Chapter - II

Materials and Methods
2.1 MATERIALS

2.1.1 Test compounds

Polyphenolic compounds Catechin, Epicatechin, Rutin, Quercetin, Ellagic acid, Naringin, Naringenin and Morin used for the study were purchased from Sigma Chemicals, St. Louis, USA.

Curcumin (99%) pure was gifted by Kancor Ltd., Angamaly.

Isoflavones - Genistein and Daidzein were gifted by Dr. Muraleedharan G. Nair, Bioactive Natural Products Laboratory, Department of Horticulture, Michigan State University, USA.

Antiinflammatory drugs used for the study:

- Beta-Methazone - (Celestone, U.S. Scheming)
- Phenyl butazone - (Esgipyrene, S.G. Pharmaceuticals)
- Ibuprofen - (Brufen, Boots, India Ltd.)
- Mefanamic acid - (Meftal, Blue Cross India)
- Paracetamol - (Calpol, Themis)

These compounds were purchased from Amala Cancer Hospital, Pharmacy.

Rasayanas

Amritha Prasam (AP)
Aswagandha Rasayana (AR)
Brahma Rasayana (BR)
Chyavanaprasam (CP)
Narasimha Rasayana (NR)

were purchased from Thaikkat Mooss’s Vaidyaratnam Oushadhasala, Ollur, Thrissur, Kerala.
2.1.2 Chemicals

Dulbecco's Modified Eagles Medium (DMEM)
Eagles Minimum Essential Medium (MEM)
Rosewell Park Memorial Institute Medium (RPMI)-1640
Hanks Balanced Salt Solution (HBSS)
Trypsin
Haematoxylin
Chloramine-T
Fluid Thioglycollate medium

Acrylamide
Bis-acrylamide
Tris-base
Tris-HCl
Folin-Ciocaltaue reagent
Coomassie Brilliant Blue

Foetal Calf Serum

Paraffin Wax

Nitrocellulose filters 0.22 μm pore, 13mm diameter)

Polycarbonate filters (8 μm pore, 13 mm diameter)

Hi-media, Laboratories, Bombay, India

Sisco Research Laboratories Pvt. Ltd., Bombay, India

Biological Industries, Kibbutz bet haemek, Israel

E.Merck (India) Ltd., Bombay

Millipore Pvt. Ltd., Bangalore

Costar Co, Cambridge, MA USA
Dimethyl Benz(a)anthracene  
20-Methylcholanthrene

Sigma Chemicals,  
St. Louis, USA.

Collagen Type - I (Sigma)

Gifted by  
Dr.Muraleedharan Nair  
Michigan State University

Hydroxyproline

Gifted by  
Dr.K.Balasubramaniam,  
Welcome Research Lab,  
CMC, Vellore, Tamil Nadu,  
India

Sialic Acid

Gifted by  
Dr.A.S.Balasubramaniam,  
Department of  
Neurological Sciences,  
CMC, Vellore

Croton Oil

Prepared in the laboratory by evaporating the petroleum-benzene extract of crushed seeds of *Croton tiglium* (26), which were obtained from the local market

All other chemicals used were of Analytical reagent grade.
2.1.3 Reagents

Buffer solutions

a) PBS
   NaCl - 8.00g
   KCl - 0.20g
   Na₂HPO₄·2H₂O - 1.44g
   KH₂PO₄ - 0.20g
   Distilled water - 1000ml
   pH was adjusted to 7.2 with 1N HCl or NaOH.

b) PBS-EDTA solution
   EDTA - 20mg
   PBS - 100ml

c) Trypsin Solution (0.2%)
   Trypsin - 200mg
   Glucose - 20mg
   PBS-EDTA - 100ml

d) Alsever’s Solution
   Dextrose - 2.05g
   Sodium citrate - 0.80g
   NaCl - 0.42g
   Distilled water - 100ml
   pH was adjusted to 6.1 with 10% citric acid

e) Neutral buffered formalin solution
   Na₂HPO₄·2H₂O - 16.37g
   NaH₂PO₄·H₂O - 100mg
   Formalin - 100ml
   Distilled water - 900ml
Stains

f) Trypan Blue
   Trypan blue - 100mg
   Saline (0.9%) - 100ml

g) Eosin
   Eosin Y - 500mg
   Ethanol - 100ml (Final volume)
   Eosin was dissolved in 5ml of distilled water and made up to 100 ml with ethanol

h) Harris Haematoxylin
   Haematoxylin - 5g
   Ethyl alcohol - 50ml
   Potassium alum - 50mg
   Potassium iodide - 50mg
   Distilled water - 950ml

