (S-180) was obtained from Cancer Research Institute, Bombay, India, in ascites form and was maintained by serial transplantation.

Ehrlich ascites was obtained from Amla Cancer Research Centre, Trichur, India, in ascites form and was maintained by serial transplantation.

**Tumor propagation:**

The different steps involved in tumor propagation are as follows:

a. Ascites fluid from the intraperitoneal cavity of the donor animal was aseptically aspirated using hypodermic syringe with 18 guage needle, 7-8 days after tumor inoculation.

b. A small portion of ascites fluid (100 µl) was tested for bacterial contamination.

c. Tumor viability was determined by the tryphan blue dye exclusion test and cells were counted using electronic cell counter (Sysmex F300, Toa Medical Electronics Co., Kobe, Japan).
d. The aspirate was diluted in Dulbecco's modified Eagle's medium (DMEM) to get a concentration of $5 \times 10^6$ cells/ml and 0.2 ml of this tumor cell suspension ($1 \times 10^6$) was injected intraperitoneally to generate ascites tumor. After 7-8 days, the mice were used for the next propagation or transplantation.

Ehrlich ascites was also propagated and maintained on similar lines.

*Development of solid tumor:*

Solid tumors for the experiments were produced by the intradermal inoculation of $5 \times 10^5$ viable tumor cells on the dorsum of mice. Once the palpable tumor appears, the tumor diameter in 3 perpendicular planes ($D_1$, $D_2$, $D_3$) were measured thrice a week. The tumor volume ($V$) was calculated from the standard formula:

$$V = \frac{\pi}{6} (D_1 \cdot D_2 \cdot D_3)$$

Tumors of the size $100 \pm 10 \text{ mm}^3$ (10-14 days after inoculation) were taken for the experiments (Plate-1).

*Cryopreservation of tumor:*

Cells from the first two passages were stored in liquid nitrogen and used as tumor bank. After every 10 sub-passages, the tumor line was discarded and replaced by a new passage generated from cells removed from the bank. For this, tumor cells (stock) from the liquid nitrogen were taken out, thawed and injected into fresh animals.

*2.3 Hyperthermia treatment (HT):*

For hyperthermia treatment, a thermostat controlled circulating water bath with a overall dimension of $535 \times 330 \times 320 \text{ mm}$, bath opening of $360 \times 300/150 \text{ mm}$ and bath liquid
capacity of 14-20 litre (Julabo PC 20B, Germany) was used. Distilled water was used as the bath liquid. The working temperature range of water bath was 22-100°C, with an accuracy of ± 0.02°C and a LED display. Water bath was set at the desired temperature. The set temperature was achieved and stabilized within 10 minutes and maintained throughout the experiment.

Mice were anaesthetized with ketamine (50 mg/kg body weight) and diazepam (0.5 mg/mouse), injected intraperitoneally, the animals were then placed on a perspex tray with longitudinal slits. The tumors were pulled out through the slits in such a way that only the tumors protruded out to the water below the tray, while the animal remained above the tray (Plate-2c). Thus the tumors could be heated without affecting the other parts of the body. The tray was then placed in the water bath with the tumors immersed 1.5 to 2.0 cm below the surface. Five to seven tumors were heated at a time. A table fan was used to ventilate the air so as to control humidity above the water bath (Plate-2a,b).

**Temperature monitoring:**

All the temperature measurements were done using copper constantan thermocouple microprobes. The rectal temperature was measured using a rectal probe (RET-3, 1.0 cm long, Sensortek, USA). The intratumor temperatures at three points, i.e. at the base, center and outer periphery of the tumor, were measured using hypodermic needle microprobes (29 gauge, 0.33 mm diameter, 1.0 cm long) in one of the animals from each group. The temperature of the water bath was also continuously monitored using a microprobe. All the microprobes were connected to a digital thermometer sensor (Sensortek, ING, BAT, USA) bypassed through a multichannel thermocouple selector (Sensortek, SWT-5, USA).

Hyperthermia at 43°C, 30 min. was applied to the solid tumors, 15 minutes after i.v. injection of FBLM or NBLM. This HT timing is usually used in HT therapy (Marmor, 1979).
Plate 1: Sarcoma-180 tumor bearing mouse.
Plate 2a: Experimental setup showing local hyperthermia treatment.

Plate 2b: Experimental setup showing local hyperthermia treatment: close up view.
Plate 4a, b: Electron micrographs of a niosome showing lipid bilayer. The niosomes were mixed with 2 or 3 drops of neutral 2% phosphotungstic acid. The copper-coated grid was then immersed into the above suspension. The drop was then absorbed with a filter paper. A JEOL 100C transmission electron microscope (TEM) was used. x 1,00,000.
Plate 5a: Electron micrograph of a niosome observed after a week. x 26,000

Plate 5b: Electron micrograph of a niosome observed after a month. x 83,000.
Plate 6a: This picture shows intact lumen (L), alveolar space (Als), nucleus (N), pinocytic vesicles (V), endothelial cells (EN), Type I (EP1) and Type II (EP2) epithelial cells.

