CHAPTER II

MATERIALS AND METHODS
2.1 MATERIALS

2.1.1 Plants

*B. racemosa* seed was collected from its natural habitat in the forest of Trivandrum District, Kerala, India (Plate 1&2). The seeds were locally available. A voucher specimen of the plant was identified by taxonomists of Kerala University. A sample specimen is preserved in the herbarium of Botany Department, University College, Thiruvananthapuram. [Specimen no: 1149]

2.1.2 Experimental animals

Inbred strains of Swiss albino mice were obtained from the Sree Chithira Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram, Kerala.

2.1.3 Tumour Cell Lines

**Daltons Lymphoma ascities (DLA):** which arose as a spontaneous carcinoma of the mouse thymus (DBA mice) in 1947.

**Ehrlich ascities carcinoma (EAC):** The initial tumours for the Ehrlich solid adeno carcinoma was found by Paul Ehrlich as spontaneous cancer of the mammary gland of mice tat developed in 1905. The ascitic variant Ehrlich tumour was produced by the i.p. transplantation of Ehrlich solid adenocarcinoma in 1932.

**HeLa cell-line:** These cells have been reported to contain human papilloma virus 18 (HPV-18) sequences. $p^53$ expression was reported to be low, and normal levels of $p^{RB}$ (retinoblastoma suppressor) were found. (Gey GO *et al* 1952).

**SiHa Cell-line:** This line was established from fragments of a primary tissue sample obtained after surgery from a Japanese patient. Electron microscopic observations revealed presence of typical desmosomes at the cell junctions and as abundance of tonofilaments in the cytoplasm. Mycoplasma
Plate No. 1

Barringtonia racemosa plant

Barringtonia racemosa seeds
Barringtonia racemosa plant bearing flowers and fruits

A close up of the flower of Barringtonia racemosa
contamination was detected and eliminated in 1975. The line is reported to contain an integrated human papilloma virus type 16 genome (HPV-16, 1 to 2 copies per cell). (Baker C C et al/1987).

**Vero cell line:** The Vero cell line was initiated from the kidney of a normal adult African green monkey on March 27, 1962, by Y. Yasumura and Y. Kawakita at the Chiba University in Chiba, Japan. (Vanderzant et al 1996). The cell line was brought to the Laboratory of Tropical Virology, National institute of Allergy and Infectious Diseases, National Institute of Health in the 93rd passage for Chiba University by B. Simizu on June 1964.

**L929 cell line:** L929 cell line was initiated from the normal subcutaneous areolar and adipose connective tissue of a male C3H/An mouse; used as target in TNF detection assays confirmed as mouse with IEF of AST, MDH, PEP B. Morphology consists of fibroblasts growing as monolayer.

DLA and EAC cell lines were obtained from Amala Cancer Research Center, Trichur, Kerala. HeLa, SiHa and L929 cell lines were kindly provided by Dr. K. Karunakaran, Dept. of Ethnopharmacology, Rajiv Gandhi Institute, Poojappura, Thiruvananthapuram, Kerala. Vero cells were kindly provided by Dr. Molly Thomas, Dept. of Microbiology, Sree Chithira Tirunal Institute, Thiruvananthapuram.

### 2.1.4 Chemicals

Various chemicals were procured from different sources as stated below.

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPMI-1640</td>
<td></td>
</tr>
<tr>
<td>Eagle’s minimum essential medium (MEM)</td>
<td>Hi-Meia Laboratories</td>
</tr>
<tr>
<td>Dulbeccos modified eagle’s medium (DMEM)</td>
<td></td>
</tr>
<tr>
<td>Antibiotics</td>
<td></td>
</tr>
<tr>
<td>Chemical</td>
<td>Source</td>
</tr>
<tr>
<td>------------------------------------------</td>
<td>-------------------------------------------------</td>
</tr>
<tr>
<td>Foetal Calf Serum (CS)</td>
<td></td>
</tr>
<tr>
<td>Trypsin</td>
<td></td>
</tr>
<tr>
<td>EDTA</td>
<td>-Sigma Chemical Company, USA</td>
</tr>
<tr>
<td>Trypan blue</td>
<td></td>
</tr>
<tr>
<td>Ethidium bromide</td>
<td></td>
</tr>
<tr>
<td>Acridine Orange</td>
<td></td>
</tr>
<tr>
<td>Phytohaemagglutinin</td>
<td>- DIFCO, USA</td>
</tr>
<tr>
<td>Colchicine</td>
<td>- DIFCO, USA</td>
</tr>
<tr>
<td>Vincristine</td>
<td>- Cipla</td>
</tr>
<tr>
<td>Giemsa</td>
<td>- Qualigens Finechemicals</td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>- Khandelwal Laboratories, Bombay</td>
</tr>
<tr>
<td>Silica gel</td>
<td>- Merck, India</td>
</tr>
</tbody>
</table>