   Haematoxylin was dissolved in alcohol using gentle heat. The alum was dissolved in distilled water by heating with frequent stirring and kept overnight at 4°C. Alcoholic haematoxylin was added to the alum solution. The mixture was cooled and potassium iodide was added and filtered.

i) Crystal violet

   Crystal violet - 50mg
   Methanol - 20ml
   Distilled water - 80ml
### 2.1.4 Instruments and devices

<table>
<thead>
<tr>
<th>Instrument/Device</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disc electrophoresis tank</td>
<td>Balaji Scientific Services, Madras</td>
</tr>
<tr>
<td>Blind Well Chamber (Modified Boyden Chamber)</td>
<td>Nuclepore, Cambridge, USA</td>
</tr>
<tr>
<td>CO₂ Incubator</td>
<td>Napco, Canada</td>
</tr>
<tr>
<td>Cooling Centrifuge</td>
<td>Remi, Special instruments consortium, Madras</td>
</tr>
<tr>
<td>Inverted Microscope</td>
<td>Wilovert, Germany</td>
</tr>
<tr>
<td>Laminar Flow Hood</td>
<td>Klenzaids Contamination Control Pvt. Ltd., Gujarat</td>
</tr>
<tr>
<td>Spectrophotometer</td>
<td>SL150, ELICO Pvt. Ltd., India</td>
</tr>
<tr>
<td>Research Microscope</td>
<td>Meiji, Japan</td>
</tr>
<tr>
<td>Sonicator</td>
<td>Labline Instruments, Illinois, USA</td>
</tr>
<tr>
<td>Tissue Homogenizer</td>
<td>York Scientific Industries, Delhi</td>
</tr>
<tr>
<td>Rotary Microtome (Radical)</td>
<td>Lab Agencies, Kerala</td>
</tr>
</tbody>
</table>

### 2.1.5 Animals

C57BL/6 mice, Swiss albino mice and BALB/c mice, 4-5 weeks old, weighing 20-25 g, were purchased from National Centre for Laboratory Animal Sciences (NCLAS), National Institute of Nutrition, Hyderabad, India.

... pressure (under Nitrogen) using a 0.22 µm cellulose filter. The sterility of the medium was tested using fluid thioglycollate medium. For this 10 ml thioglycollate 929.8 g/L, sterile) was inoculated with 1 ml of medium and incubated at 37°C for 7 day and checked for contamination. Antibiotics -
2.1.6 Tumour cell lines

B16F-10 melanoma cells, L-929 (mouse lung fibroblast) cells were obtained from National Facility for Animal Tissue and Cell Culture, Pune.

Dalton's lymphoma ascites (DLA) cells (arose as a spontaneous carcinoma of thymus) were initially obtained from Cancer Institute, Adayar and Ehrlich ascites tumour (EAT) cells were initially obtained from Cancer Institute, Bombay. The cell lines were maintained in the laboratory by transplanting in the peritoneal cavity of mice.

2.2 METHODOLOGY

2.2.1 Sterilization of glasswares

All glassware and filtration apparatus used for tissue culture purposes, were soaked in a solution of Extran (1%) overnight, cleaned using brush and washed thoroughly under running water. These were then rinsed several times in distilled water. The glasswares were oven dried autoclaved at a pressure of 15 pounds/square inch for 15-20 minutes, dried and used for experiments.

2.2.2 Preparation of culture media

DMEM (9.98 g/l), MEM (10.3 g/l) and RPMI (10.3 g/l), pH 7.2, were prepared in sterile double distilled water and filtered under positive pressure (under Nitrogen) using a 0.22 μm cellulose filter. The sterility of the medium was tested using fluid thioglycollate medium. For this 10 ml thioglycollate 929.8 g/L, sterile) was inoculated with 1 ml of medium and incubated at 37°C for 7 day and checked for contamination. Antibiotics - penicillin (100 lu/ml) and streptomycin (100 μg/ml) were added to the medium, prior to use.
2.2.3 Maintenance of cell lines in tissue culture

a. B16F-10 melanoma cells

The spent medium was removed from confluent bottles and the cells were washed three times with PBS. One ml of trypsin solution was overlaid and incubated for 3-4 minutes at 37°C and the bottles were tapped to dislodge the cells. DMEM (5 ml) containing 10% FCS and antibiotics (complete medium) were added and cells were dispersed to single cell suspension by repeated pipetting. An aliquot of cell suspension was added to fresh tissue culture bottles containing 10 ml of complete medium and incubated at 37°C. The cells were subcultured every week.

b. L-929 Cells

The same procedure as that for B16F-10 melanoma cells were followed, except that cells were washed with PBS-EDTA and maintained in MEM containing 10% goat serum and antibiotics.