Plate 6b: This picture shows intact alveolar macrophages (Alm), nucleus (N), nucleolus (NL), Type I (EP1) and Type II (EP2) epithelial cells.
Hyperthemic conditions of 43°C for 30 minutes was earlier standardised and established in our laboratory setup by Uma Devi and Satish (1993).

2.4 Statistical analysis:

Statistical significance of difference between control and test (treated) groups were calculated by one factor analysis of variance (ANOVAR), multiple comparisons were made with Dunetts test, with the help of a software programme GWBASIC - PROG. 5B-"Foundations in pharmacology"; Copyright: R.B. Barlow; August 1991, CA, USA.

3.1 Preparation of niosomal bleomycin:

Non ionic surfactants like sorbitan esters (Span 20, 40 and 60), polyoxyethylene sorbitan esters (Tween 20 and 80) polyoxyethylene ethers (Brij 35 and 78) and polyoxypropylene -polyoxyethylene block co polymer (PF 108) were chosen for the preparation of niosomes. Different methods and techniques employed for the preparation of niosomes of bleomycin have been outlined.

3.1.1 Lipid layer hydration method (Azmin et al, 1985).

Niosomes were prepared by dissolving cholesterol, surfactant and dicetyl phosphate (47.5:47.5:5) (71.25:71.25:5 mg), in chloroform and evaporating the solvent using rotary flash evaporator under low pressure at 40-50°C. Niosomes were formed by adding part by part of phosphate buffered saline containing BLM to the dried thin film formed on the walls of the round bottom flask heated to about 40-50°C on a water bath with gentle agitation. The mixture was intermittently mixed on a vortex to get a good dispersion of the mixture. Sonic dispersion of the mixture was carried out at 25°C using a probe sonicator, 20 KHz, 500 watts, (Vibra cell, Sonics and Materials Inc. Co., USA) for 30 seconds at one minute intervals for a period of 4 minutes. After sonication, the suspension was maintained at room temperature for
two hours to allow niosomes to form and seal. The BLM entrapment was determined by column chromatography.

*Comment:*

With span 40, 60 and Tween 20, 80 good thin films could be formed and thereby niosome preparation with these surfactants by the method of lipid hydration was possible. However, with Brij 35, 78 and Poloxamer F108 perfect thin films could not be made and consequently niosome formulation was not successful.

### 3.1.2 Reverse phase evaporation (Pidgeon et al, 1987):

Several formulations were prepared using different surfactants. The surfactant, cholesterol and dicetyl phosphate (47.5:47.5:5) (71.25:71.25:5 mg), were placed in a tube (12.5mm x 75mm) with the ground glass joint and dissolved in a mixture of ether-chloroform (1:0.25). Aqueous phase containing drug was added to this and the resulting two phase system was sonicated at 4-5°C using a probe sonicator at 20 KHz, 500 watts for period of 2 to 3 minutes. The clear gel formed was further sonicated after the addition of 500 μl of phosphate buffered saline (PBS). The organic phase was removed at 40°C under low pressure in two stages. The resulting viscous niosome suspension was diluted to above 2 ml with PBS such that each ml contains 75 mg of lipid vesicles. The suspension was then heated on a water bath at 60°C for 10 minutes. The BLM entrapment was determined by column chromatography.

*Comment:*

This technique for the preparation of niosomes was found suitable with the surfactants Span 40 and 60, Brij 35 and 78 and a combination of Span 60 and Poloxamer F108. With Span 20, Tween 20 and 80 and Poloxamer F108, alone a clear single phase dispersion did not form and hence niosome preparation was not successful.
3.2 Characterisation of BLM - containing niosomes and thermo-sensitive niosomes:

The mean vesicle diameter of the prepared niosomes before and after sonication was determined using Droplet and particle size analyser (Malvern 2600, UK) and later characterised by transmission electron microscopy (JEOL 100C, Japan).

3.3 Physical stability:

Size distribution analysis was the parameter chosen to assess the physical stability of the prepared niosomes. The mean vesicle diameter of the prepared niosomes was determined on day one before sonication and was determined on the 7th and 30th day after sonication using a Droplet and particle size analyzer (Malvern 2600, UK). The niosomes were then characterised by transmission electron microscopy (JEOL 100C, Japan).

3.4 Determination of vesicular drug entrapment efficiency:

Unentrapped BLM was separated from niosomes by gel filtration on Sephadex G-50 column. The column was prepared using 1.5g of Sephadex G-50 powder kept in double distilled water for 48h for swelling.

One ml of prepared niosome suspension was placed on the top of the column and elusion was carried out using normal saline. Niosome encapsulated BLM elutes out first as a slightly dense, white opalescent suspension followed by free drug. The absorbance of the unentrapped BLM was measured at 254 nm (UV spectrophotometer) after suitable dilution and on determination of the volume of BLM solution collected.
3.5 Study of drug leakage from the vesicles:

Vesicle stability for drug leakage was studied at room temperature and at 4°C in phosphate buffered saline and in plasma at 37°C and 43°C.

