Chapter – 2

MATERIALS AND METHODS
2.1. Experimental animals:

In the present investigation Swiss albino mice (*Mus musculus*) with diploid chromosome number 40 were used as test animals. The animals were bred and maintained in the departmental animal house, following the standard ethically accepted procedures. Healthy virgin male and female Swiss albino mice 8-10 weeks of age, with average body weight (b.w) of 25 ±3 g were used for the experiments. Animals were housed in polypropylene shoe box type cages with steel mesh tops and rice husk bedding in a standard environmental conditions of 23± 2°C temperature and, 55-65% relative humidity. They were provided with drinking water and laboratory rodent diet pellets (Lipton, India Ltd) *ad libitum*. All studies were conducted as per the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India.

The mice are the animals of first choice for genetic toxicology studies because of their well defined cytogenetic, physiological and metabolic system. Mouse has 20 pairs of acrocentric chromosomes, which makes scoring of the structural chromosomal aberrations easier. It has well defined physiological and metabolic system and being a mammal, many of its physiological functions and biochemical pathways are similar to man, making the study particularly relevant to human studies.

2.2. Preparation of the extract:

Fresh, sun dried *Garcinia indica* fruit rind was purchased from the dealer in Mangalore. The plant material was identified by a taxonomist, Dr Suryaprakash Shenoy, Department of Applied Botany, Mangalore University. A voucher specimen was kept in Applied Zoology museum (*AZ -007-G ind*). Fruit rind was cleaned by using distilled water and dried in an oven at 40°C. The extract was prepared by soaking about 500gms of fruit rind in one liter of double distilled water overnight. Next day the rind was ground to a paste using kitchen mixer. The content obtained was filtered using double layered clean fine muslin cloth. The aqueous extract thus obtained was again filtered using filter paper. Then the filtrate was evaporated in a water bath maintained at 40°C. The thick concentrate obtained after the evaporation was further dried in a desiccator containing fused calcium chloride. The final yield of the extract thus obtained was weighed and kept in deep freezer (-25°C) for future use.
500gms of *Garcinia indica* extract in one litre of distilled water yielded 38 gms of crude aqueous extract

2.3. Phytochemical study:

Phytochemical analysis of crude extracts was done using standard chemical tests (Harnborne, 1973, Farnsworth and Bingel 1977, Kokate, 1985) to assess the presence of following phytochemicals

- Alkaloids
- Steroids
- Glycosides
- Proteins and amino acids
- Carbohydrates
- Saponins
- Flavones
- Oils and fats
- Phenolic compounds and tannins

2.3.1. Chemicals and Reagents:

**Molisch’s reagent:** 10gms of Naphthol dissolved in 100ml of 95% of ethanol

**Anthrone reagent:** 0.2g of anthrone dissolved in 100ml of concentrated H$_2$SO$_4$ and mixed thoroughly

**0.1% Ninhydrin reagent:** 0.1 gm of ninhydrin powder dissolved in 100ml of acetone

**Dragendorff’s reagent**

50 gms of tartaric acid was dissolved in 250ml of distilled water. 4.5 g of basic bismuth nitrate was added to the solution and mixed well for 2 hrs. 100ml of 40% Potassium iodide was then added to the solution and shaken vigorously, allowed to stand for 24 hrs and filtered

**Buret reagent**

1.5 gms of copper sulphate and 6gms of sodium potassium tartarate were dissolved in 500ml of water and the total volume was made up to one litre
Phenolphthalein reagent: 1gm of phenolphthalein powder dissolved in 100ml of ethanol

Lead acetate (10% solution): 10 gms of lead acetate dissolved in 100ml of methanol

Alcoholic KOH (0.1N): 5.6 gms of KOH dissolved in 100ml of ethanol

HCl solution: 50ml of conc HCl dissolved in 50ml of water

NaOH solution (10%): 10 gms of NaOH dissolved in 100ml of distilled water

Copper sulphate solution: 24.7 gms of copper sulphate dissolved in 100ml of distilled water

Iodine in KI: 20 gms of KI and 12.7 gms of iodine were dissolved in 30ml of distilled water and total volume was made up to one litre

2.3.2. Test protocol:

i) Alkaloids:

Alkaloids are basic nitrogenous plant products mostly optically active and possess nitrogen heterocycles as their structural units with pronounced physiological action

<table>
<thead>
<tr>
<th>Test</th>
<th>Observation</th>
<th>Inference</th>
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<tbody>
<tr>
<td><strong>a. Dragendorff's test</strong></td>
<td>Orange colored precipitate</td>
<td>Alkaloids present</td>
</tr>
<tr>
<td>1ml extract + 4.5 ml diluted HCl + few drops of Dragendorff's reagent</td>
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<td></td>
</tr>
<tr>
<td><strong>b. Wagner's test</strong></td>
<td>Brown colored precipitate</td>
<td>Alkaloids present</td>
</tr>
<tr>
<td>1ml of extract mixed with 4.5 ml dHCl shaken well and few drops of iodine in KI was added</td>
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ii) Steroids:
These are large class of organic compounds They occur widely in plants and animals
and are characterized by the presence of 1,2-cyclopentanophenanthrene ring system,
which may be partially reduced or otherwise modified