2.2.4 Maintenance of experimental animals

C57BL/6 mice, Swiss albino mice/BALB/c mice were used for the experiment. They were housed in ventilated cages and allowed free access to pelleted mouse chow (Lipton, India) and water ad libitum, throughout the experimental periods.

2.2.5 Maintenance of tumours in animals

DLA and EAT cells were maintained by transplanting the cells every two weeks. Tumour cells were aspirated and washed with PBS and 1 x 10^6 cells were injected intraperitoneally to induce ascites tumour in Swiss albino mice.
B16F-10 melanoma cells were propagated in C57BL/6 mice as transplantable solid tumours, 1x10^6 cells were injected subcutaneously to the hind limb of mice. After 10-15 days, when the tumour was visible, the animal was sacrificed, tumour mashed and processed in PBS and 1 x 10^6 viable cells were injected to another set of animals.

2.2.6 Preparation of B16F-10 melanoma cells for in vivo studies

Tumours were propagated in vivo in C57BL/6 mice as solid tumours to provide fresh tumour cells. For experimental purpose subcutaneous tumour was resected, and a single cell suspension was prepared by mincing the tissue and forcing pieces through a fine steel mesh. The cells were separated from RBC's and then suspended in PBS to required cell number and they were used for in vivo experiments after determination of viability (2.2.8).

2.2.7 Preparation of B16F-10 melanoma cells for in vitro studies

The tumour cells were harvested from subconfluent cultures (50-80% confluency). The spent medium was removed, the monolayers were washed three times with PBS and the cells were harvested by mechanical dissociation using a rubber policeman. The cell concentration was adjusted to required number. Cell suspensions with 90% viability were used for in vitro experiments.

2.2.8 Determination of cell viability

Cell viability was determined by the trypan blue dye exclusion method (192). 1 ml of cell suspension was mixed with 0.1 ml of 1% trypan blue, kept for 2-3 minutes and loaded on a haemocytometer.
Viable cells exclude trypan blue dye, while non-viable cells take up the dye and thus appeared blue in colour. The number of stained and unstained cells were counted separately.

\[
\text{% Dead Cells} = \frac{\text{No. of dead cells}}{\text{No. of viable cells} + \text{No. of dead cells}} \times 100
\]

2.2.9 In vitro cytotoxicity studies

a. Short term in vitro cytotoxicity assay

Cytotoxic activity of the test compounds towards B16F-10 melanoma cells, DLA cells and EAT cells were studied by the short term in vitro assay.

Tumour cells (1 x 10^6 cells) were added to test tubes containing various concentrations of the test compounds, and the final volume was made upto 1 ml with PBS. Control tubes contained tumour cells only. The assay mixture was then incubated at 37°C for 3 h, and the percentage of dead cells were determined by trypan blue dye exclusion method (2.2.8).

b. Long term in vitro cytotoxicity studies by tissue culture

B16F-10 melanoma cells and L-929 cells growing in log phase were used for the experiment. Cells were collected by trypsinization and 50,000 cells were seeded into tissue culture bottles containing 10 ml complete medium and incubated at 37°C. After 24 h, various concentrations of the test compounds were added to the bottles and incubated for six more days. After incubation, the viable cells were collected by trypsinization and counted using a haemocytometer.
Percentage viable cells = T/C x 100,

where ‘T’ is the number of cells in the treated bottles and ‘C’ the number of cells in control bottles.

2.2.10 Determination of the effect of compounds on the solid tumour development

The effect of test compounds on solid tumour development was studied using B16F-10 melanoma cells in C57BL/6 mice. The animals (six per group) were injected subcutaneously with B16F-10 melanoma cells (1 x 10^6 cells/0.1 ml PBS/animal) on the right hind limb. Oral administration of the test compounds were started 24 h after tumour inoculation and continued for ten alternate days. From 7th day onwards, two dimensional tumour diameter was measured every 3rd day for 30 days, using vernier calipers and the tumour volume was calculated using the formula,

\[ V = \frac{4}{3} \pi r_1^2 r_2^2, \]

where ‘r_1’ and ‘r_2’ are the two dimensional radii of the tumour mass.