The separated niosomes equivalent to 5 mg/ml bleomycin was divided into two portions. One portion was kept at room temperature and the second at 4°C. At definite time intervals, they were again passed through sephadex G-50 column and the amount of drug that has leached out of the vesicle was determined spectrophotometrically. The amount of drug still remaining within the vesicle was further confirmed by treating a known quantity of the separated niosomes with 1% triton X-100 and sonicated briefly to disrupt the vesicles. The resulting mixture was centrifuged and the supernatant assayed for drug content spectrophotometrically (254 nm) against a suitable blank.

3.6 Determination of the vesicle stability:

For the determination of the vesicle stability, self quenched (100 mmol/L) purified 6-CF in PBS was entrapped instead of BLM, during the rehydration of the dry lipid film, the resultant 6-CF niosomes was used for further studies.

Thermal stability was performed by measuring the leakage of 6-CF from the niosomes incubated at 37 or 43°C in PBS with without 1% plasma collected from S-180 bearing mice. Self quenched within the niosomes, the 6-CF was not fluorescent but developed an intense fluorescence when leaching out of the niosome and diluted into the dispersion medium.

The analysis were performed on a spectrofluorimeter (Kontron, SFM 25, Germany) fitted with a thermostable cell. Excitation and emission wave lengths were 485 and 515 nm respectively. Temperatures (37 and 43°C) were programmed and controlled (within 0.1°C) by a water bath (Julabo PC 20B, Germany).
The fluorescence monitoring began when the experimental temperature was reached in the analytical cell as measured by an immersed thermocouple and was performed for 3 hours. Results were expressed as a percentage of the maximal values which were obtained by disruption of the niosomes by addition of 1% solution of Triton X-100. The 'zero' value was measured on an intact suspension at 4°C.

pH stability was assessed by incubating the 6-CF niosomal suspension diluted in 4 ml of PBS. The pH of the PBS was ranging from 6.00 to 8.00, at 37°C and 43°C, with and without 1% plasma collected from S-180 bearing mice.

4.0 Antitumor efficacy of niosomal bleomycin with and without hyperthermia:

Experimental design:

Tumor bearing animals were divided into different groups (10 animals per group) for different treatment as shown in Tables-4,5.

In case of solid tumor, treatment was started after the tumor size reached 100 ± 10 mm³. Indicated doses of free BLM (FBLM), niosomal BLM (NBLM) in saline and empty niosomes were injected intravenously and the tumor growth was monitored. While mice bearing Ehrlich ascites were treated (intravenous) 48h after tumor inoculation.

Hyperthermia at 43°C, 30 min. was applied on solid tumor bearing mice described under section 2.3.
<table>
<thead>
<tr>
<th>Sl.no.</th>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>Control</td>
<td>-</td>
</tr>
<tr>
<td>Group II</td>
<td>Empty niosomes</td>
<td>-</td>
</tr>
<tr>
<td>Group III</td>
<td>Free BLM</td>
<td>10</td>
</tr>
<tr>
<td>Group IV</td>
<td>Free BLM</td>
<td>15</td>
</tr>
<tr>
<td>Group V</td>
<td>Free BLM</td>
<td>20</td>
</tr>
<tr>
<td>Group VI</td>
<td>Niosomal BLM</td>
<td>10</td>
</tr>
<tr>
<td>Group VII</td>
<td>Niosomal BLM</td>
<td>15</td>
</tr>
<tr>
<td>Group VIII</td>
<td>Niosomal BLM</td>
<td>20</td>
</tr>
</tbody>
</table>
Table - 5

Sarcoma-180 bearing BALB/c mice for treatment with
Free BLM or Niosomal BLM or hyperthermia or in combination

<table>
<thead>
<tr>
<th>Sl.No.</th>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>Control</td>
<td>-</td>
</tr>
<tr>
<td>Group II</td>
<td>Empty niosomes</td>
<td>-</td>
</tr>
<tr>
<td>Group III</td>
<td>43°C, 30 min.</td>
<td>-</td>
</tr>
<tr>
<td>Group IV</td>
<td>Free BLM</td>
<td>10</td>
</tr>
<tr>
<td>Group V</td>
<td>Free BLM</td>
<td>15</td>
</tr>
<tr>
<td>Group VI</td>
<td>Free BLM</td>
<td>20</td>
</tr>
<tr>
<td>Group VII</td>
<td>Niosomal BLM</td>
<td>10</td>
</tr>
<tr>
<td>Group VIII</td>
<td>Niosomal BLM</td>
<td>15</td>
</tr>
<tr>
<td>Group IX</td>
<td>Niosomal BLM</td>
<td>20</td>
</tr>
</tbody>
</table>

Bimodality Treatment

| Group X     | FBLM + 43°C, 30 min.        | 10           |
| Group XI    | NBLM + 43°C, 30 min.        | 10           |

FBLM: Free bleomycin
NBLM: Niosomal bleomycin
Parameters compared:

4.1 Volume doubling time:

The time required to double the tumor volume (VDT) from 100 to 200 cu mm was taken as criterion to assess the antitumor efficacy of free and niosomal BLM in S-180 bearing mice.