All other chemicals, solvents and reagents used of analytical reagent grade.

2.1.5 Instruments

Following instruments were used for the present study.

a. Spectrophotometer -Baush and Lomb, Spectronic 100%

b. Inverted microscope -Leitz, Germany

c. Laminar flow hood -Klenziads, Bombay

d. Deep freezer (-80°C, -40°C and -20°C)

e. Freeze-Dryer -LABCONCO

f. Vacuum rotary evaporator -York, Scientific Industries, Delhi

g. Remi cooling centrifuge -Consortium, Madras-2
h. Incubator - NSW, India
i. Rotary microtome - Reichert Histostat, USA
j. Rota vapour - York Scientific Industries, Delhi

2.2 METHODS

2.2.1 Methods for separation and purification of phytochemicals

Phytochemical methods involve techniques for separation, purification and identification of the many different constituents present in plants. It is mainly carried out by using one or other or a combination of four chromatographic techniques, paper chromatography (PC), thin layer chromatography, column chromatography (CC) and gas liquid chromatography (GLC). The choice of the technique depends largely on the solubility properties and volatilities of the compounds to be separated. PC is applicable to water-soluble plant constituents, like carbohydrates and phenolic compounds. TLC and CC are used for separating all liquid soluble components like lipids, steroids, terpenoids etc. GLC finds its main application with volatile compounds, fatty acids etc. All the above techniques can be used on a micro and macro-scale. For preparative work, TLC is carried out on thick layer of adsorbents. For isolation on an even larger scale than this, it is usual to use column chromatography to yield purified components in gram amounts (Harbone 1973).

2.2.2 Extraction and Purification of *B. racemosa* seeds

The *B. racemosa* seeds were subjected to extraction procedures using various solvents in the order of their polarity. The screening for cytotoxic effect was carried out after evaporating the solvents completely and reconstituting the residue in Phosphate buffered saline.

2.2.2.1 *B. racemosa* seed extract

The seeds were washed, dried and powdered. The seed powder (300 gm) was subjected to extraction with various solvents of hexane,
chloroform, ethyl acetate, methanol and water. The extract in each solvent was filtered and residue re-extracted with the same solvent (thrice). The pooled extracts in each solvent were then evaporated by rota vapour. The yield of solvent free extracts for each solvent as percentage w/w relative to dry starting material were hexane (0.2%), chloroform (0.67%), ethyl acetate (1.7%), methanol (6.7%) and water (8.3%). The extracts in each solvent is referred to as the crude extract. Among this methanol, extract was found to be most cytotoxic against DLA and EAC cell lines. Hence, this extract was purified and used for further studies.

2.2.2.2 Purification of the methanol extract of B.racemosa seed extract

The crude methanol extract (7 gm) was subjected to column chromatography (50cmx15cm) over 150 gm of silica gel (60-120 mesh, Merck) and eluted with hexane, chloroform, chloroform: methanol in different ratios, methanol and water as eluents.

Different types of mobile phases were used for purification; Fraction 1-5 is hexane extractable portion; Fraction 6-10 is chloroform extractable portion, Fraction 11-54 is chloroform:methanol extractable portion in different ratios; Fraction 55-59 is methanol extractable portion; Fraction 60-70 is water extractable portion.

