Chapter 2

Materials and Methods
2.1. Materials

2.1.1. Plant Materials

2.1.1.1. *Hibiscus furcatus* Roxb. ex DC
   2.1.1.1.1. Distribution
   2.1.1.1.2. Morphology
   2.1.1.1.2. Collection

2.1.1.2. *Ophiorrhiza incarnata*
   2.1.1.2.1. Distribution
   2.1.1.2.2. Morphology
   2.1.1.2.3. Collection

2.1.3. Chemicals

2.1.4. Instruments

2.1.5. Cell lines
   2.1.5.1. Maintenance of Cell lines

2.1.6. Animals

2.2. Methods

2.2.1. Extraction

2.2.2. Phytochemical analysis (Qualitative test for phytochemicals)
   2.2.2.1. Test for carbohydrate
   2.2.2.2. Test for terpenoids
   2.2.2.3. Test for alkaloids
   2.2.2.4. Test for phenolic compounds
   2.2.2.5. Test for flavonoids
   2.2.2.6. Test for glycosides
   2.2.2.7. Test for Saponins
   2.2.2.8. Test for tannins

2.2.3. *In vitro* Antioxidant assays
   2.2.3.1. Free radical scavenging activity by DPPH method
   2.2.3.2. 2, 2’-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) radical scavenging activity
   2.2.3.3. Superoxide radical scavenging activity
   2.2.3.4. Hydroxyl radical scavenging activity
2.2.3.5. Nitric oxide radical scavenging activity

2.2.3.6. Lipid peroxidation assay

2.2.4. Protective effect of *H. furcatus* extracts against H2O2 induced haemolysis and lipid peroxidation in RBC

2.2.4.1. Determination of haemolysis inhibition in RBC

2.2.4.2. Inhibition of lipid peroxidation of RBC

2.2.5. *In vitro* cytotoxicity

2.2.6. Apoptosis assay

2.2.6.1 Induction of apoptosis in DLA and EAC cell lines

2.2.6.2. Analysis of morphology and nuclear condensation

2.2.6.3. Measurement of apoptosis by DNA fragmentation

2.2.6.3.1. Extraction and Purification of DNA

2.2.6.3.2. Agarose Gel Electrophoresis and analysis of fragmented DNA

2.2.7. Determination of Total count and Differential Count.

2.2.8. Determination of Bone marrow cellularity

2.2.9. Determination of haemoglobin (Hb) in blood

2.2.10. Determination of serum glutamate oxaloacetate transaminase (SGOT) activity

2.2.11. Determination of serum glutamate pyruvate transaminase (SGPT) activity

2.2.12. Determination of serum alkaline phosphatase (ALP) activity

2.2.13. Determination of serum creatinine

2.2.14. Estimation of Urea

2.2.15. Determination of serum total protein

2.2.16. Determination of superoxide dismutase (SOD) activity

2.2.17. Determination of catalase (CAT) activity

2.2.18. Determination of glutathione peroxidase (GPx) activity

2.2.19. Determination of reduced glutathione (GSH)

2.2.20. Determination of lipid peroxidation

2.2.21. Determination of tissue protein

2.2.22. Histopathology

2.2.23. Explant collection and preparation for tissue cultures

2.2.24. Culture media preparation

2.2.25. Determination of camptothecin in *Ophiorrhiza incarnata* by HPLC

2.2.25.1. HPLC system and condition

2.2.25.2. Sample preparation for HPLC analysis
2.2.25.2.1. Plant sample preparation
2.2.25.2.2. Standard preparation

2.2.26. Statistical analysis
2.1. Materials

2.1.1. Plant Materials

2.1.1.1. *Hibiscus furcatus* Roxb. ex DC
Binomial: *Hibiscus furcatus*
Family: Malvaceae
Common name: Pachapuli (Malayalam)

2.1.1.1.1. Distribution
*H. furcatus* (*Family: Malvaceae*) is distributed throughout India, common in the Western Ghats and plains, usually straggling extensively our forest thickets or hedges and bushes in the waste land in plains. Flowering is from November to February.