4.2 Animal survival:

Mortality at different post-treatment days was recorded for each group. The actuarial survival curves were drawn by Kaplan-meier method. The last point for the evaluation in this study was 120 day survival, which is roughly equivalent to 5 year survival in man.

4.3 Life span:

For Ehrlich ascites, percent increase in life span, ILS (animal survival rate) was considered to determine the antitumor efficacy. Mortality at different post treatment drug was recorded for each group. The actuarial survival curves were drawn by Kaplan-meier method.

4.4 Assessment of cytogenetic damage in solid tumor:

The chromosome damage after the different treatment was assessed on the basis of micronucleus (MN) assay. The method of Van Beuningen et al, (1981), with some modifications as adopted by Uma Devi and Sharma et al, (1990) was followed.

Tumor was removed in toto soon after the animals were moribund by cervical dislocation. The necrotic tissue was separated and discarded. Single cell suspension of the tumor was prepared by mechanical dispersion in physiological saline and filtered through nylon mesh (45 μ) and centrifuged at 500g for 5 minutes. The pellet was resuspended in water bath at 37°C for 15 minutes. After centrifugation (500 g, 5 min), the pellet was suspended in hypotonic saline (physiological saline: DDW::1:3) and incubated at 37°C for 12 minutes. The cells were
centrifuged and the pellet was vortexed well and fixed in Carnoy's fixative (1:3: acetic acid: methanol) for 30 minutes. The cells were given more changes of fixative and finally resuspended in 30 ml of fixative, vortexed well to get a uniform suspension. Four drops of the suspension was spread over a clean slide, prechilled in acetone, and flame fixed.

**Fluorescence microscopy:**

The flame fixed MN slides were stained with two drops of 0.001% ethidium bromide, covered with a coverslip and observed under binocular fluorescence research microscope (Carl Zeiss, Switzerland). The ethidium bromide stained cell and MN give orange fluorescence. MN were scored according to the criteria of Heddle et al, (1979). The MN counted includes only those which were.

i. round in shape and had the fluorescent intensity similar to the main nucleus.

ii. lying around and within a three-diameter distance of the main nucleus; and

iii. of a size ranging from 1/5 to 1/10th of the main nucleus (Plate 3).

5.0 **Toxicity studies after treatment with free and niosomal BLM:**

5.1 **Haematological toxicity:**

Haematological toxicity of the free and niosomal BLM was determined by injecting (i.v.) 10 mg/kg of free and niosomal BLM to BALB/c mice (5 mice/group) bearing sarcoma-180, and the haematological measurements were done on 1, 3, 7 and 14 post-treatment days using a Blood analyser (Technicon H1, Belgium).

Haematological toxicity of the free and niosomal BLM was also determined by injecting (intravenous) 10 mg/kg of free and niosomal BLM to BALB/c mice. The peripheral and bone marrow WBC counts were also carried out at definite time intervals (1, 3, 5, 7 and 14 days) post injection.
Peripheral WBC were obtained by collecting the blood of BALB/c mice (5 mice/group) from the retro-orbit puncture. Total WBC count was determined using automatic blood cell counter (Sysmex, Toa Medical Electronics Co. Ltd., Kobe, Japan, Model F300).

Bone marrow WBC were obtained by flushing the tibia and femur from one leg of the mice (5 mice/group) with isotonic minimal essential media. A cell suspension was prepared by passing the bone marrow three times through a 22 guage needle and counting the WBC with a haemocytometer after suspending the pelleted cells (500 x g) in minimal essential media/acetic acid (50:50).

5.2 Pulmonary toxicity:

Free and niosomal BLM were tested for pulmonary toxicity.

Animals:

BALB/c mice weighing between 20 and 25 g (6-10 weeks old) were used for this experiment.

Treatment:

Group I served as a control and received empty niosomes containing saline injected i.v. via the tail vein. Group II was treated with free BLM and Group III with NBLM. 20 mg/kg free or niosomal BLM was injected intravenously, twice a week for a period of 4 weeks.

Four weeks after the treatment, the animals were moribund by cervical dislocation, the chests were opened and the lungs were perfused via the right ventricle with 3 ml of saline and removed and fixed in 3% glutaraldehyde buffer.
**Pathogenesis:**

The pathogenesis of BLM induced pulmonary fibrosis was investigated in detail with particular emphasis on the sequential cellular responses. There are many cell types in the lung and their reactions to injury are known to be variable. Most biochemical - toxicology evaluations treat the lung as a whole, and specific cell types responding to the injurious agent and the cellular process of repair, including cytodynamic studies of cell turnover, are not made. Hence, it is better to study these cellular events in detail by electron microscopy.