All the 70 collected fractions were evaporated by rota vapor and tested in vitro for their cytotoxicity which appeared increase in the chloroform: methanol extractable portions 26-29 (F5-7:3) and 47-53 (F8-2:8). These were combined and evaporated by rota vapor (Roussakis et al 1994; T.D Babu et al 1995; Kuo-Hsiung Lee et al 1987). These two fractions such as F5 (7:3) and F8 (2:8) are referred to as the purified fractions.
Table - 6
Column chromatographic purification of *B.racemosa* seed extract

<table>
<thead>
<tr>
<th>Sl.No.</th>
<th>Mobile Phase</th>
<th>Ratio</th>
<th>Fractions</th>
<th>Total No.of Volume Collected</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Hexane</td>
<td>1:0</td>
<td>1-5</td>
<td>500ml (100ml each)</td>
</tr>
<tr>
<td>2.</td>
<td>Chloroform</td>
<td>1:0</td>
<td>6-10</td>
<td>500ml (*)</td>
</tr>
<tr>
<td>3.</td>
<td>Chloroform: Methanol</td>
<td>9:1</td>
<td>11-17</td>
<td>700ml (*)</td>
</tr>
<tr>
<td>4.</td>
<td>Chloroform: Methanol</td>
<td>8:2</td>
<td>18-24</td>
<td>700ml (*)</td>
</tr>
<tr>
<td>5.</td>
<td>Chloroform: Methanol</td>
<td>7:3</td>
<td>25-31</td>
<td>600ml (*)</td>
</tr>
<tr>
<td>6.</td>
<td>Chloroform: Methanol</td>
<td>6:4</td>
<td>32-36</td>
<td>500ml (*)</td>
</tr>
<tr>
<td>7.</td>
<td>Chloroform: Methanol</td>
<td>4:6</td>
<td>37-43</td>
<td>700ml (*)</td>
</tr>
<tr>
<td>8.</td>
<td>Chloroform: Methanol</td>
<td>2:8</td>
<td>44-54</td>
<td>1100ml (*)</td>
</tr>
<tr>
<td>9.</td>
<td>Methanol</td>
<td>1:0</td>
<td>55-59</td>
<td>500ml (*)</td>
</tr>
<tr>
<td>10.</td>
<td>Methanol: Water</td>
<td>1:1</td>
<td>59-66</td>
<td>800ml (*)</td>
</tr>
<tr>
<td>11.</td>
<td>Water</td>
<td>1:0</td>
<td>67-70</td>
<td>400ml (*)</td>
</tr>
</tbody>
</table>

### 2.3 Toxicity studies

#### 2.3.1 Acute toxicity
Six groups of mice weighing 20-25gm were used for the study. Various concentrations of *B.racemosa* seed extract were given intraperitoneally to each group containing 10 mice (5 males and 5 females). The behavioral parameters of mice were observed for 1 hr and intermittently for the next 6 hrs after dosing. LD$_{50}$ was calculated graphically. Detailed procedure was mentioned in section 3.2.4.1.

#### 2.3.2 Short-term toxicity
Four groups of mice weighing 20-25gm were used for the experiment. The first group was used as control. The remaining groups were given various concentrations of *B.racemosa* seed extract i.p. daily for 15
days. Body weight, food and water intake and general behavior were monitored. Haematological and biochemical parameters were also evaluated. Detailed procedure was mentioned in section 3.2.4.2.

2.4 Assessment of anticancer activity

The crude and purified fractions of *B. racemosa* seed extract were obtained by the phytochemical procedures (see 2.2.2.2 and 2.2.2.1) were tested for *in vitro* cytotoxicity assays, as stated in 3.2.3 and 3.2.5; using ascitic tumour cells, cells in culture.

2.4.1 Experimental Animal Maintenance

Inbred strains of Swiss albino mice (7-8 weeks old, male) weighing 20-25gm were used for all experiments. They were maintained on normal mouse chow (Lipton, India) and water *ad libitum* and were housed in ventilated cages.

2.4.2 Maintenance of tumour cell lines in mice

Ehrlich ascities carcinoma (EAC) tumour cells were gifted by the Cancer Research Institute, Bombay. Daltons Lymphoma ascetics (DLA) were obtained from Cancer Institute, Adayar, Madras. EAC and DLA cells were propagated in the peritoneal cavity of mice by transplanting 1 million DLA cells/ml of PBS. Briefly, the tumour cells were aspirated from the tumour bearing mice, aseptically. The cells were then washed with phosphate buffered saline (PBS) thrice. One million cells in 0.5 ml PBS were injected i.p. to obtain ascites tumours. Palpable tumours appear in a span of 7-12 days in case of these tumours. The untreated control mice survived a maximum up to 23 to 25, 15-16 in the case of both DLA and EAC, respectively. One million cells in 0.1 ml PBS were injected to obtain subcutaneous (solid) tumours in mice.