2.1.1.1.2. Morphology
Rambling shrubs, stem, petiole and pedicels armed with pustular base aculei. Leaf blade entire to 3 - 5 lobed or angled, palmately 3-5 nerved at base, aculei more dense on the nerve beneath. Petiole long with pubescence and aculei as in stem. Flowers axillary, solitary. Bracts involcellular, bifurcate, slightly shorter than calyx with hairs. Calyx prominent, with long stellar hairs. Corolla yellow with purple centre. Fruit ovoid or conical, enclosed within a accrescent calyx. Seeds 2-3 per cell, dark brown, concentrically tuberculed.

2.1.1.1.2. Collection
*H. furcatus* plants were collected from the outskirts of our campus, Amala Cancer Research Centre, Amala Nagar, Thrissur, Kerala, India.

2.1.1.2. *Ophiorrhiza incarnata*
Binomial: *Ophiorrhiza incarnata*
Family: Rubeaceae

2.1.1.2.1. Distribution
*O. incarnata* is herbaceous plant distributed in southern Western Ghats, in kerala it is in Vyanad district. Flowering season is from June to July.
2.1.1.2.2. Morphology
Herbaceous plant with brown pubescent stem. Leaves narrowly elliptic, dark green above. Inflorescence terminal capitate cymes, pedicels glabarous, flowers heterostylous with pinkish white in colour. Calyx lobes ovate-lanceolate, acute glabarous; corolla infundibulum, wide at the mouth, glabarous outside with a villous ring at the insertion of the filaments within. Seeds angular, glabarous, brown; wall of the areole thick with a number of tubercles on it.

2.1.1.2.3. Collection
*O. incarnata* plants were collected from Wynad district of Kerala, India.

2.1.3. Chemicals

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Supplier/Manufacturer</th>
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<tbody>
<tr>
<td>Doubliscos modified eagles Medium (DMEM)</td>
<td>Himedia Laboratories Pvt Ltd, India</td>
</tr>
<tr>
<td>Rosewell Park Memorial Institute medium (RPMI-1640)</td>
<td>Gene laboratories, Pvt Ltd</td>
</tr>
<tr>
<td>RNase A</td>
<td>Merck specialities Pvt Ltd</td>
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<tr>
<td>Steptomycin</td>
<td>Merck specialities Pvt Ltd</td>
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<tr>
<td>Benzylpenicillin</td>
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<td>Agar agar</td>
<td>Dabur India Ltd, India</td>
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<td>Agarose</td>
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<td>Cyclophosphamide (CP)</td>
<td>Nice chemicals Pvt Ltd</td>
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<td>Spectrum Pvt Ltd</td>
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<tr>
<td>DPPH</td>
<td>Sigma Aldrich, Pvt Ltd</td>
</tr>
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2, 2' zino-bis(3-ethylbenzthiazoline-6-sulphonicacid)  
Nitro blue tetrazolium(NBT)  
Riboflavin  
Sisco Research Laboratories, India  
Ethylene diaminetetra aceticacid (EDTA)  
Sodium dihydrogen phosphate dehydrate (Na₂HPO₄.2H₂O)  
Copper sulphate (CuSO₄.5H₂O)  
Sodium bicarboate (Na₂CO₃)  
Sodium hydroxide (NaOH)  
Potassium hydroxide (KOH)  
Di-potassium hydrogen phosphate (KH₂PO₄)  
Hydrogen peroxide (H₂O₂)  
Sodium Dodeciyl Sulphate (SDS)  
Ascorbic acid  
Ferric chloride  
Ferrous ammonium sulphate (FeSO₄)  
Sulphanilamide  
Sisco Research Laboratories, India  
Ortho-Phosphoric acid (H₃PO₄)  
Meta-Phosphoric acid (HPO₃)  
Trichloro acetic acid  
Naphthylethylene diamine dihydrochloride  
Deoxyribose  
Sisco Research Laboratories, India  
Thiobarbituric acid (TBA)  
Tris-HCl  
5,5'-dithio-bis 2-nitrobenzoic acid (DTNB)  
Sisco Research Laboratories, India  
Reduced glutathionine (GSH)  
Tris buffer  
Sodium nitopruusside  
Mercuric chloride (HgCl₂)  