<table>
<thead>
<tr>
<th>Tests</th>
<th>Observation</th>
<th>Inference</th>
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<tbody>
<tr>
<td>Solkowski’s test</td>
<td>Red colored precipitate</td>
<td>Steroids present</td>
</tr>
<tr>
<td>1ml of extract dissolved in equal volume of chloroform + 2ml of conc H₂SO₄</td>
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iii) Glycosides:
Hemiacetyl form of a sugar reacts with a molecule of an alcohol to form acetyl
derivatives, which are generally known as glucosides and of fructose are known as
fructosides

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<tr>
<th>Tests</th>
<th>Observation</th>
<th>Inference</th>
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</thead>
<tbody>
<tr>
<td>a. Sulphuric acid test</td>
<td>Reddish colored precipitate</td>
<td>Glycosides present</td>
</tr>
<tr>
<td>1ml of extract and few drops of conc H₂SO₄ were mixed, shaken and the contents were allowed to stand for a few minutes</td>
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</table>

iv) Proteins and aminoacids:
Proteins and complex nitrogenous compounds, occur in plants and animal cells
Proteins on hydrolysis with strong inorganic acids or enzymes yield a mixture of
amino acids Following tests
were conducted

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<tr>
<th>Tests</th>
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<th>Inference</th>
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</thead>
<tbody>
<tr>
<td>a. Ninhydrin test:</td>
<td>Blue color</td>
<td>Aminoacids present</td>
</tr>
<tr>
<td>2-3 drops of Ninhydrin reagent was added to 1ml of extract</td>
<td></td>
<td></td>
</tr>
<tr>
<td>b. Millon’s test</td>
<td>White precipitate which turned to red on heating</td>
<td>Amino acids present</td>
</tr>
<tr>
<td>To 1ml of extract few drops of Millon’s reagent was added</td>
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</tbody>
</table>
v) **Carbohydrates**

Substances with the general formula of \( C_n(H_2O)_n \) are called as carbohydrates (Hydrates of carbon) because they contain hydrogen and oxygen in the same proportion as in water.

<table>
<thead>
<tr>
<th>Tests</th>
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<th>Inference</th>
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</thead>
<tbody>
<tr>
<td><strong>a. Molisch's test:</strong></td>
<td>Formation of reddish violet color at the junction of two liquids</td>
<td>Carbohydrates present.</td>
</tr>
<tr>
<td>1 ml extract+2 ml Molisch's reagent mixed and shaken carefully 2 ml of conc. H(_2)SO(_4) added along the side of the test tube and allowed it to stand for 2 minutes</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>b. Anthrone test:</strong></td>
<td>Green or blue color precipitate</td>
<td>Carbohydrates are present</td>
</tr>
<tr>
<td>1 ml of extract+1-2 ml of water +1-2 ml of anthrone reagent were mixed well, shaken and the contents were warmed gently</td>
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</table>

vi) **Saponins:**

These are plant steroidal glycosides which have the property of foaming in water (like soap solution) They act by lowering the surface tension of water.

<table>
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<tr>
<th>Tests</th>
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<tbody>
<tr>
<td><strong>a. Aqueous test</strong></td>
<td>Formation of lather (foam)</td>
<td>Saponins present</td>
</tr>
<tr>
<td>To small quantity of extract few drops of water was added and shaken well</td>
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</tbody>
</table>
vii) Flavones:
The flavones, which are also known as anthoxanthins, are yellow pigments, which occur in the plant kingdom

<table>
<thead>
<tr>
<th>Tests</th>
<th>Observation</th>
<th>Inference</th>
</tr>
</thead>
<tbody>
<tr>
<td>a. Aqueous test</td>
<td>Formation of yellow color</td>
<td>Flavones present</td>
</tr>
<tr>
<td>To 1ml of extract, equal quantity of aqueous NaOH was added</td>
<td></td>
<td></td>
</tr>
<tr>
<td>b. H₂SO₄ test:</td>
<td>Appearance of yellowish red color</td>
<td>Flavones present</td>
</tr>
<tr>
<td>To Small quantity of extract, few drops of conc H₂SO₄ was added</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

eviii) Oils and Fats:

<table>
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<tr>
<th>Tests</th>
<th>Observation</th>
<th>Inference</th>
</tr>
</thead>
<tbody>
<tr>
<td>a. Spot test:</td>
<td>Oils stain on the paper</td>
<td>Oils and fats present</td>
</tr>
<tr>
<td>Small quantity of extract was pressed with filter paper</td>
<td></td>
<td></td>
</tr>
<tr>
<td>b. Saponification test</td>
<td>Formation of soapy solution</td>
<td>Oils and fats present</td>
</tr>
<tr>
<td>1ml of extract + few drops of 0.5 N alcoholic KOH + a drop of phenolphthalein were mixed Heated on water bath for 1-2hrs</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ix) Phenolic compounds and Tannins:

<table>
<thead>
<tr>
<th>Tests</th>
<th>Observation</th>
<th>Inference</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>Precipitation/ appearance of greenish color</td>
<td>Tannins present</td>
</tr>
<tr>
<td>1ml of extract and equal amount of FeCl₃ solution were mixed</td>
<td></td>
<td></td>
</tr>
<tr>
<td>b</td>
<td>White precipitate appeared</td>
<td>Phenolic compounds present</td>
</tr>
<tr>
<td>Lead acetate test 1ml of extract and 2ml of 10% lead acetate solution were mixed</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2.4. Toxicity studies:

Before selecting the doses of *Garcinia* aqueous extract *in vivo* toxicity of the extract was determined in Swiss albino mice by following the method of Ghosh (1984). For this the mice were fasted by withdrawing the food and water for 18 hours. Then they were divided into different groups of 10 animals (5 males and 5 females in each group). Various doses of the extract (100, 200, 300, 400, 500, 1000, 2000, 3000, b w) were prepared in saline and administered to different groups of fasting animals through oral gavage method. After administering the extract feeding was resumed and animals were kept under observation for 72 hrs. No toxicity signs were observed in the tested doses during this period. The animals were further observed for 14 days to determine the toxic or lethal effects of the extract. None of the doses of *Garcinia* induced mortality in the experimental animals. From the above tested doses three doses (i.e., 100, 200, 400 mg/kg b w) were selected for further study.