2.5 Tissue Culture

Dulbeccos MEM and RPMI 1640 were the medium used for culturing the cells. Medium was prepared by dissolving the medium in sterile triple
distilled water. Sodium bicarbonate was added to the medium with stirring and the pH adjusted to 7.2. Foetal calf serum (15%) was added to the medium and the medium was then filter-sterilized under positive pressure using nitrogen gas. Maintenance and sub culturing of the cells were done according to the standard procedure (Freshney et al). In brief, medium was drained from confluent bottles and monolayer of cells was rinsed with PBS-EDTA (0.02%). Trypsin (0.2%) was added to the cells and incubated for 5-10 min. at 37ºC. After the cells were detached, fresh medium supplemented with 10% FCS, 1000 IU/ml of penicillin and 0.1mg/ml streptomycin were added and cells were dispersed by repeated pipetting. The cell number were counted using a haemocytometer and 0.2 million cells were added to fresh culture bottles containing the complete medium and incubated at 37ºC. The cells would become confluent and ready for subculture by days.

2.5.1 Determination of cytotoxicity using cell culture

The antitumour activity of *B.racemosa* seed extract against four different tumour cell-lines in vitro was determined by an MTT based chemosensitivity assay (Mosmann 1983). The human cell lines HeLa and SiHa were grown in Dulbeccos modified eagles medium (DMEM) supplemented with 10% FCS. Vero cells were grown in modified Eagle medium (MEM) supplemented with 10% FCS. L929 cell line was maintained in RPMI 1640 medium supplemented with 15% goat serum. The cells (0.3-5x10^4/ml) were plated in a 96-well micro plate in a volume of 180µl. 20µl of various concentrations of drugs were added to each well and the cells were cultured for 4 days at 37ºC in an atmosphere of 5% CO₂ in air. MTT (20µl, 5mg/ml dissolved in RPMI medium without FCS) was added to each well and the plates were incubated for 4 hours. The plates were then centrifuged (820g, 15min.) prior to removal of the medium and MTT, and then the MTT-formozan crystals were dissolved in DMSO (100µl/well) and the absorbance was measured at 570nm in a multi-well plate render (Geran *et al* 1972).
2.5.2 Short-term Cytotoxicity

Short-term cytotoxicity (in vitro) of the drug was tested using Dalton's lymphoma ascites (DLA) and Ehrlich ascites carcinoma (EAC) cells. Tumour cells were aspirated from ascites tumour bearing mice (Swiss albino mice for DLA and EAC). The cells were washed thrice with normal sterile phosphate buffered saline and the viability checked by the Trypan blue exclusion method (Kaltenbach et al 1958). One million cells were incubated with various concentrations of the drug in a total volume of 1 ml phosphate buffered saline (PBS) at 37°C for 3 hours. After incubation, the percentage of live cells was evaluated by the Trypan blue exclusion method.

2.6 Anti tumour testing using murine models

Inbred strains of Swiss albino mice (males) weighing 18-20gm and 8-10 weeks old were used for testing the antitumour efficiency of drugs. Ascities (fluid) tumours (DLA and EAC) were induced in these animals by transplanting 1 million tumour cells i.p. The control (untreated) group received sterile phosphate buffered saline after tumour inoculums for a period of 14 days. Oral administration of the extracts involved the use of a gavage. Solid tumours were induced by subcutaneous injection of one million tumour cells on the right hind leg of mice (Belijanski et al). Tumour volume was measured to assess the inhibition in the growth of tumour. The detailed procedure is given in 3.2.3. In the ascites model, the survival rate and the increase in life span were the parameters used to assess the anti tumour efficacy of the drug. The increase in the life span (ILS) of mice was calculated using the formula

\[ \text{ILS} \% = \frac{T-C}{C} \times 100 \]

where \( T \) = Mean survival time of treated mice

\( C \) = Mean survival time of control mice, expressed in days.
2.7 Determination of Apoptosis

2.7.1 In vitro study

It was carried out by incubating $1 \times 10^6$ DLA cells in RPMI 1640 with 5% FCS containing various concentrations of the purified fraction $F_8 (2:8)$ in a CO$_2$ incubator at 37°C for 6 and 12 hrs.