**Preparation of the sample:**

The samples fixed in 3% glutaraldehyde were then precooled and post fixed with 1% Osmium tetraoxide followed by dehydration using graded alcohols. The specimens were cleared in propylene oxide and after impregnation they were embedded in epoxy resin (Polar bed 812) in plastic moulds kept in incubator at 60°C for 24h. Ultra thin sections preceded by semi thin sectioning were done using Ultatome-5 (LKB, Ulratome-5, Sweden). Semi thin section were stained using toluene blue stain. The copper girds were stained with uranyl acetate and lead nitrate. The electron micrographs were taken using transmission electron microscope (Philips 420 ST, Netherlands). The histopathological changes in the endothelium of the pulmonary arteries, the epithelial cells, interstitium, fibrosis etc. were observed.

5.3 **Gastro intestinal toxicity (G.I. toxicity):**

Free and Niosomal BLM were tested for G.I. toxicity in BALB/c mice.

**Treatment:**

Group I served as a control and received empty niosomes containing saline injected i.v. via the tail vein. Group II was treated with free BLM and Group III with NBLM. 20 mg/kg free or niosomal BLM was injected intravenously. Each group consisted of 5 animals.
All the animals were sacrificed by cervical dislocation three days after treatment. Jejunum was excised fixed in Bouin's fluid for 24h and processed routinely for histological studies. Five micron sections were stained with haematoxyline and eosin and surviving crypts were counted under light microscope using the microcolony survival assay developed by Withers and Elkind (1970). Ten circumferences were examined for each mouse. Data were statistically evaluated by the students 't' test.

6.0 Plasma kinetics and tissue distribution profiles of free and niosomal BLM:

Plasma kinetics and tissue distribution studies were carried out in BALB/c mice, bearing sarcoma-180 tumor. Mice weighing 20-25g were distributed in two groups, each group containing 20 animals. The mice were then randomly sorted into 2 groups and treated as follows:

Group I  Free BLM
Group II  Niosomal BLM

Both the groups received drug equivalent to 10 mg/kg body weight, intravenously. Following the treatment blood samples were collected from the retro orbit puncture into heparin rinsed haematocrit tubes (from four animals in each group for each point) at predetermined time intervals of 5, 10, 15, 20, 30, 45 min. and 1, 1.5, 2, 3, 5, and 8h. The blood was allowed to stand for 5 min immediately after collection and then centrifuged to get the plasma. The plasma was treated as described earlier under the determination of calibration curve for BLM in plasma by HPLC (see Section 2.1.3).

Separate group (five mice per group) of mice were sacrificed at 4, 8 and 12h after injection of free and niosomal BLM and the various tissues - tumor, liver, kidney, lung, spleen, brain, skin and small intestine were removed and assayed for BLM.
To one gram of the tissue (weighed after drying the tissues on a coarse filter paper) one ml of water was added and tip sonicated in an ice bath for half a minute to two minutes depending on the hardness of the tissue in question. Sonication was carried out at maximum frequency tolerable by the tip, and 100% duty cycle.

The homogenate was vortexed for 5 minutes on a vortex. On vortexing 200 µl of 20% trichloroacetic acid was added to precipitate the proteins. The rest of the steps followed were similar to that adopted for determination of calibration curve for BLM in plasma of S-180 bearing mice.

7.0 Antitumor efficacy of niosomal BLM subsequent to activation of macrophages by niosomal muramyl dipeptide and tuftsin:

Niosomes containing muramyl dipeptide (MDP) and tuftsin was prepared by adopting lipid hydration technique as described under preparation of niosomes. Ehrlich ascites tumor model was used to assess the antitumor efficacy.

Treatment:

BALB/c mice bearing Ehrlich ascites were divided into 3 groups (10 animals per group). Group I received niosomal BLM 10 mg/kg (i.v.) on the seventh day post tumor inoculation. Group II and Group III were treated with 2 doses of niosomal tuftsin (50 μg/mouse/dose, i.v.) and niosomal MDP (100 μg/mouse/dose, i.v.) respectively on fifth and sixth day post tumor inoculation and on the seventh day both the groups received 10 mg/kg niosomal BLM through lateral tail vein injection. Mortality at different post treatment days was recorded for each group and the results were statistically analysed using Kaplan-meier survival test.
To activate and exploit macrophages in delivering the NBLM more quantitatively to the tumor site, three groups of animals (ten animals per group weighing between 22-25 g) bearing S-180 solid tumor were taken for the study. Solid tumor for the experiment was produced as per the method described in section 2.2. Animals bearing tumors of size 100 ± 10 mm³ were selected for the experiment.

Treatment regimen:

Group I received one injection (10 mg/kg, i.v.) of FBLM. Group II received one injection (10 mg/kg, i.v.) of NBLM. Group III was injected two doses of MDP (100 μg/mouse/dose) on two consecutive days and followed by a single injection of NBLM (10mg/kg, i.v.) on the third day.