2.2.11 Determination of the survival rate of tumour bearing animals

The mortality of animals of each experiment were noted and the percentage increase in life span (ILS) was calculated from the formula,

\[ \% \text{ ILS} = \frac{([T-C]/C) \times 100,} \]
where "T" is the number of days treated animals survived and 'C' is the number of days control animals survived.

2.2.12 Study on the *in vivo* invasion of B16F-10 melanoma cells in C57BL/6 mice.

Studies on the *in vivo* invasion of tumour cells were done in C57BL/6 mice, using B16F-10 melanoma cells. Pulmonary colonization was carried out as described by Fidler (32). C57BL/6 mice (8 per group) were inoculated with $1 \times 10^6$ B16F-10 melanoma cells (2.2.7) (in 0.05 ml PBS) through the lateral tail vein. Simultaneously, test compounds (200 μmoles/kg body weight/animal, suspended in 1% gum acacia) were administered orally for 10 alternate days. The animals were sacrificed on the 21st day and the rate of pulmonary metastasis was determined:

a. by counting the metastatic focii on the surface of the lungs (Fig 6).
b. by measuring the lung collagen hydroxyproline content (2.2.19)
c. by estimation of sialic acid levels in the serum (2.2.20) and
d. by histopathological analysis of the lungs (2.2.21).

2.2.13 Determination of the survival rate of metastatic tumour bearing animals.

C57BL/6 mice (6 per group) were inoculated with $1 \times 10^6$ B16F-10 melanoma cells, through the lateral tail vein. Simultaneously, test compounds (200 μmoles/kg body weight/animal, suspended in 1% gum acacia) were administered to the respective group, orally for 10 alternate days. The animals were observed for their survival and percentage increase in life span was calculated (2.2.11).
Fig. 6

A  Normal lungs of C57BL/6 mice.
B  Lungs of metastatic tumour bearing C57BL/6 mice.
2.2.14 Collagen matrix invasion assay

Invasion of collagen matrix by tumour cells was carried out using modified Boyden Chambers (Blind well) as described by Albini et al., (5).

The lower compartment of the chamber was filled with DMEM without FCS and the nitrocellulose filter was placed above. Polycarbonate filters (8 μm pore, 13 mm diameter), coated with 25 μg collagen type I, were placed above nitrocellulose filters (0.22 μm pore size, 13 mm diameter) in Blindwell chamber. B16F-10 melanoma cells (1 x 10^5 cells) were prepared and suspended in DMEM without FCS. The cell suspension was added to the upper chamber and incubated at 37°C for 24 h, in a 5% CO₂ atmosphere. After the incubation period, the cells on the upper surface of the filter were completely removed by wiping with cotton swab, and the filters were fixed with methanol for one minute and stained with crystal violet for one minute. The cells that had penetrated through the polycarbonate filter were counted in 10 fields, under a microscope. All the assays were performed in duplicate. Results were calculated as percentage inhibition of invasion, using the formula,

\[
\% \text{ Inhibition of invasion} = 100 - \frac{\text{Mean number of migrating cells in test}}{\text{Mean number of migrating cell in control}} \times 100
\]

2.2.15 Tumour cell adhesion experiment

Cell attachment to the collagen matrix was assayed as described by Inokuchi et al., (165). B16F-10 melanoma cells (5 x 10^3 cells/well) were added to flat bottom wells of titre plates precoated with collagen type I, and incubated for 1 h at 37°C. After incubation, medium was removed and wells were washed with PBS. Adhering cells were fixed with formaldehyde (5%), for 20 minutes and stained with crystal violet for 20 minutes. The cells,
under ten microscopic fields per well, were counted. Each experiment was done in triplicate.