2.7.2 In vivo study

A group of 18 male mice was divided into three groups containing 6 mice each. The first group was used as control. The second and third was given 3 and 6mg/kg respectively for 16 days. On the 14$^{th}$ day, the cells were aspirated from the peritoneal cavity of mice 4hrs after drug administration using a syringe. Similarly on the 16$^{th}$ day, the same procedure was repeated and evaluated apoptotic cells.

The microscopic observation of apoptotic cells was performed as previously reported by Kirsh Volders et al, 1997. Briefly, after 6 and 12 hours of treatment, the cells were methanol-fixed and seeded on glass slides, then stained with Giemsa 10% solution and examined by optical microscope. At least 1000 cells were blind scored for each experimental point. The same DLA cells were also stained by Ethidium bromide and Acridine orange (Haque et al 1997). Detailed procedure is mentioned in 5.4.6 b.

2.8 Chromosomal aberration studies

Chromosomal aberration studies were carried out by using human blood obtained from five healthy donors (non-smokers). The samples were collected in heparinised tube and processed for anticlastogenic effect. There are two culture conditions. In culture A, cells were treated with various concentrations of $B.racemosa$ seed extract in different time intervals to study the chromosomal aberrations. In culture B, the mutagen bleomycin was added to induce chromosomal breakage according to the method of Hsu et al and this was considered as a control or standard condition. The total
incubation time for all these cultures were 72hrs at 37°C in a 5% CO₂ incubator (Hsu TC et al 1991). The detailed procedure was mentioned in 6.2.

2.9 Modulation of the toxicity induced by the antitumour drugs
cyclophosphamide and vincristine

The modulatory effects of B. racemosa seed extract on cyclophosphamide and vincristine induced toxicity were studied as follows.

2.9.1 Modulation of Vincristine induced toxicities in normal mice

Four groups of mice as stated in 2.8.2 were used for this study. The details of the procedure for the experiment are mentioned in 7.2.5.

2.9.2 Modulation of toxicities in tumour bearing mice treated
Vincristine

The protocol was followed as given in 7.2.6. All the mice were inoculated with 1x10⁶ Daltons Lymphoma ascites cells i.p. on day 0. Treatment schedule was started twenty-four hours after the tumour inoculums.

2.9.3 Modulation of cyclophosphamide induced toxicities in normal
mice

Four groups of mice as stated in 2-6-2 were used for this study. The details of the procedure for the experiment are mentioned in 7.2.3.

2.9.4 Modulation of toxicities in tumour bearing mice treated with
Cyclophosphamide

The protocol was followed as given in 7.2.4. All the mice inoculated with 1x10⁶ DLA cells i.p. on day 0. Treatment schedule was stated 24 hours after tumour inoculums.

The blood for haematological and biochemical estimations was collected from the caudal vein of 4 animals from each of the treated and control group. The details of the haematological and biochemical parameters are as follows.
2.9.5 Haematological parameters

Haematological parameters were determined by Coulter Counter.

2.9.5.1 Method

The Coulter method accurately counts and sizes cells by detecting and measuring changes in electrical resistance. When a particle (such as a cell) in conductive liquid passes through a small aperture. As each cell goes through the aperture, it impedes the current and causes a measurable pulse. The number of pulse signals the number of particles. The height of each pulse is proportional to the volume of that particle while the number of pulses indicates particle count, the amplitude of the electrical pulse produced depends n the cell’s volume. Theoretical analysis of the behavior of particles within an aperture shows that the height of the electrical pulse produced by the cell is the characteristic that most nearly shows proportional to the cell volume (Eckhoff RF 1967, Grover NB 1972, Waterman CS et al 1974.

2.9.5.2 Red and White cell counting

Each bath has an aperture one for counting RBC/Plt and one for counting WBC. The counts take place concurrently the system draws the WBC dilution through the WBC aperture while it draws the RBC/Plt aperture. The system counts for 3 consecutive periods of 4 seconds each.