2.5. Anticlastogenicity studies:

2.5.1. Metaphase Chromosome Analysis:

The preparation of bone marrow metaphase chromosome was done by following the method of Tjio & Whang (1962).

Principle:

In order to obtain well-spread metaphase chromosomes, animals were treated with colchicine and cells were harvested in hypotonic solution. Colchicine is a mitotic arrestant and it inhibits the spindle fiber formation thereby arresting the mitosis at metaphase stage. Hypotonic solution causes the swelling of cells, enabling the *in situ* spreading of the chromosomes.

Chemicals and Reagents:

a) Colchicine 0.025% colchicine (Batch No T 823279, SRL, Mumbai, India) prepared in distilled water was used for the experiment. Based on the body weight of the animal, 0.2-0.3 ml of colchicine was administered intraperitoneally.
b) 0.56% Potassium chloride (KCl) was freshly prepared at the time of experiments by dissolving the required amount of the salt in distilled water.

c) Acetic-methanol (fixative) 1 part of glacial acetic acid is mixed with 3 parts of absolute methanol. Fixative was freshly prepared at the time of experiments.

d) Giemsa Stain (Stock) Giemsa stain (E-Merck, Batch No DB2 DR 52285, Mumbai, India) 1 gm of Giemsa powder was dissolved in 54 ml of glycerol and mixed thoroughly. The content was then kept at 60°C oven for 2 hrs with intermittent stirring. The mixture was then cooled to room temperature. To this cooled mixture 84 ml of distilled methanol was added stirred well and filtered. The filtrate thus obtained is the stock Giemsa.

Staining Buffer (Stock Solution) Disodium hydrogen phosphate - Na$_2$HPO$_4$ (0 2M) 2.366 g was dissolved in 250 ml of double distilled water.

Potassium dihydrogen phosphate - KH$_2$PO$_4$ (0 2M) 2.27 g was dissolved in 250 ml of double distilled water. Buffer solution was prepared by mixing equal quantity of Na$_2$HPO$_4$ and KH$_2$PO$_4$. For 1000 ml of buffer solution, 50 ml each of these salts were used.

Preparation of bone marrow slides

The experimental animals were intraperitoneally injected with 0.025% colchicine (0.2 - 0.3 ml). After 1-1½ hours, the animals were killed by cervical dislocation, and the marrow cells from femur and tibia bones were flushed with 0.56% KCl taken in a syringe. Marrow suspension was thoroughly mixed and kept at room temperature for 20 minutes. After 20 minutes, the suspension was centrifuged at 1000 rpm and the pellet was resuspended in 2 ml of fixative (1:3 acetic-methanol), and kept at room temperature for 45 minutes and centrifuged. The supernatant was discarded, and fixative was again added to the pellet and mixed thoroughly, incubated for 10 minutes at room temperature and centrifuged. This step was repeated three times. Finally, the pellet was suspended in appropriate amount of fixative, dropped onto clean, pre-chilled slides and flame dried.
Staining

20-25 drops of stock Giemsa stain was diluted with 30-35 ml of working buffer (pH 6.8) and filtered through Whatmann No 1 filter paper. The diluted stain was taken in a coplin jar and slides were kept in the stain for 20 minutes. The excess stain in the slides was washed with phosphate buffer and air-dried.

Scoring of aberrations

Slides were screened under Olympus BX51 microscope. One hundred well-spread metaphases were examined from each animal. A total of 2000 dividing and non-dividing cells were also scored from each animal to determine the mitotic index values.

2.5.2. Bone marrow micronucleus assay:

Bone marrow micronucleus test was conducted according to the method described by Schmid (1973) and modified by Seetharam et al (1983). Here, bovine serum albumin (3%) prepared in phosphate buffered saline (pH 7.2) was used as suspending medium instead of fetal calf serum of Schmid’s method.

Chemicals and Reagents:

Buffered saline

Reagents

Disodium Hydrogen Phosphate (Na$_2$HPO$_4$) - 1.065 gm dissolved in 50 ml of double distilled water

Sodium Dihydrogen Phosphate (NaH$_2$PO$_4$) - 1.17 gm dissolved in 50 ml of double distilled water

0.9% NaCl - 0.9 g of NaCl in 100 ml of double distilled water

Buffered saline (pH - 7.2) was prepared by mixing 41 ml of Na$_2$HPO$_4$+ 9 ml of NaH$_2$PO$_4$ and 50 ml of saline.

Preparation of 3% bovine serum albumin (BSA) 3 gms of BSA – Fraction V (Himedia, Mumbai, India) was dissolved in 100 ml of PBS by slowly adding the powder with constant stirring, to avoid clump formation.
Preparation of stains

Giemsa stain Already explained

May-Grunwald’s stain. 0.2 gm of stain powder was dissolved in 100 ml of distilled methanol, mixed well and filtered

Preparation of micronucleus slides and staining

The experimental mice were killed by cervical dislocation. The bone marrow cells from femur and tibia bones were flushed with 3% bovine serum albumin (BSA) taken in a syringe. A total of 2 ml of cell suspension thus obtained was dispersed by gentle pipetting and centrifuged at 1000 rpm for 10 minutes. The supernatant was discarded and pellet was resuspended in small quantity of BSA to get a suspension of appropriate thickness. A drop of suspension was smeared on clean slide and air dried. Immediately after drying the slides were fixed in distilled methanol for 10 minutes.