2.2.16 Tumour cell motility assay

In motility assay, B16F10 melanoma cells (1 x 10^5/150 µl DMEM) were seeded onto the upper compartment of Blindwell chamber, containing only polycarbonate filters without any collagen coating. The lower chamber was filled with DMEM without FCS. The chambers along with cells were incubated for 24 h at 37°C in 5% CO2 atmosphere and the number of cells migrating to the lower compartment were determined, by counting the migrating cells in the lower chamber.

\[
\% \text{ Motility} = \frac{\text{Mean number of migrating cells in test}}{\text{Mean number of migrating cells in control}} \times 100
\]

2.2.17 Gelatin Zymography

Principle

Proteases of cell extracts, were initially resolved on SDS-polyacrylamide gels which were incorporated with gelatin. Following incubation of the gel in the activation buffer, protease separated on the gel were breakdown the gelatin and appear as transparent zones or clearings against a dark background (upon staining).

Requirements

a. 0.25 M Sucrose - 0.01 M Tris-HCl buffer, pH 7.4

<table>
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<th>Component</th>
<th>Quantity</th>
</tr>
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<tbody>
<tr>
<td>Sucrose</td>
<td>85.57 g</td>
</tr>
<tr>
<td>Tris-HCl</td>
<td>1.21 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>
b. **0.1 M Tris-HCl, pH 8.0, 10 mM CaCl₂**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl₂·2H₂O</td>
<td>1.47 g</td>
</tr>
<tr>
<td>Tris</td>
<td>12.1 g</td>
</tr>
<tr>
<td>pH adjusted with conc. HCl.</td>
<td></td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml (Final volume)</td>
</tr>
</tbody>
</table>

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**Trypsin Solution**

Trypsin - 75 μg/ml in 0.1 M Tris-HCl, with 10 mM CaCl₂, pH 8.

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**Activation Buffer (0.1 M Tris-HCl, 10 mM CaCl₂, pH 7.8)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
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</thead>
<tbody>
<tr>
<td>Tris-HCl</td>
<td>12.10g</td>
</tr>
<tr>
<td>CaCl₂·2H₂O</td>
<td>1.47g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml (Final volume)</td>
</tr>
</tbody>
</table>

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**Preparation of gels**

11% polyacrylamide gels with 0.1% SDS and 0.6% gelatin

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
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<tbody>
<tr>
<td>29.2% acrylamide + 0.5% bisacrylamide</td>
<td>11 ml</td>
</tr>
<tr>
<td>0.1 M Tris-HCl, pH 8.8</td>
<td>1.2 ml</td>
</tr>
<tr>
<td>20% SDS</td>
<td>0.15 ml</td>
</tr>
<tr>
<td>20% Ammonium per sulphate</td>
<td>0.10 ml</td>
</tr>
<tr>
<td>Gelatin (180 mg/2 ml distilled water, heated to dissolved)</td>
<td>2 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>6.505 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.045 ml</td>
</tr>
</tbody>
</table>

Mix and pour at room temperature.
5% stacking gel
29.2% acrylamide + 0.5% bisacrylamide - 1.67 ml
0.1M Tris-HCl, pH 8.8 - 1.75 ml
20% SDS - 0.10 ml
20% Ammonium per sulphate - 0.10 ml
Distilled water - 6.36 ml
TEMED - 0.020 ml

Mix and pour above the 11% gel, at room temperature

f. Sample Buffer [2X]
Glycerol - 1 ml
1M Tris-HCl, pH 6.8 - 0.25 ml
20% SDS - 1 ml

Make upto 5 ml with distilled water.

g. Running Buffer
Tris Base - 3 g
SDS - 2 g
Glycine - 14.2 g

Made upto 1 L with distilled water.

h. 2% Triton X-100
Triton X-100 - 2 ml
0.1 M Tris-HCl, pH 7.8 - 100 ml (Final volume)

i. 10 mM EDTA Solution
EDTA.Na₂ - 372.24 mg
0.1 M Tris-HCl, pH 7.8 - 1000 ml (Final volume)
j. **Destaining solution**

Methanol : Acetic acid: Water - 50:10:40

k. **0.2% Coomassie Blue**

Coomassie Blue - 0.2 g
Destaining Solution - 100 ml
(Methanol:Acetic acid: Water - 5:1:4)