During the RBC count, pulses that represent cells of 36FL or greater are classified as red cells. During the WBC count, pulses that represent cells of 35FL or greater are classified as white cells. Both counts then go to the computer for coincidence correction and voting. The count cycle is mentioned for abnormal variations using the aperture alert.

2.9.5.3 Platelets count and size distribution

During the RBC sensing pulses from 2FL to 20FL are classified as platelets. To ensure that platelet count accurately reflect the cell population whenever the platelets data accumulation is below a pre determined value, platelet sensing is extended for up to 3 second sensing period. The
extended time is taken in to consideration in the platelet calculations. Platelet pulses are sorted by size in to 64 channels to produce a platelet histogram. The computer then checks to see if platelet distribution fits the curve criteria that represent the platelets from 0FL to 70FL. If the curve criteria are not met, there is a no fit condition and an *flag (Review results) appears in the flag area.

2.9.5.4 Measurement of hemoglobin concentration

The system uses the lysed WBC dilution to measure Hgb. The absorbance of light from an incandescent lamp is measured at 525nm through the optical path length of the bath. A beam of light from the lamp passes through the samples, through a 525nm filter and is measured by a photodiode. The signal is amplified and the voltage is measured and compared to the blank reference reading.

2.9.5.5 Derivation of parameters

**WBC count:** WBC is the number of leukocytes measured directly, multiplied by a calibration constant. Expressed in thousands of leukocytes per μl of whole blood.

\[
\text{WBC} = n \times 10^3 \text{ cells/μl}
\]

**RBC count:** RBC is the number of erythrocytes measured directly, multiplied by a calibration constant. Expressed in thousands of erythrocytes per μl of whole blood.

\[
\text{RBC} = n \times 10^6 \text{ cells/μl}
\]

**Platelet (Plt) count:** Plt is number of thrombocytes derived from directly measured plt pulses, multiplied by a calibration constant. Expressed in thousands of thrombocytes per μl of whole blood.

\[
\text{Plt} = n \times 10^3 \text{ cells/μl}
\]
Hemoglobin (Hgb) concentration: Hemoglobin is determined from the absorbance computed from the ratio of the blank to the sample photocurrent reading. This number is multiplied by a constant. Expressed in grams of hemoglobin per deciliter of whole blood.

\[ \text{Hgb (g/dL)} = \text{calibration factor} \times \text{calibration constant} \times \text{absorbance} \]

Absorbance = \( \log_{10} \frac{\text{blank photocurrent}}{\text{Sample photocurrent}} \)

Hematocrit (HCT): This is the computed relative volume of erythrocytes expressed in percentage.

\[ \text{HCT (\%)} = \frac{\text{RBC} \times \text{MCV}}{10} \]

Mean corpuscular Hemoglobin (MCH) concentration: This is the computed average weight of hemoglobin in a measured dilution, expressed in grams of hemoglobin per deciliter of erythrocytes,

\[ \text{MCHC} = \frac{\text{Hgb} \times 100}{\text{HCT}} \]

Biochemical parameters

2.9.5.6 Determination of serum alkaline phosphatase

Serum alkaline phosphatase (SAP) was determined photometrically.

Principle

\[ 4\text{-nitrophenylphosphate} + \text{H}_2\text{O} \xrightarrow{\text{AP}} \text{Phosphate} + 4\text{ nitrophenolate} \]

The rate of increase in 4-nitrophenolate is determined photometrically and is directly proportional to the AP activity in the sample material.

Test concentration

Diethanolamine HCL buffer, pH 9.8 -1.0 mol/ l

Magnesium chloride -0.5 mol/ l
Procedure
Reagent temperature - +25, +30 or +37°C
Wave length - 405 nm
Light path - 1 cm
Measuring temperature - +25, +30 or +37°C
Serum/plasma - 20 µl
Reaction solution - 1000 µl

Mix after one minute. Measure the increase in absorbance every minute for 3 minutes.

Calculation
Enzyme activity (U/l) = (∆A/min).2754

Dilution limit

If with a measurement period of 3 minutes the differences in absorbance per minute (∆A/min) at the beginning of the measurement are greater than 0.250, the determination is repeated using sample material diluted 1+5 with sodium chloride solution (154m mol/l=9gm/l), and the result multiplied by 6.

2.9.5.7 Determination of SGPT

It was determined by the method of Reitman and Frankel (1960).