Tumor was excised from all the animals at 1, 2, 4, 8, 12, and 24h after the injection (in cases of Group III after the last injection). Tumor was processed similarly as done earlier in section 6.

8.0 Preparation of thermosensitive niosomes encapsulating BLM:

The non-ionic surfactant Span 60 was chosen for the preparation because it showed better entrapping efficiency and was found to form good thin film. Lipid hydration method was found to be suitable since it is more stable over a period of time.

8.1 Lipid hydration method (Azmin et al, 1985):

Thermosensitive niosomes were prepared exactly as that of thermosensitive liposomes but with the addition of a surfactant. Earlier, phase transition temperature of DPPC/DSPC/Cholesterol/surfactant (5:4:2:1) was estimated by monitoring the leakage of encapsulated 6-CF throughout the vesicles when exposed to a temperature increasing from 30° to 50° at 1°C/min. rate as programmed on the temperature controller. The phase transition temperature was estimated and found to be 42.7°C.
Thermo-sensitive niosomes were prepared by dissolving Dipalmitoyl phosphatidyl choline (DPPC), distearoyl phosphatidyl choline (DSPC), cholesterol (CHOL) and surfactant (Span 60) (5:4:2:1) in chloroform and the method followed for the preparation is similar to that mentioned in section 3.1.1.

8.2 Size analysis of the prepared niosomes:

The mean vesicle diameter of the prepared niosomes before and after sonication was determined using Drop and particle size analyser and latter characterised by electron microscopy (JEOL 100C, Japan).

8.3 Determination of vesicular drug entrapment efficiency:

The entrapment efficiency of thermosensitive was determined on the same lines as described under section 3.4.

8.4 Study of drug leakage from the vesicles:

Vesicle stability for drug leakage was studied at room temperature and at 4°C in phosphate buffered saline and in plasma at 37 and 43°C. The separated thermo-sensitive BLM niosomes equivalent to 5 mg/ml was divided into 2 portions. One portion was kept at room temperature and the other at 4°C. The drug leakage studied thereafter was studied as per the method reported in section 3.5.

8.5 Determination of the vesicle stability:

For the determination of vesicle stability, self quenched (100 mmol/L) purified 6-CF in PBS was entrapped instead of BLM, during the rehydration of the dry lipid film. The resultant 6-CF was used for further studies. The thermal and pH stability studies was performed on the
9.0 Antitumor efficacy of thermosensitive niosomal BLM (Th.sen NBLM) with and without hyperthermia:

**Experimental design:**

Tumor bearing animals were divided into different groups (10 animals per group) of different treatment as shown in Table-6,7. In case of solid tumor, treatment was started after the tumor size reached $100 \pm 10 \text{ mm}^3$. Indicated doses of Free BLM and Th.sen NBLM in saline and empty niosomes were injected intravenously via the tail vein and the tumor growth monitored. While mice bearing Ehrlich ascites were treated (intravenous via tail vein) 48h after tumor inoculation. Hyperthermia was induced in solid tumor bearing mice as reported in section 2.3.

**Parameters compared:**

9.1 Volume doubling time.
9.2 Animal survival.
9.3 Life span.

The methods to assess the above were done on the same lines described in sections 4.1, 4.2 and 4.3.

10.0 Plasma kinetics and tissue distribution profiles of free and thermosensitive niosomal BLM, with and without HT:

Plasmakinetics and tissue distribution studies were carried out in BALB/c mice bearing sarcoma-180 tumor. Hyperthermia at $43^\circ\text{C}$, 30 min. was applied to the tumor as described under section 2.3.
Table - 6

Ehrlich ascites bearing BALB/c mice for treatment with Free BLM and Thermo-sensitive Niosomal BLM

<table>
<thead>
<tr>
<th>Sl.No.</th>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>Control</td>
<td>-</td>
</tr>
<tr>
<td>Group II</td>
<td>Empty niosomes</td>
<td>-</td>
</tr>
<tr>
<td>Group III</td>
<td>Free BLM</td>
<td>10</td>
</tr>
<tr>
<td>Group IV</td>
<td>Th.sen NBLM</td>
<td>10</td>
</tr>
</tbody>
</table>

Th.sen NBLM : Thermo-sensitive niosomal bleomycin
Table - 7

Sarcoma-180 bearing BALB/c mice for treatment with Free BLM or Thermo-sensitive Niosomal BLM or hyperthermia or in combination

<table>
<thead>
<tr>
<th>Sl.No.</th>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>Control</td>
<td>-</td>
</tr>
<tr>
<td>Group II</td>
<td>Empty niosomes</td>
<td>-</td>
</tr>
<tr>
<td>Group III</td>
<td>43°C, 30 min.</td>
<td>-</td>
</tr>
<tr>
<td>Group IV</td>
<td>Free BLM</td>
<td>10</td>
</tr>
<tr>
<td>Group V</td>
<td>Th. sen NBLM</td>
<td>10</td>
</tr>
</tbody>
</table>

Bimodality Treatment

<table>
<thead>
<tr>
<th>Sl.No.</th>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group VI</td>
<td>Free BLM + 43°C, 30 min.</td>
<td>10</td>
</tr>
<tr>
<td>Group VII</td>
<td>Th. sen NBLM + 43°C, 30 min.</td>
<td>10</td>
</tr>
</tbody>
</table>

Th. sen NBLM : Thermo-sensitive niosomal bleomycin
Mice weighing 20-25 g were distributed in two groups, each group containing 20 animals. The mice were then randomly sorted into two groups and treated as follows.