Merck specialities Pvt Ltd  
Merck specialities Pvt Ltd
Camptothecin  
10-Hydroxy camptothecin  
Sucrose  
2,4-Dichloro phenoxy acetic acid (2,4-D)  
Naphthalene acetic acid (NAA)  
Indole 3 Acetic acid (IAA)  
Indole butric acid (IBA)  
6 Benzy adenine (BA)  
Kinetin (KN)  
Silica gel G  
Dimethyl sulfoxide (DMSO)  
Acetic acid  
Sulfuric acid  
Hydrochloric acid  
Pyridine  
Butanol  
Triton X-100  
Phenol  
Chloroform  
Isoamyl alcohol  
Tween 20  
Acetonitrile (HPLC)  
Water (HPLC)  
Methanol (HPLC)  

2.1.4. Instruments

Laminar Flow chamber  
Refrigerated centrifuge  
Freeze drier  
Microscope  
Inverted microscope  
UV chamber  
UV spectrophotometer  
Incubator  

Sigma Aldrich, Pvt Ltd  
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Merck Specialities Pvt Ltd  
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Kemi Pvt Ltd, India  
Remi Pvt Ltd, India  
Labconco, USA  
Meiji, Japan  
Willvert Will  
Commag  
Elico India Ltd.  
Rotex Instruments Pvt
2.1.5. Cell lines
Dalton’s Lymphoma Ascites (DLA) cells Amala Cancer Research Centre
Ehrlich Ascites Carcinoma (EAC) cells

2.1.5.1. Maintenance of Cell lines
The cells (DLA and EAC) were maintained in the intraperitoneal cavity of mice. At first 1x10^6 (100µl) cells were injected into the intraperitoneal cavity of mice. After days the cells were aspirated using a 1ml syringe and Phosphate Buffered Saline (PBS). The cells were washed in PBS and the number of cells was counted using a haemocytometer and makes up as 100µl PBS containing 1x10^6 cells. Then the cells were injected into the intraperitoneal cavity of other mice and continued in every 15 days intervals.

2.1.6. Animals
Male Swiss Albino and Balb/c mice (25-30) were purchased from the Small Animal Breeding Station, Agricultural University, Mannuthy, Kerala, India. The animals were maintained under standardized environmental conditions (22-280C, 60-70% relative humidity, 12hr dark/light cycle) and fed with standard rat feed (Lipton India) and water ad libitum. All animal experiments were conducted during the present study got prior permission from Institutional Animal Ethics Committee (IAEC approved) and followed the guidelines of IAEC.

2.2. Methods
2.2.1. Extraction
_H. furcatus_ plants were collected and the flowers, leaves and root bark were separated and each part was dried in hot air oven at 40oC. Powdered parts (100g each) were extracted with 70% methanol and water using soxhlet apparatus. Extracts were further evaporated using rotary evaporator under reduced pressure and temperature. While _O.
incarnata after collection was air dried and powdered. The plant powder was then extracted with 100% methanol and used for further studies.

2.2.2. Phytochemical analysis (Qualitative test for phytochemicals)

2.2.2.1. Test for carbohydrate
Qualitative determination of carbohydrates was performed by Benedict's test: to 0.5ml of the filtrate. 0.5ml Benedict’s reagent was added. The mixture was heated on boiling water bath for 2 minutes. A characteristic red coloured precipitate indicates the presence of sugar.

2.2.2.2. Test for terpenoids (Salkowski test)
Libermann-Buchard test: Crude extract was mixed with few drops of acetic anhydride, boiled and cooled, conc. H2SO4 was then added from the sides of the test tube. A brown ring at the junction of two layers was formed. The upper layer turned green which showed the presence of steroids and formation of deep red colour indicated the presence of triterpenoids.
Salkowski test: 5ml of the extract was mixed with 2ml of chloroform and concentrated sulphuric acid to form a layer. A reddish brown colouration of the interface showed the presence of terpenoids.

2.2.2.3. Test for alkaloids
Mayer’s test: The alcoholic extract was evaporated to dryness and the residue was heated on a boiling water bath with 2% hydrochloric acid. After cooling, the mixture was filtered and treated with a few drops of Mayer's reagent. The samples were then observed for the presence of turbidity or yellow precipitation.
Dragendorff’s test: Crude extract was mixed with Dragendorff’s reagent (potassium bismuth iodide solution). Reddish brown precipitate was formed which suggested the presence of alkaloids.