Principle

Based on the reference method of the International Federation of Clinical chemistry (IFCC)

2-oxoglutanate + l-alanine \text{ALAT}\rightarrow\text{glutamate + pyruvate}
Pyruvate + NADH + H^+ \text{LDH}\rightarrow\text{lactate + NAD}^+
The rate of NADH consumption is measured photometrically and is directly proportional to the ALAT activity in the sample.

**Test concentrations**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris buffer, pH 7.5</td>
<td>100 m mol/l</td>
</tr>
<tr>
<td>L-alanine</td>
<td>500 m mol/l</td>
</tr>
<tr>
<td>2-oxoglutarate</td>
<td>15 m mol/l</td>
</tr>
<tr>
<td>LDH</td>
<td>≥1.2</td>
</tr>
</tbody>
</table>

**Procedure**

- **Reagent temperature**: 25, +30 or +37°C
- **Wavelength**: 334nm, 340nm, 365nm
- **Light path**: 1 cm
- **Measuring temperature**: +25, +30 or +37°C
- **Serum or plasma**: 50 μl
- **Reaction solution**: 500 μl

Mix, after approximately 1 minute measure the decrease in absorption every minute for 3 minutes.

**Calculations**

Enzyme activity (U/l) = (ΔA/min) F

**Dilution limit**

If the initial readings exceed the following values, dilute 1 part serum or plasma with 10 parts physiological sodium chloride solution (154 mmol/l = 9g/l), then repeat the determination and multiply the result by 11.

**2.5.9.8 Determination of SGOT**

It was also determined by the same procedure that mentioned above for SGPT (Reitman and Frankel 1960).
2.5.9.9 Determination of Urea

Urea was estimated by enzymatic method (Tietz 1987)

METHOD

Urea is hydrolysed in presence of water and urease to produce ammonia and carbon dioxide. The ammonia from this reaction combines with 2-oxoglutarate and NADH in presence of glutamate dehydrogenase (GLDH) to yield glutamate and NADH. The decrease in absorbance is proportional to the urea concentration within the given time intervals.

Reaction principle

\[
\text{Urea} + 2 \text{H}_2\text{O} \xrightarrow{\text{Urease}} 2\text{NH}_4^+ + \text{CO}_2
\]

\[
2\text{oxoglutarate} + \text{NH}_4^+ + \text{NADH} \xrightarrow{\text{GLDH}} \text{L-glutamate} + \text{H}_2\text{O} + \text{NAD}^+
\]

Assay

Wave length 340nm

Optical path 1 cm

Temperature 25°C, 30°C or 37°C

Measurement against reagent blank.

Procedure

<table>
<thead>
<tr>
<th>Pipette into test tube</th>
<th>Reagent Blank (Rb)</th>
<th>Sample or Standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent Mixture</td>
<td>1000 µl</td>
<td>1000 µl</td>
</tr>
<tr>
<td>Sample/Standard</td>
<td>--</td>
<td>10µl</td>
</tr>
</tbody>
</table>

Mix, read absorbance of sample/standard after 30 seconds (A1) Start timer simultaneously and read again after exactly 1 minute (A2). Calculate the absorbance difference: \( \Delta A \text{Sample/Standard} = (A2 + A1) - \Delta A\text{Rb} \)
2.5.9.10 Quantitative determination of inorganic phosphate in serum

Phosphorus was estimated by calorimetric method (Henry 1974)

Test summary

Phosphate reacts in acid medium with ammonium molybdate to form a phosphomolybdate complex with a yellow colour. The intensity of this colour is proportional to the concentration of inorganic phosphate in the sample; the colour formed is measured at 340 nm or 360 nm. The final colour is formed within one minute and is stable for one hour.

Reagent composition

The concentrations of the active ingredients in the reagents are

<table>
<thead>
<tr>
<th>Components</th>
<th>Approximate concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium Molybdate</td>
<td>0.6mmol/L</td>
</tr>
<tr>
<td>Sulfuric acid</td>
<td>0.26mol/L</td>
</tr>
</tbody>
</table>

Test procedures

Wave length: 340 nm
Temperature: 25°C, 30°C into a series of matched cuvettes, pipette:

Blank: 2ml reagent + 20 µl water
Standard: 2ml reagent + 20 µl standard
Sample: 2ml reagent + 20 µl sample

Calculations

\[ A_{\text{of sample}} \times \text{concentration of standard} = \text{mg/ dL phosphate} \]

\[ A_{\text{of standard}} \]
2.5.9.11 Estimation of calcium [O- cresolphthalein Complexone Method]

Calcium was estimated by O-cresolphthalein method (Tietz 1987).