**Procedure**

Gelatin zymography was followed according to the procedure of Habres and Billings (34) with some modifications. The serum free medium samples were collected as follows. Medium from subconfluent (70%) B16F-10 tumour cells were removed, cells were then washed with serum free medium and resupplied with fresh serum-free DMEM (5 ml), at 37°C for 24 h. Later the cells were detached by mechanical dissociation, with a rubber policeman and the cells were suspended in 0.5 ml sucrose buffer (2.2.17.a) and lysed using sonicator with 6 burst for 10 seconds each on ice. The tumour cell lysate was centrifuged at 4°C, 5000 g for 10 minutes and after determining the protein concentration, supernatant (equivalent to 100 µg protein) containing the proteases were activated with trypsin [5 µl from the stock] (2.2.17.c), for 30 minutes at room temperature. Trypsin treated and untreated samples (equivalent to 100 µg protein) were mixed with an equal volume of sample buffer [2X] (2.2.17.f), lacking reducing agent or heat, were loaded on 0.1% SDS - 11% polyacrylamide gels containing 0.6% gelatin (2.2.17.e) and electrophoresed at 10°C for 3 h with a constant current of 2 mA per tube until the bromophenol blue tracking dye reached the periphery of the gels.

The gels were then washed with 2% Triton X-100 (2.2.17.h), on a shaker at 20-25°C for three changes of 30 minutes each, to remove the SDS.
which could interfere with proteolytic activity. This was followed by 2 h washing with activation buffer (2.2.17.d) and the gels were finally incubated in the same buffer at 37°C for 18 h, in the presence and absence of test compounds. Following staining with 0.2% Coomassie Brilliant blue (2.2.17.k) for 2 h, the gels were destained in the destaining solution (2.2.17.j) to visualize the clear protease band against a dark background.

To characterize the nature of proteases, 10 mM EDTA was added to the incubation buffer and incubated for 18 h. The gels were stained and then destained. EDTA is a specific inhibitor of metalloproteases (collagenases).

2.2.18 Estimation of protein

Principle

This assay relies on the formation of a protein-copper complex (Biurete reaction) and reduction of phosphomolybdate-phosphotungstate reagent (Folin-Ciocalteu’s reagent) by tyrosine and tryptophan residues of protein, to form coloured products, by Lowry’s method (213).

Reagents

Solution A: 98 ml of 2% Na₂CO₃ (in 0.1N NaOH) +
1 ml of 1% CuSO₄·5H₂O +
1 ml of 2% Sodium potassium tartarate

Solution B: Commercial Folin-Ciocalteu’s reagent
(diluted 1:1 with distilled water)
**Procedure**

Sample was diluted with distilled water to a final volume of 1.2 ml and was mixed with 6 ml of solution A. Mixture was incubated at room temperature for 10 minutes. To this mixture 0.3 ml of solution B was added, mixed well and incubated at room temperature for 30 minutes. The absorbance was measured at 660 nm. The amount of protein was calculated from the standard curve using bovine serum albumin (BSA) as standard.

**2.2.19 Estimation of tissue hydroxyproline content**

The determination of collagen in biological tissues usually requires acid hydrolysis and measurement of the hydroxyproline released.

The chloramine-T method by Bergman and Loxley (27) was for the estimation of tissue hydroxyproline content.

**Principle**

The oxidation of hydroxyproline by Chloramine-T gives a stable product, which reacts with Para dimethyl amino benzaldehyde (PDMAB) to form a chromophore. The presence of isopropanol during colour development increase yield and stability of colour.

**Reagents**

a. Oxidant Buffer

\[
\begin{align*}
\text{CH}_3\text{COONa.}2\text{H}_2\text{O} & \quad - & \quad 57 \text{ g} \\
\text{Na}_3\text{C}_6\text{H}_5\text{O}_7.2\text{H}_2\text{O} & \quad - & \quad 37.5 \text{ g} \\
\text{C}_6\text{H}_5\text{O}_7\text{H}_2\text{O} & \quad - & \quad 5.5 \text{ g} \\
isopropanol & \quad - & \quad 385 \text{ ml}
\end{align*}
\]
Made upto 1 L with distilled water. (This buffer is stable indefinitely).

b. Chloramine T

7% w/v in aqueous solution (Prepared at the time of experiment).

c. Oxidant Solution

Chloramine T and oxidant buffer were mixed in the ratio of 1:4 prepared just before use.

d. Ehrlich Reagent

Para Dimethyl Aminobenzaldehyde - 17.6 g
60% Perchloric acid (S.G. 1.54) - 40.8 g
Isopropanol - made upto 100 ml.

Prepared on the day of use, just before the experiment.