**Group I**  Thermosensitive NBLM
**Group II**  Thermosensitive NBLM + 43°C, 30 min.

Both the groups received drug equivalent to 10 mg/kg body weight, intravenously.

Following the treatment blood samples were collected from retro orbit puncture into heparin rinsed haemtocrit tubes (from 4 animals in each group for each time point) at predetermined time intervals of 5, 10, 15, 20, 30, 45 min and 1, 1.5, 2, 3, 5, and 8h.

The blood was allowed to stand for 5 min. immediately after collection and then centrifuged to get the plasma. The plasma was treated as described earlier under the determination of calibration curve for BLM in plasma by HPLC.

Separate groups (five mice per group) of mice were sacrificed at definite time intervals (4 and 24h) after injection of free and thermosensitive niosomal BLM and then various tissues - tumor, liver, kidney, lung, spleen, brain, skin and small intestine were removed and assayed for BLM. The organs removed were processed in similar lines as described under section 6.

11.0 Antitumor efficacy of thermo-sensitive niosomal BLM subsequent to activation of macrophages by niosomal muramyl dipeptide and tufts:in:

Thermo-sensitive niosomes containing muramyl dipeptide (MDP) and tuftsin was prepared by adopting lipid hydration technique as described under preparation of niosomes. Ehrlich ascites tumor model was used to assess the antitumor efficacy.
**Treatment:**

BALB/c mice bearing Ehrlich ascites were divided into 3 groups (10 animals per group). Group I received thermo-sensitive niosomal BLM 10 mg/kg (i.v.) on the seventh day post tumor inoculation. Group II and Group III were treated with 2 doses of thermo-sensitive niosomal tuftsin (50 µg/mouse/dose, i.v.) and thermo-sensitive niosomal MDP (100 µg/mouse/dose, i.v.) respectively on fifth and sixth day post tumor inoculation and on the seventh day both the groups received 10 mg/kg niosomal BLM through lateral tail vein injection. Mortality at different post treatment days was recorded for each group and the results were analysed using Kaplan-meier survival test.

To activate and exploit macrophages in delivering thermo sensitive NBLM more quantitatively to the tumor site, three groups of animals (ten animals per group weighing between 22-25 g) bearing S-180 solid tumor were taken for the study. Solid tumor for the experiment was produced as per the method described in section 2.2. Hyperthermia at 43°C, 30 min. was applied to the tumor as described under section 2.3.

Animals bearing tumors of size 100 ± 10mm³ were selected for the experiment.

**Treatment regimen:**

Group I received one injection of Th.sen NBLM (10 mg/kg, i.v.). Group II received one injection (10 mg/kg, i.v.) of thermo sensitive NBLM in combination with HT. Group III was injected two doses of MDP (100 µg/mouse/dose) on two consecutive days and followed by a single injection of thermo sensitive NBLM (10 mg/kg, i.v.) in combination with HT on the third day.

Tumor was excised from all the animals at 1, 2, 4, 8, 12, and 24h after the injection (in case of Group III after the last injection). Tumor was processed similarly as done earlier in section 6.
12.0 Plumbagin (PLM):

12.1 Preparation of plumbagin niosomes (NPLM):

The non ionic surfactant Span 60 was chosen for the preparation. Lipid hydration of technique was followed for the preparation of niosomal PLM but with certain modifications.

*Preparation of drug solution:*

A known quantity (10 mg) of PLM was dissolved in a minimum quantity of absolute alcohol (300 μl) and the volume was made up to 3 ml with phosphate buffered saline containing 30% PEG 400.

*Lipid hydration method (Azmin et al, 1985):*

Niosomes were prepared by dissolving cholesterol, surfactant (Span 60) and dicetyl phosphate (47.5:47.5:5) (71.2:71.2:7 mg) in chloroform and evaporating the solvent using rotary flash evaporator, under low pressure at 40 - 50°C. Niosomes were formed by adding the above mentioned PLM solution part by part to the dried thin film formed on the walls of the round bottom flash heated to about 40-50°C on a water bath with gentle agitation. The mixture was intermittently mixed on a vortex to get a good dispersion of the mixture. Sonic dispersion of the mixture was carried out at 25°C using a probe sonicator, 20 KHz, 500 watts vibra-cell, (Sonics and Materials Inc. Co., USA) for 30 seconds at one minute intervals for a period of four minutes. After sonication, the suspension was maintained at room temperature for 2 hours to allow niosomes to form and seal.