Principle

Calcium in alkaline medium reacts with O-cresolphthalein Complexone to form a purple coloured complex whose absorbance is proportional to the calcium concentration. The interference due to magnesium and iron is eliminated by using 8-hydroxy quinoline.

Procedure

Label three clean test tubes as Blank (B), Standard (S) and Test (T). Pipette into each test tube as shown below:

<table>
<thead>
<tr>
<th></th>
<th>(B)</th>
<th>(S)</th>
<th>(T)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Working Reagent</td>
<td>2.5ml</td>
<td>2.5ml</td>
<td>2.5ml</td>
</tr>
<tr>
<td>Calcium standard</td>
<td>--</td>
<td>0.02ml</td>
<td>--</td>
</tr>
<tr>
<td>Serum</td>
<td>--</td>
<td>--</td>
<td>0.02ml</td>
</tr>
</tbody>
</table>

Mix well and allow them to stand at room temperature for 5 minutes. Then measure the absorbance of Standard (S) and Test (T) against Blank (B) on Photo colorimeter with yellow green filter or on a spectrophotometer at 570nm (hg 578 nm) within 30 minutes.

Calculations

Calcium in mg % = \( \frac{A_{of \ T}}{A_{of \ S}} \)

2.6 IR Spectroscopy

2.6.1 Mice

Swiss albino male mice weighing 25-30 gm reared in the animal house of Regional Cancer Centre, Thiruvananthapuram were used. Animals
were given standard commercial pellet diet and water *ad libitum* and maintained at 20-28°C and relative humidity 60-70 % with 12 hours dark light cycle.

### 2.6.2 Preparation of cell lines

The cell lines were propagated in the peritoneal cavity of mice transplanting one million Daltons Lymphoma Ascitic (DLA) cells per ml of Phosphate Buffered Saline (PBS). The cells were collected from the peritoneal cavity of mice having sufficient cell growth as evidenced by ascetics and washed thrice with PBS and suspended in the same solvent to have a concentration of 1x10^6 cells/ml.

### 2.6.3 Solid tumour growth formation

The detailed procedure are mentioned in section 4.2.5.

**Infrared Spectroscopic analysis of DLA induced tumour tissue, drug treated tumour and control (normal thy muscle)**

The mice were randomly divided into 4 groups of six mice each. Group 1 was kept as control. Group 2 was kept as tumour control. Group 3 was given the purified fraction of *B.racemosa* seed extract F$_8$ (2:8) at a dose of 6mg/kg subcutaneously, once a day, using a day 1-14 regimen. Fourth group was also given the purified fraction at a dose of 9mg/kg. On the 20th day, the tumour was operated from two of each from all the three groups except group 1. Fresh thy muscle tissue was operated from the first group of mice. Detailed procedure is mentioned in section 8.2.4.

### 2.6.4 Histopathological studies

The operated tumour tissues were fixed in 10% buffered formalin and 3-5micromolar sections were made using a microtome and stained with H & E. Detailed procedure are mentioned in section 8.2.5.
2.7 Statistical analysis

Students 't' test was used for the statistical evaluation of the data. To determine the statistical significant between two values in the control (X) and treated (Y) group, 't' value was calculated using the equation (Snedecor and Cochran 1967).

\[
t = \frac{x - y}{\sqrt{V[(1/n_x) + (1/n_y)]}}
\]

where \(X\) and \(Y\) are the means of the two samples \(X\) and \(Y\), \(n_x\) and \(n_y\) are the sample size and \(S\) was found out using the equation.

\[
S = \sqrt{n_x (S_x^2 + (n_y - 1) S_y^2)}
\]

\[
\frac{1}{n_x + n_y - 2}
\]

where \(S_x\) and \(S_y\) are the standard deviation of the two samples. Statistical significance was deduced from 't' distribution tables.