Staining of the MN slides should be done between 3-24 hours after preparation. Slides were kept in May-Grunwald’s stain diluted with equal quantity of phosphate buffer (pH-6.8) for 15 minutes. They were then transferred to Giemsa diluted with phosphate buffer (1:6) for 10 minutes. The slides were then rinsed in phosphate buffer giving 2-3 changes and then they were kept in distilled water for proper differentiation of the young and mature erythrocytes. With this method, polychromatic erythrocytes stain bluish and normochromatic erythrocytes reddish pink while nuclear maternal stain dark purple.

Scoring of micronucleus

Slides were screened under Olympus BX51 microscope for the presence of micronuclei (MN) in polychromatic erythrocytes (PCE) and normochromatic erythrocytes (NCE). 2000 PCE and the NCE in the corresponding field were screened from each animal to score the MN and to determine P/N ratio.

2.5.3. Peripheral blood micronucleus assay:

Peripheral blood micronucleus test was carried out according to the method described by Schlegel and MacGregor (1982). The blood was drawn from tail vein.
from different groups of experimental mice. A drop of blood was placed on absolutely clean slide and a thin smear was made by using a spreader slide. The smears were air dried and fixed in absolute methanol for 5 minutes. After drying slides were stained by using Wright’s – Giemsa method.

**Chemicals and reagents:**

**Reagents**

Buffer solution (pH- 6 8) Method of preparation already explained in this chapter.

**Preparations of stains**

Giemsa stain was prepared as per the procedure already given in this chapter.

Wright’s stain was prepared by dissolving 250mg of Wright’s stain powder in 50ml of methanol. The stain was mixed well and filtered.

**Staining of the slides and scoring**

After fixation of the smear, the slides were kept in undiluted Wright’s stain for 6 min and then transferred to Wright’s stain freshly diluted with equal volume of phosphate buffer (pH- 6 8) and kept for 4 min. Again the slides were washed in phosphate buffer for about 2 min and then transferred to Giemsa’s stain, diluted with phosphate buffer (1 6) for 12 minutes. The slides were then washed in phosphate buffer for 2 min and air-dried.

Slides were screened under Olympus BX51 microscope for the presence of micronuclei (MN) in normochromatic erythrocytes (NCE). About 2000 NCE per animal were scanned for the presence of MN and number of PCE present in the corresponding focus were scored to determine the effects of agents on the erythropoiesis.

**2.5.4 Sperm abnormality assay:**

In this assay control and test agents were administered to the male mice and after 35 days sperm preparations were made and analyzed for abnormalities and variations in the sperm count. The analysis was done according to the criteria of Wyrobek and Bruce (1975) and Vega et al.,(1988).
Reagents used:
Buffered saline (pH-7.2) was prepared as per the procedure given in the MN test protocol.
1% aqueous eosine stain was prepared by dissolving 1 gm of stain powder (Hi media-Mumbai) in 100ml of distilled water.

Procedure
Eight week old male Swiss albino mice were used for the experiments. 5 animals were used for each treatment and control groups. Initial body weight of the animals was taken before starting the experiments. One post treatment sampling time; i.e., 5 weeks was used for the study. It is because the germ cells which are exposed at late spermatogonia to the mutagen would reach the cauda epididymis after undergoing a series of changes during the course of development into a sperm. As per the time schedule given by Oakberg (1956) for the development of sperm from spermatogonial stage to sperm in mice is around five weeks (Wyrobek and Bruce, 1978).

On 35th day after the last treatment, the body weight of the animals was noted before sacrificing the animals. The animals were sacrificed by cervical dislocation and the reproductive tract was exposed. Both the testes were removed from the scrotal region and weighed. Both the cauda epididymis were also removed and placed in a petridish containing 1 ml of phosphate buffer saline (pH-7.2). The cauda epididymis were minced thoroughly using wire mesh and the suspension obtained was filtered through a muslin cloth to remove tissue debris. The filtered suspension was stained with 1% aqueous eosin (10 1) for about 20 minutes. A drop of the sperm suspension was taken on a clean slide and smeared. Slides were air dried and observed under the microscope. Two thousand sperms per animal were examined from each treatment and control groups for abnormalities. Testes weight and sperm count were also determined. Sperm count was done by using haemocytometer. 1ml of the sperm suspension was diluted (1 40) with PBS and mixed thoroughly. A sample of the diluted sperm suspension was introduced into the Neubaur counting chamber and the total sperm count in 8 squares of 1mm³ each was determined and multiplied by 5x10⁴ to calculate the number of sperms per epididymis (Vega et al., 1988).
2.6. Antioxidant studies:

In the present study possible antioxidant effects of solvent fractions of *Garcinia indica* was determined by estimating the SOD, GSH and Catalase in different groups of animals. The liver tissue was used for this analysis.

2.6.1. Collection of tissue sample:

The animals were anesthetized using chloroform. The abdominal cavity was opened by longitudinal incision and liver was exposed, perfused with cold saline. The liver tissue was removed and washed first in ice-cold distilled water and then in normal saline. The tissue samples from different groups of animals were stored at -25°C deep freezer until use. UV-visible double beam spectrophotometer (UV-1601, Shimadzu, Japan) was used to take the optical density readings.