2.2.2.4. Test for phenolic compounds
Qualitative determination of phenolic compound was performed by ferric chloride test (Mace 1963). The extract was diluted to 5ml with distilled water. To this add a few drop of neutral 5% ferric chloride solution. The appearance of dark green colour indicates the presence of phenolic compounds.
2.2.2.5. Test for flavonoids

**Alkaline reagent test:** Crude extract was mixed with few drops of sodium hydroxide solution. An intense yellow colour was formed. Yellow colour turned to colorless on addition of few drops of diluted acid, marked the presence of flavinoids.

To 5ml of the dilute ammonia solution a portion of the aqueous extract was added, followed by addition of concentrated sulphuric acid. Appearance of yellow colouration indicates the presence of flavonoids.

Four milliliters of extract solution was treated with 1.5 ml of 50% methanol solution. The solution was warmed and metal magnesium was added. To this solution, 5-6 drops of concentrated hydrochloric acid was added and red color was observed for flavonoids and orange color for flavones.

2.2.2.6. Test for glycosides

Glycosides are compounds which upon hydrolysis give rise to one or more sugars (glycones) and a compound which is not a sugar (aglycone or genine). To the solution of the extract in glacial acetic acid, few drops of ferric chloride and concentrated sulphuric acid are added, and observed for a reddish brown coloration at the junction of two layers and the bluish green color in the upper layer.

2.2.2.7. Test for Saponins

Froth test, Crude extract was mixed in 1 ml water in a semi-micro tube, shaked well and noted the stable froth. Stable froth indicated the presence of saponins.

2.2.2.8. Test for tannins

For tannins about 0.5mg of dried powdered samples was boiled in 20 ml of water in test tubes then filtered. Few drops of 0.1% ferric chloride was added and observed for brownish green or blue black colouration.

2.2.3. *In vitro* Antioxidant assays

2.2.3.1. Free radical scavenging activity by DPPH method

The free radical scavenging activities were determined by 1, 1-diphenyl-2-picrylhydrazyl (DPPH) method with some modifications of the method proposed by Coruh et al (2007). DPPH solution (0.05 mg/ml) in methanol was prepared and a series of extract solutions with varying concentrations were prepared by dissolving
the dried extracts in methanol and 0.1 ml of solutions from each extract was added to 1.4 ml of DPPH solution. The absorbance at 517 nm was recorded after 5 min of incubation at room temperature. Radical scavenging capacity of each extract has been calculated as the percent DPPH radical scavenging affect which is:

\[
\% \text{ of inhibition} = \left(\frac{\text{Absorbance of control} - \text{Absorbance of extract}}{\text{Absorbance of control}}\right) \times 100
\]

2.2.3.2. 2, 2’-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) radical scavenging activity

The assay was carried out by interacting the extract with a model stable free radical derived from 2,2’-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS). The production of radical cation was accomplished as described by Long and Halliwell (2001) with some modifications. Briefly a stock solution of ABTS (7 mM) was prepared in water. To this solution ammonium persulphate (2.45 mM final conc) was added and the solutions were allowed to react, leading to an incomplete oxidation of ABTS to generate ABTS radical. The ABTS radical solution was diluted to an absorbance of 0.75 at 734 nm in phosphate buffer saline (PBS, pH 7.4) and 10µl of different concentrations of the extract were added to 1ml of ABTS radical solution. Absorbance was measured spectrophotometrically at 6 minutes after initial mixing, using PBS as reference. Percentage of inhibition was calculated using the equation:

\[
\% \text{ of inhibition} = \left(\frac{\text{Absorbance of control} - \text{Absorbance of extract}}{\text{Absorbance of control}}\right) \times 100
\]

2.2.3.3. Superoxide radical scavenging activity

The reaction mixture contained 3mg KCN dissolved in EDTA (6 µM), riboflavin (2 µM) NBT (50µM) and various concentrations (10 – 1000 µg/ml) of the extract and phosphate buffer in a final volume of 3ml. The tubes containing the reaction mixture were uniformly illuminated with an incandescent lamp for 15min and the absorbance was measured at 530 nm before and after the illumination (McCord and Fridovich, 1969). Percent inhibition of superoxide radical was calculated using the equation:

\[
\% \text{ of inhibition} = \left(\frac{\text{Absorbance of control} - \text{Absorbance of extract}}{\text{Absorbance of control}}\right) \times 100
\]