Procedure

One gram tissue was homogenized in 4 ml of isotonic saline and hydrolyzed at 110°C for 24 h with 6N HCl. 1 ml of hydrolysate was neutralized with KOH solution and made upto 5 ml with distilled water.

To a known volume of hydrolysate sample, isopropanol was added to make a final volume of 3 ml. 1 ml of oxidant solution was added while stirring and kept at room temperature for 4 minutes. After adding Ehrlich reagent, the tubes were heated to 60°C in a water bath for 21 minutes and incubated at room temperature for 1 h. The absorbance was read at 560 nm, and the amount of hydroxylproline was calculated from the standard curve.
2.2.20 Estimation of serum sialic acid

The thiobarbituric acid method of Skoza and Mohos (320) was used for serum sialic acid estimation.

Sialic acid on periodate oxidation yields formylpyruvic acid which with thiobarbituric acid forms the chromophore. The presence of dimethyl sulphoxide, intensifies and stabilizes the colour.

Reagents

Periodate Solution : 25 μM periodic acid/ 62.5 mM H₂SO₄
Arsenite Solution : 2% (w/v) sodium arsenite/ 0.5M HCl
Thiobarbituric acid : 6% (w/v) aqueous, adjusted to pH 9 with NaOH.

Serum samples were hydrolysed in 0.05M H₂SO₄ (pH between 1.6 and 2.0) at 80°C for 60 minutes. These samples (0.2 ml) were oxidized by adding periodate solution (0.05 ml) and incubated at 37°C for 30 minutes. Followed by the termination of oxidation by adding arsenite solution (0.05 ml), 0.1 ml of thiobarbituric acid was added. The mixture was heated in a boiling water bath for 7.5 minutes. The colour developed was intensified by adding equal volume of DMSO and the absorbance was read at 549 nm. The sialic acid content was calculated from the standard curve using N-acetyl neuraminic acid, as standard.

2.2.21 Histopathological Analysis

Tissue specimens were fixed in 10% buffered formalin immediately after sacrifice. Specimens were dehydrated by passing through ascending grades (50 - 100%) of alcohol, then cleared in xylene, impregnated and
embedded in paraffin. Thin paraffin mounted sections were cut (4µm) and the slides were stained using haematoxylin (2.1.3.h) and eosin (2.1.3.g) and mounted in DPX.

2.2.22 Two-stage carcinogenesis

Inbred Swiss albino mice, male (7-8 weeks old) were used for the experiment. The mice were shaved on the dorsal surface, 2 days prior to the experiment, and DMBA and croton oil was used as standard initiator and promoter respectively (363).

Mice were divided into groups of 15 each and were topically applied, with subcarcinogenic dose of initiator DMBA (470 nmol for mouse in 0.2 ml acetone), on the shaved area. After two weeks the animals were treated with croton oil (10% in 0.2 ml acetone), a known promoter, twice weekly for 6 weeks. 30-40 minutes prior to each croton oil application, the experimental mice were topically treated with the test compounds in 0.2 ml acetone. Skin tumour formation in each animal was recorded weekly for 20 weeks and tumours having sizes greater than 2 mm in diameter were included in the cumulative total, if they persisted two weeks or more. The animals (10 nos./group) were observed for the following parameters.

a. the mean number of tumour per mouse
b. the percentage number of mice with tumour and
c. onset of tumour

From each group, 5 animals were sacrificed at the onset of papilloma, and the skin collagen hydroxyproline content was estimated as given in section (2.2.19).
2.2.23 Methylcholanthrene induced chemical carcinogenesis

20-methylcholanthrene induced sarcoma development was studied in Swiss albino mice. Inbred mice (7-8 weeks old) male, were shaved on the dorsal surface 2 days before experiment. 20 methylcholanthrene (200 µg/0.2 ml DMSO) were injected subcutaneously on the dorsal side. Test compounds were administered, orally twice a week for 10 weeks. The animals were observed for the onset of sarcoma as well as their survival for 6 months.

Immunological Parameters

2.2.24 Preparation of spleen cells (229)

BALB/c mice were sacrificed by cervical dislocation and an incision was made on the left side just below the last rib and spleen was removed without any adherent tissues. Spleen was cut into small pieces and squeezed over a sieve in cold HBSS. Clumps were allowed to settle in centrifuge tube at 4°C for 20 minutes. The supernatant was collected, washed twice in HBSS, and resuspended in MEM at required cell number.