2.6.2. Methodology:

i) Superoxide dismutase (SOD):

Estimation of the superoxide dismutase activity was done following the method of Beauchamp and Fridovich (1971). Illumination of riboflavin in presence of oxygen and electron donors like methionine or EDTA causes the reduction of flavin. It then reoxidizes and simultaneously reduces oxygen to superoxide anion O$_2^-$. This anion reacts with detector molecule nitro-blue tetrazolium,

\[
\text{NBT} + \text{O}_2^- \rightarrow \text{NBT}^* \text{ radical} + \text{O}_2
\]

The reduction of NBT by superoxide anions was assessed by taking the OD at 560nm using a spectrophotometer. This principle has been used as the basis for SOD assay.

Chemicals and Reagents:

Potassium phosphate buffer (0.2 M, pH-7.8)

Reagents

Potassium dihydrogen phosphate (KH$_2$PO$_4$) [0.2 M] 5.44g of KH$_2$PO$_4$ in 1 litre of double distilled water
Dipotassium hydrogen phosphate (K$_2$HPO$_4$) [0.2 M]: 6.96 g of K$_2$HPO$_4$ in one litre of double distilled water

200 ml of KH$_2$PO$_4$ was mixed with 20 ml of K$_2$HPO$_4$ and the pH was adjusted to 7.8

Potassium phosphate buffer (0.05 M, pH -7.8): 25 ml 0.2 M potassium phosphate buffer was diluted to 100 ml with double distilled water

Potassium phosphate buffer (10 mM, pH - 7.8): 5 ml of 0.2 M potassium phosphate buffer was diluted with double distilled water to 100 ml

Nitro blue tetrazolium chloride (NBT): NBT (SRL, Mumbai,) was dissolved in 0.05 M potassium phosphate buffer and the concentration obtained must be 1 mg/ml

Riboflavin: Riboflavin (SRL, Mumbai) was dissolved in 0.05 M potassium phosphate buffer and the concentration obtained must be 1 mg/ml

Superoxide Dismutase (SOD) substrate: To 25 ml of 0.2 M potassium phosphate, 149 mg of Methionine (SRL, Mumbai, Batch No.), 4.93 ml NBT and 0.63 ml of riboflavin were added and the volume was made up to 100 ml with double distilled water

Preparation of the homogenate:

10 % w/v homogenate was prepared in cold, 10mM potassium phosphate buffer. The homogenate was centrifuged and the supernatant was used for SOD assay. 0.2 ml of the supernatant was diluted to 2.0 ml with 0.05 M potassium phosphate buffer.

Assay method:

- To 0.1 ml of diluted homogenate, 2.9 ml of SOD substrate was added.
- The control mixture was prepared by adding 0.1 ml of potassium phosphate buffer (0.05 M) to 2.9 ml of SOD substrate. 3 ml of potassium phosphate buffer alone was kept as blank.
- The control and test samples were illuminated in an illumination chamber (aluminum foil lined box fitted with 15V fluorescent lamp) for 10 minutes.
• The optical density was read at 560 nm. The enzyme activity was expressed as units/mg protein.

ii. Reduced Glutathione (GSH):

The GSH activity was assessed following the method of Ellman (1959). This method is based on the development of a stable yellow color when 5, 5'-dithio-bis-2-nitrobenzoe acid (DTNB/ Ellman's reagent) is added to a solution containing sulphydryl compounds. The advantages of using this reagent are its water solubility, reactivity at neutral pH, rapid reaction with thiol groups, and high sensitivity.

Chemicals and Reagents:

Precipitating solution: 1.67 g glacial metaphosphoric acid, 0.2 g EDTA, and 30 g NaCl were dissolved in 100 ml double distilled water.

Ellman Reagent (DTNB): 40 mg of DTNB (SRL, Mumbai, Batch No T 824330) was dissolved in 100 ml phosphate buffer (pH 7.8).

Phosphate solution (0.3 M): 3.67 g of Na₂HPO₄ dissolved in 100 ml of double distilled water.

Preparation of the homogenate:

Homogenate was prepared as per the procedure given for SOD assay.

Assay method:

2 ml of diluted supernatant was mixed with 3 ml of precipitating solution and mixed thoroughly. The mixture was allowed to stand for 10 minutes at room temperature for complete precipitation and centrifuged. 1 ml of the supernatant was mixed with 4 ml of phosphate solution. To this, 0.5 ml of Ellman's reagent was added, mixed well and kept for 10 minutes for color development (stable yellow color). Optical density was read at 412 nm. The GSH content was expressed as μ moles of GSH/mg protein.

iii. Catalase test:

Catalase activity was estimated using the method of Abei (1984). In the ultraviolet range H₂O₂ shows a continual increase in absorption with the decreasing...
wavelength The decomposition of H$_2$O$_2$ can be followed directly by the decrease in absorbance at 240 nm. The difference in absorbance per unit time is a measure of catalase activity.