2.2.3.4. Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity of the extract was measured by studying the competition between deoxyribose and test compounds for the hydroxyl radicals
generated from Fe\textsuperscript{3+}/ascorbate/EDTA/H\textsubscript{2}O\textsubscript{2} system (Fenton reaction). The hydroxyl radicals attack deoxyribose, which eventually results in the formation of thiobarbituric acid reacting substances (Elizabeth and Rao, 1990).

The reaction mixture contained deoxyribose (2.8mM), ferric chloride (0.1mM) EDTA (0.1mM), H\textsubscript{2}O\textsubscript{2} (1mM), ascorbate (0.1mM), KH\textsubscript{2}PO\textsubscript{4}-KOH (20mM, pH 7.4) and various concentrations of the sample in a volume of 1ml was incubated for 1hr at 370°C. Deoxyribose degradation was measured as thiobarbituric acid reactive substrate by the method of Ohkawa et al (1979). The inhibition produced by different concentration was calculated compared to control. Percent inhibition of hydroxyl radical was calculated using the equation:

\[
\% \text{ inhibition} = \frac{(\text{Absorbance of control} - \text{Absorbance of extract})}{\text{Absorbance of control}} \times 100
\]

### 2.2.3.5. Nitric oxide radical scavenging activity

Nitric oxide, generated from sodium nitroprusside in aqueous solution at physiological pH, interacts with oxygen to produce nitrite ions, which were by Griess reaction (Green et al., 1982). The reaction mixture (3 ml) containing sodium nitroprusside (10 mM) in phosphate buffered saline (PBS) and the EBM extract (from 1µg to 1000 µg/ml) was incubated at 25°C for 150 minutes. After incubation, 0.5 ml of Griess reagent (1% sulphanilamide, 2% H\textsubscript{3}PO\textsubscript{4} and 0.1% naphthylethylene diamine dihydrochloride) was added. The absorbency of the chromophore formed was reviewuated at 546 nm.

\[
\% \text{ inhibition} = \frac{(\text{Absorbance of control} - \text{Absorbance of extract})}{\text{Absorbance of control}} \times 100
\]

### 2.2.3.6. Lipid peroxidation assay

The level of lipid peroxidation was measured by the method of Ohkawa et al (1979). 10 - 1000µg/ml of extract was incubated with 0.1ml rat liver homogenate (25%) containing 30 mM KCl, Tris-HCl buffer (0.04 M, pH 7.0), ascorbic acid (0.06 mM) and ferrous ion (0.16 mM) in a total volume 0.5ml for 1hr. After incubation, 0.4ml of reaction mixture was treated with 0.2ml SDS (8.1%), 1.5 ml TBA (0.8%) and 1.5 ml acetic acid (20 %, pH 3.5) distilled water were kept for 1hr in a boiling water bath at 1000°C. After 1 h, the reaction mixture was removed from the water bath, cooled and added 5ml of pyridine : butanol (15:1 ratio), mixed thoroughly and centrifuged at 3000 rpm for 10min. Absorbance of the clear supernatant was measured at 532 nm.
against pyridine : butanol. Percent inhibition of lipid peroxidation was calculated using the equation:

\[
\% \text{ inhibition} = \frac{(\text{Absorbance of control} - \text{Absorbance of extract}) \times 100}{\text{Absorbance of control}}
\]