2.2.25 Preparation of peritoneal macrophages (229)

BALB/c mice were injected intraperitoneally with sodium caseinate (0.5% in saline pH 7.2). Rest of the procedures were done under sterile conditions. Mice were sacrificed by cervical dislocation and abdomen was cleaned with spirit. Skin was removed and the peritoneal cavity was distended by injecting 5 ml of DMEM. Peritoneal cavity was gently prodded and aspirated to collect the peritoneal fluid which contained macrophages. The cells were washed and suspended in DMEM to desired cell density.
2.2.26 Collection and preparation of SRBC (229)

Sheep blood was freshly collected from the slaughter house in equal volumes of Alsever's solution (2.1.3.d) and stored at 4°C for not more than 1 week. Cells were washed three times with PBS before use and suspended in medium or HBSS to the final desired cell density.

2.2.27 Trypsinization of SRBC

10 parts of 4% SRBC and one part of trypsin solution (1% in distilled water) were incubated at 37°C for 30 minutes. Cells were washed three times with PBS and resuspended at a concentration of 1%.

2.2.28 Determination of circulating antibody titre

The non-agglutinated SRBC will settle at the bottom of the round bottom well of a titreplate as a clear "button", while agglutinated SRBC settle as a diffused mat. The maximum dilution of antisera at which clear agglutination was observed gives the titre of the antibody (241).

Antisera (0.1 ml) was serially diluted in round bottom 96 well titre plates in PBS (pH 7.2) and an equal volume of trypsinized SRBC (0.1 ml) (2.2.27) was added and incubated at room temperature for 3 h. The dilution at which clear agglutination observed was noted.

2.2.29 Determination of antibody forming cells

Antibodies produced from the lymphoid cells, from animals immunized with SRBC, causes lysis of sheep red blood cells in its vicinity (plaques) in a semi-solid support, in presence of complement (230).
Agarose (0.5 ml, 0.5%) was distributed into several tubes kept at 45°C to avoid solidification. The tubes were taken out and 0.05 ml of SRBC (7%) and 0.05 ml spleen cells (8 x 10^6 cells per ml) were added. The contents were thoroughly mixed and spread on an area of 10 cm² on a glass slide and was allowed to solidify. The slides were incubated with rabbit serum (source of complement) diluted 1 to 10 with PBS at 37°C for 1 h. The number of plaques were counted using a colony counter and represented as plaque forming cells (PFC) per 10⁶ spleen cells.

2.2.30 Determination of macrophage-mediated cytotoxicity

Activated macrophages can mediate cytotoxicity towards tumour cell lines such as L-929 cells, which can be assessed by staining with crystal violet (2.1.3.i) (91).

L-929 cell suspension (2.2.7/2.2.8) (5 x 10^3 cells/0.1 ml) was cultured in 96 well microtitre plates for 24 h 37°C in 5% CO₂ atmosphere.

Macrophages were induced in BALB/c mice by injecting 0.2 ml of sodium caseinate (0.5% in isotonic saline, pH 7.2) intraperitoneally in the presence and absence of test compounds. Macrophages (2.5 x 10⁴ cells/0.1 ml) were harvested on 5th day (2.2.25), and suspended in RPMI (2.B.1.b) with 10% FCS and added to each well. The plates were incubated at 37°C and 5% CO₂ atmosphere for 48 h. After incubation, medium was removed, washed three times with PBS, fixed with 0.1 ml of 5% formaldehyde solution for 20 minutes and stained with 0.05% solution of crystal violet (2.1.3.i) for 20 minutes. Excess stain was washed and the morphology of the cells were observed under a microscope.
2.2.31 Statistical analysis

Students 't' test (214) was used for the statistical evaluation of the data. To determine the statistical significance between two values in the control (x) and treated (y) group, 't' value was calculated using the equation,

\[ t = \frac{\bar{x} - \bar{y}}{S \sqrt{\left(\frac{1}{n_x} + \frac{1}{n_y}\right)}} \]

where \(\bar{x}\) and \(\bar{y}\) are the means of two samples x and y; \(n_x\) and \(n_y\) are the sample size and \(S\) was found out using the equation.

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

where, \(S_x\) and \(S_y\) are the standard deviations of the two samples, \(\text{df}\) - the degree of freedom \((n_x + n_y - 2)\).

By knowing 't' value and degree of freedom, statistical significance (P value) was deduced from 't' distribution table.