**Chemicals and Reagents:**

**Phosphate buffer** (50mM, pH 7.0)

**Solution A**  6.8 g of KH$_2$PO$_4$ was dissolved in 1000 ml of double distilled water

**Solution B**  8.9 g of Na$_2$HPO$_4$ 2H$_2$O was dissolved in 1000 ml of double distilled water

Working buffer was prepared by mixing solutions A and B in the proportion of 1:1:5 (V/V)

**Hydrogen peroxide** (30 mM)  0.34 ml of 30% hydrogen peroxide (Qualigens) was dissolved in phosphate buffer to make 100 ml

**Triton-X (1%)**  1 ml of Triton-X (BDH, England, Lot No K25908352 927) was made up to 100 ml with phosphate buffer

**Preparation of the homogenate:**

10% w/v homogenate was prepared in 1% triton-X prepared in phosphate buffer. The homogenate was centrifuged and the supernatant was diluted with Triton X (1 50) and used for the assay

**Assay method:**

The reduction in the optical density was followed for the reaction mixture containing 2 ml of diluted homogenate and 1 ml of H$_2$O$_2$ solution, over a period of 30 seconds. The blank contained 1 ml of phosphate buffer and 2 ml diluted homogenate. The optical density was read at 240 nm.

**2.6.3. Protein Estimation:**

In all the above mentioned assays, the quantities of enzyme/molecules were expressed as units/mg of protein. Therefore, each time protein estimation was done following the method of Lowery et al. (1951). The protein reacts with Folin-Ciocalteau reagent to give a colored complex. The color formed is due to the
formation of alkaline copper with protein as in biuret test and reduction of phosphomolybdate by tyrosine and tryptophan present in the protein. The intensity of the color depends on the amount of these aromatic amino acids present in the proteins.

**Chemicals and Reagents:**

- **2% Sodium Carbonate** (Reagent A) 2g of Na₂CO₃ was dissolved in 100ml of 0.1N NaOH
- **0.5% Copper Sulphate** (Reagent B) 500mg of copper sulphate was dissolved in 100ml of sodium potassium tartarate solution
- **Reagent C** 50ml of Reagent A was mixed with 1ml of Reagent B
- **Folin-Ciocalteau (FC) Reagent** Concentrated Folin’s reagent was diluted with double distilled water (1:2)
- Bovine serum albumin was used as the standard

**Preparation of the homogenate:**

Preparation of the homogenate was done as per the procedure given for SOD assay.

**Assay method:**

0.5ml of tissue homogenate was made up to 1ml with 0.1N sodium hydroxide. To this mixture, 5ml of Reagent C was added, mixed and incubated at room temperature for 10 minutes. Then 0.5ml of FC reagent was added, mixed and incubated at room temperature for 30 minutes for the development of color. Optical density was read at 600 nm using spectrophotometer.

**2.7. In vitro anticancer studies:**

The cell lines used here were mouse melanoma cells (B-16) and human breast adenocarcinoma cells (MCF-7).

**2.7.1 Reagents and methods:**

Preparation of Dulbecco’s modified eagle’s media (DMEM). DMEM (Batch No. MO268-10L(95gm/L) Sigma laboratories,USA) with L-glutamine, without sodium bicarbonate (E-Merck, Mumbai) powder in the given vial was dissolved in 1 litre of...
autoclaved double distilled water. To 900 ml of autoclaved double distilled water (DDW), contents of 1 unit vial was added at room temperature with constant stirring. The vial was rinsed with small amount of DDW to remove traces of powder and added to the above solution. 1500 mg NaHCO₃ (Himedia) was added, stirred well. The pH of the solution was adjusted to a range of 7.1 – 7.4 using 1N HCl or 1N NaOH. It should be noted that, pH tends to rise during filtration and hence, adjust it 0.2-0.3 units below the final desired pH. Final volume was made up to 1000 ml with DDW sterilized by filtering through autoclaved membrane filter (Cellulose acetate filter, 0.2 μm pore size, 47 mm diameter (Sartorius AG, Germany) in a filter unit, using vacuum pump. 2.5 ml of antibiotic-antimycotic solution, gentamycin, 2 ml t.e., 80 mg/ml (Himedia laboratories, Mumbai, India) was added and stored in a refrigerator at 2-8°C until further use.

**Preparation of Trypsin**

Trypsin (Batch No P-10-025025p Pan biotech GMBH.) The chemicals mentioned below were dissolved in 100 ml of DDW, filtered and stored in the refrigerator.

NaCl-800 mg, Na₂HPO₄-115 mg, KH₂PO₄-20 mg, KCl-20 mg, Glucose-50 mg, Trypsin-100 mg,

EDTA (Na₂salt)- 0.02 mg

**2.7.2. Maintenance of B-16 cell and MCF-7 cell lines:**

B-16 is mouse melanoma cell line and MCF-7 is human breast cancer cell line. Both are adherent type of culture.

After the culture attains confluence, the media was removed and the cells were treated with trypsin, incubated for 2-3 min. Then the trypsin was removed by aspirator and the culture was washed with media and 2 ml fresh media was added. Bottom of the flask (Nunclon R -25 cm, Denmark) was tapped so that all the cells get detached. Then the cells were mixed gently in order to get single cell suspension and cells were counted (1x10⁶/ml) in a haemocytometer using trypan blue dye exclusion method, transferred to a fresh culture flask containing DMEM supplemented with 10% Fetal Bovine Serum (FBS, Himedia laboratories Pvt Ltd, Mumbai, India) and antibiotic-antimycotic solution (Himedia laboratories Pvt Ltd, Mumbai, India). Flask was tilted in order to distribute the cells uniformly, and kept at 37°C in humidified.
atmosphere with 5% CO₂. Media was changed every 3 days and after 6 days when culture attained confluence it was sub-cultured or used for the experiments.