12.2 Determination of vesicular drug entrapment efficiency:

Unentrapped PLM was separated from niosomes by the method described under section 3.4.
13.0 Acute toxicity studies with PLM:

The toxicity of various doses of plumbagin formulation was investigated by determining the apparent LD$_{50}$ of drug administered via the tail vein injection to BALB/c mice (20-25g). Group of 10 mice per dose were monitored over 14 days and the deaths were noted.

Group 1 Solvent control was injected i.v. with 0.2 ml of PEG 400 empty niosomes.

Group 2-6 were injected i.v. with plumbagin at single doses of 4, 6, 8, 10 and 12 mg/kg.

Group 7-12 were injected i.v. with niosomal plumbagin (NPLM) at single doses of 4, 6, 8, 10, 12 and 14 mg/kg.

Following the injections, the animals were placed in separate cages for close observation. The toxic effect was assessed on the basis of mortality.

14.0 Antitumor efficacy of niosomal plumbagin:

Experimental design:

Tumor bearing animals were divided into different groups (20 animals per group in case of Ehrlich ascites and 10 animals per group for solid tumor) for different treatment as shown in Table-8,9.

In case of solid tumor, treatment was started after the tumor size reached 100 ± 10 mm$^3$. Indicated doses of free PLM (FPLM), niosomal PLM (NPLM) in saline and empty niosomes were injected intravenously and the tumor growth was monitored, while mice bearing Ehrlich ascites were treated (intravenous) 48h after tumor inoculation.
Table - 8

Ehrlich ascites bearing BALB/c mice for treatment with Free PLM and Niosomal PLM

<table>
<thead>
<tr>
<th>Sl.No.</th>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>Control</td>
<td>-</td>
</tr>
<tr>
<td>Group II</td>
<td>Empty niosomes</td>
<td>-</td>
</tr>
<tr>
<td>Group III</td>
<td>Free PLM</td>
<td>2</td>
</tr>
<tr>
<td>Group IV</td>
<td>Free PLM</td>
<td>3</td>
</tr>
<tr>
<td>Group V</td>
<td>Free PLM</td>
<td>4</td>
</tr>
<tr>
<td>Group VI</td>
<td>Free PLM</td>
<td>5</td>
</tr>
<tr>
<td>Group VII</td>
<td>Free PLM</td>
<td>6</td>
</tr>
<tr>
<td>Group VIII</td>
<td>Niosomal PLM</td>
<td>2</td>
</tr>
<tr>
<td>Group IX</td>
<td>Niosomal PLM</td>
<td>3</td>
</tr>
<tr>
<td>Group X</td>
<td>Niosomal PLM</td>
<td>4</td>
</tr>
<tr>
<td>Group XI</td>
<td>Niosomal PLM</td>
<td>8</td>
</tr>
<tr>
<td>Group XII</td>
<td>Niosomal PLM</td>
<td>10</td>
</tr>
</tbody>
</table>

Number of animals used per group: 10
### Table - 9

**Sarcoma-180 bearing BALB/c mice for treatment with Free PLM and Niosomal PLM**

<table>
<thead>
<tr>
<th>Sl.No.</th>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>Control</td>
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<tr>
<td>Group II</td>
<td>Empty niosomes</td>
<td>-</td>
</tr>
<tr>
<td>Group III</td>
<td>Free PLM</td>
<td>2</td>
</tr>
<tr>
<td>Group IV</td>
<td>Free PLM</td>
<td>3</td>
</tr>
<tr>
<td>Group V</td>
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<td>4</td>
</tr>
<tr>
<td>Group VI</td>
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<td>6</td>
</tr>
<tr>
<td>Group VII</td>
<td>Niosomal PLM</td>
<td>2</td>
</tr>
<tr>
<td>Group VIII</td>
<td>Niosomal PLM</td>
<td>3</td>
</tr>
<tr>
<td>Group IX</td>
<td>Niosomal PLM</td>
<td>4</td>
</tr>
<tr>
<td>Group X</td>
<td>Niosomal PLM</td>
<td>6</td>
</tr>
<tr>
<td>Group XI</td>
<td>Niosomal PLM</td>
<td>8</td>
</tr>
<tr>
<td>Group XII</td>
<td>Niosomal PLM</td>
<td>10</td>
</tr>
</tbody>
</table>
Parameters compared:

14.1 Volume doubling time:

The time required to double the tumor volume (VDT) from 100 to 200 cu mm was taken as criterion to assess the antitumor efficacy of free and niosomal PLM in S-180 bearing mice.

14.2 Life span:

For Ehrlich ascites, percent increase in life span ILS (animal survival rate) was considered to determine the antitumor efficacy. Mortality at different post treatment days was recorded for each group.