**Counting.**

100μl of each of the cell suspension and trypan blue dye were mixed and loaded on the hemocytometer, the viable and dead cells were counted in total 10 squares (4 larger corner and 1 middle squares) of both sides of the hemocytometer. Then calculation was done using following formula:

\[
\text{Average no of cells} \times \text{dilution factor} \times 10,000 = X \text{cells/ml}
\]

Required amount of cells were taken for different experiments.

**Preparation of the extract:**

The aqueous extract was first dissolved in 0.1% DMSO. Then it was diluted with respective media in which cells were to be grown. Following two methods were used for studying the growth inhibitory effects on the tumor and normal cells.

2.7.3. Trypan blue dye exclusion test:

**Principle**

This is a simple method to determine immediate cell death. This method is based on the finding that the membrane of healthy cells is not permeable to trypan blue and actively repulses the entry of the dye from the medium. But the dye freely permeates the dead cells, staining them blue. Trypan blue (0.4%) is the most commonly used dye for this purpose.

**Preparation of trypan blue.** 0.4 g of trypan blue (Batch No 0-0307, Himedia, Mumbai, India) was dissolved in 100 ml of PBS and filtered through Cellulose Acetate filter, 0.2μm pore size, 47mm diameter, (Sartorius AG, Germany).

After adding the trypan blue, the percent dead cells were counted in a haemocytometer as mentioned earlier and calculation was done using the formula,
Dead cells counted

------------------------------------------ X 100

Total no of live and dead cells

From these values IC₅₀ for each treatment was determined

The IC₅₀ can be defined as the drug concentration, which causes 50% growth inhibition. The values for each cell line were determined graphically by plotting % of dead cells via Y axis vs the concentration of the extract along the X axis (Carmichael et al, 1985, Zheng et al, 2000)

2.7.4. MTT assay:

A number of microtitre assays have been developed to assess the cell survival. An assay which has been the subject of much recent study is the quantitative colorimetric assay using tetrazolium salt as originally developed by Mosmann in 1983 and usually referred as the 'MTT assay'. The assay quantitates living but not dead cells and has the advantage of being rapid, precise and semi-automatable (Wasserman and Twentyman, 1988). In this assay a tetrazolium salt 3-(4,5-dimethyl-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) is reduced to a purple formazan product by mitochondrial dehydrogenase that are active in living cells. The intensity of the purple color developed is a measure of cell viability. The % of cells surviving is determined by comparing the absorbance of the treated cells with that of control.

**Principle:**

MTT 3-(4,5-dimethyl-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) is a yellow water soluble salt which is biologically active. MTT enters the proliferating cells and it requires mitochondrial enzymes, where it is reduced to a purple colored formazan. It is usually carried out on cells growing in multiwell plates. The formazan precipitate extracted with an organic solvent, Dimethyl sulphoxide (DMSO) and the optical density of the resulting solution is measured using spectrophotometric method.

**Preparation of MTT** MTT (Batch No M2128, Sigma USA) was prepared by dissolving 1mg powder in 1ml MTT media without phenol red and FCS (fetal calf...
serum) under hood. Then it is filtered through Cellulose Acetate filter, 0.2µm pore size, 47mm diameter, (Sartorius AG, Germany) stored at 8°C away from the light.

MTT assay was done as per the modified method of Mosmann (1983). The culturing of the cells (number of the cells seeded is depending on the time interval) is done in 96 well plates for different time intervals. After incubation of the cells with test agents they were treated with 100µl of MTT and incubated for 4 hr, then MTT was removed and the formazan formed with the MTT was extracted with 100ul DMSO and after 10 minutes the absorbance was read at 550nm using multiwell spectrophotometer (Tecan, Austria).

The growth inhibition rate was calculated by using the following formula,

\[
\text{Inhibition rate} = \frac{\text{OD control well} - \text{OD treated well}}{\text{OD control well}} \times 100
\]

(Zheng et al., 2000)

### 2.8. Flow Cytometry:

Basically, a flow cytometer is a fluorescence microscope which analyses moving particles in a suspension. These are excited by a source of light (UV or laser) and in turn emit an epi-fluorescence which is filtered through a series of dichroic mirrors. Then, the in-built programme of the equipment converts these signals into a graph plotting the intensity of the epi-fluorescence emitted against the count of cells emitting it at a time given. In addition to determining the relative cellular DNA content, flow cytometry also enables the identification of the cell distribution during the various phases of the cell cycle. Four distinct phases could be recognized in a proliferating cell population: the G1, S (DNA synthesis phase), G2 and M-phase (mitosis). However, G2 and M phase, which both have an identical DNA content could not be discriminated based on their differences in DNA content. Diverse software containing mathematical models are used to calculate the percentages of cells occupying the different phases of the cell cycle. The flow cytometry method of Rao et al. (2009) was followed to study apoptotic cell cycle analysis.

**Materials required:**

DMEM, Trypsin, Trypan blue - prepared as explained for *in vitro* culture.
MTT (Sigma USA) dissolved 1mg/ml of MTT dissolving media

RNAse-1 100ug/ml dissolved in double distilled water

Propedium iodide- 50ug/ml in double distilled water

PBS-pH 7.2

Procedure:

1. The cells are seeded 1X10^6 into the culture dishes and incubated at 37°C with 5% CO₂ atmosphere for 24 hours

2. Then the plant extract or the chemical is added to the cells

3. The cells are harvested after 24 hours and transferred to the sterile centrifuge tubes

4. They are washed with PBS twice and fixed in 3ml of 70% ethanol and kept at 4°C overnight

5. After removal of ethanol, cells are resuspended in PBS and centrifuged twice

6. The pellet is resuspended in residual PBS. Then hypotonic solution (15μl of RNAase, 0.1% Triton X-100 (Merck, Germany), 0.1% sodium citrate solution) was added and incubated for one hr at 37°C. Then stained with 25 μl of Propedium iodide and kept for 1 hr. Care should be taken not to expose the content to light.

7. The stained cells are analyzed by flow cytometry. The population of G0/G1, S and G2/M are quantitated using cell quest (3.1) software.

8. Results are expressed as % of the cells in each phase

2.9. Immunomodulation assay methods:

2.9.1 Haemagglutination titre assay:
**Principle:**

The basic principle of haemagglutination titre test is based on the agglutination of RBC by the antibodies directed against them. Agglutination is the cross linking of the antigenic molecule by the antibodies to form an insoluble complex. Since RBC is the antigen agglutinated, it is called haemagglutination.

**Materials:**

Alsevier's solution is prepared by dissolving the following reagents:

- Dxtrose - 0.05 gm
- Sodium citrate - 0.80 gm
- Sodium chloride - 0.42 gm
- Distilled water - 100 ml

PH of the Alsevier's solution was adjusted to 6 by using HCl or NaOH.

Sheep blood with RBC is obtained from slaughter house and is mixed with Alsevier's solution. Then it is stored at 4°C for one week. SRBC was washed three times with normal saline (equal volume of saline and blood are taken and centrifuged. The supernatant is discarded using Pasteur pipette.) The SRBC is mixed with buffered saline, its number is counted using haemocytometer and required amount of SRBC is used for further test.

Method of preparation of phosphate buffered saline (pH-7.2) is already described earlier in this chapter.

**Haemagglutination titre procedure:** (Mediratta et al., 2002)

1. 100 µl of PBS is added to all wells of total plate (96 multiwell microtitre plates).
2. In the first well, 50 µl of serum is added and mixed well.
3. 50 µl from the first well is transferred to the next well and added to the next well to get two-fold dilutions of the antibodies present in the serum. Further twofold dilutions of this diluted serum were similarly carried out till the last well, so that the antibody concentration of any of the dilutions is half of the
previous dilution This situation is repeated to all the wells 50μl of last well should be discarded.  

4 The SRBC suspension is prepared in buffer (SRBC suspension) 50 μl of 1% SRBC suspension is added to each well  

5 The plates are shaken gently, then the plates are covered and incubated at room temperature for 2 hours and examined visually for agglutination  

6 The antibody titers are expressed in the graded manner, the minimum dilution (1/2) being ranked as 1, and mean ranks of different groups were compared for statistical significance (Deshputre and Naikwade, 2010)  

Haematological parameters such as total number of RBC, WBC, different WBCs, Hb level constitute the key components of the immune system. A rise or fall in their concentration affects the health/immune constitution of the body as they are known to recognize the foreign antigens and mount an immune response. Hence, these parameters are chosen to study the immunomodulatory activity of the aqueous extract of fruit rind of *Garcinia indica*.  

Blood samples of animals were subjected to haematological and serological studies. Blood cell count was done by using haemocytometer following Turke’s method (Dacie, and Lewis, 2001) for WBC and Hayem’s method (Easthan and Slade, 1993) for RBC. Platelet count readings were taken by using the instrument hematology analyzer. Haemoglobin concentration was determined by using Sahli’s method (Cavill et al., 1981). Thymus was weighed and relative weight was calculated using formula,  

\[
\text{Relative lymphoid organ weight} = \frac{\text{organ weight (mg)}}{\text{body weight (gm)}}  
\] (Du et al., 2010)  

2.10. Statistical analysis:  

All numerical data were calculated as mean and SD or SEM. The groups and control were compared by ANOVA followed by Dunnett’s post hoc test.
The basic principle behind the ANOVA test is as follows:

If \( E_1, E_2, \ldots, E_k \) be \( k \) samples drawn from normal distributions with identical variances, but possibly different means, ANOVA will test the null hypothesis \( H_0 \) according to which the means of these \( k \) distributions are indeed equal. So

- The null hypothesis is \( H_0: \mu_1 = \mu_2 = \mu_3 = \cdots = \mu_k \) against
- The alternative hypothesis \( H_1 \) at least one of the means is different from the others

If \( H_0 \) is rejected, the only conclusion of ANOVA is "At least one of the \( k \) groups has its mean significantly different from the mean of the total set of observations (to the chosen significance level). Many tests have been developed for the purpose of analyzing the reasons that made ANOVA reject the null hypothesis. These tests are globally known as "a posteriori" or "post-hoc" tests. The Dunnett's test is one of these tests.

Dunnett's test compares group means. It is specifically designed for situations where all groups are to be pitted against one "Reference" group. It is commonly used after ANOVA has rejected the hypothesis of equality of the means of the distributions (although this is not necessary from a strictly technical standpoint). Its goal is to identify groups whose means are significantly different from the mean of this reference group. It tests the null hypothesis that no group has its mean significantly different from the mean of the reference group.

For each pair (Reference, Group), the Dunnett's test calculates the value of a statistic "\( t_{\text{observed}} \)". This value is compared to a critical value. This critical value depends on the group sizes, the number of groups to be compared to the reference group, and the chosen significance level of the test (Dennett’s thtm).

In the present study ANOVA values were calculated using Graph Pad Prism Version 5 (Graph pad Inc, San Diedo, CA, USA).