2.2.4. Protective effect of *H. furcatus* extracts against H2O2 induced haemolysis and lipid peroxidation in RBC

2.2.4.1. Determination of haemolysis inhibition in RBC

The inhibition of human erythrocyte haemolysis by *H. furcatus* extracts was evaluated according to the procedure described by Tedesco *et al.*, 2000, with slight modifications. Human erythrocyte haemolysis was performed by with H2O2 as free radical initiator. To 100μl of 5% (v/v) suspension of erythrocyte in PBS added 50 μl of extract with different concentrations (50 to 250 μg in PBS, pH 7.4). To this, 100μl of 100μm H2O2 (in PBS, pH 7.4) was added. The reaction mixture shaken gently while being incubated at 37°C for 3 h. The reaction mixture was diluted with 3 ml of PBS and centrifuged at 2000 rpm for 10 min. The absorbance of the resulting supernatant was measured at 540nm by spectrophotometer to determine the haemolysis. Likewise, the erythrocyte was treated with 100 μm H2O2 and without inhibitors (plant extract) to obtain a complete haemolysis. The absorbance of the supernatant was measured at the same condition. To evaluate the haemolysis induced by extract, erythrocytes were pre-incubated with 50μl of extract. Percentage of haemolysis was calculated by taking haemolysis caused by 100 μm H2O2 as 100%. The IC50 values were calculated from the plots as the antioxidant concentration required for the inhibition of 50% haemolysis.

\[
\% \text{ inhibition} = \frac{(\text{Absorbance of control} - \text{Absorbance of extract}) \times 100}{\text{Absorbance of control}}
\]

2.2.4.2. Inhibition of lipid peroxidation of RBC

Lipid peroxidation was measured by the method of Stocks and Dormandy. Erythrocytes were mixed with 20% TCA (1:1). After 1 h incubation at temperature 4°C, samples were centrifuged (1500g for 20 min at 20°C). TBA was added to supernatant and samples were heated at 100°C for 15 min. TBARS (TBA reactive species) were measured spectrophotometrically at 532 nm. Results were presented as percent of lipid peroxidation in control.
\[
\text{% inhibition} = \frac{(\text{Absorbance of control} - \text{Absorbance of extract}) \times 100}{\text{Absorbance of control}}
\]

2.2.5. *In vitro* cytotoxicity

The cytotoxicity was determined by Trypan blue exclusion method (Babu et al, 1995). The exclusion assay is used to determine the number of viable cells present in a cell suspension. It is based on the principle that live cells possess intact cell membranes that exclude the dye and the dead cells do not. In the assay presented here, under light microscope a viable cell will have a clear cytoplasm whereas a nonviable cell will have a blue cytoplasm. For the assay DLA and EAC cells aspirated from mice intraperitoneal cavity and were counted to a density of 1 x 10^6 in 0.1 ml phosphate buffer saline (PBS, pH 7.4). To 0.8 ml of PBS add 0.1 ml of cell suspension containing 1 x 10^6 cells and different concentration of extract (0.01µg – 1mg/ml). These were incubated for 3 hours at 37°C. After incubation, 0.1 ml trypan blue dye was added and incubated for 2 min at room temperature. Apply a drop of trypan blue - cell mixture to a hemocytometer and count the stained (non viable) and unstained (viable) cells separately under a microscopic field.

2.2.6. Apoptosis assay

2.2.6.1 Induction of apoptosis in DLA and EAC cell lines

For apoptotic assay, the cells were plated at a density of 5 x 10^6 in 3 ml culture vials containing 1.5 ml DMEM supplemented with 10% FBS, and antibiotics (100 U/ml benzylpencillin and 100 µg/ml streptomycin). The extracts were dissolved in minimum of DMSO and the volume is made up to desired concentrations. Different concentrations of extract were added and incubated for 24 hrs at 37°C, the cells were harvested and morphology and nuclear condensation, DNA fragmentation were evaluated.

2.2.6.2. Analysis of morphology and nuclear condensation

Characteristic apoptotic morphological changes were assessed by Geimsa and May Grunwald staining detected microscopy. Briefly, the cells were harvested after 24hr incubation, and washed with PBS. Centrifuged at 1000 rpm and resuspended in PBS. The cells were mounted on a glass slide and were then fixed with 80% methanol. The slides were stained with Geimsa for 30 minutes and washed in PBS and air dried.
The slides were then stained in May Grunwald for 15 minutes and washed in distilled water. The slides were observed under microscope for morphological staining.

**2.2.6.3. Measurement of apoptosis by DNA fragmentation**

**2.2.6.3.1. Extraction and Purification of DNA**

After treatment, cells were harvested and washed with PBS by centrifuged at 1500 RPM for 5 min at 4oC. Cell pellets were incubated on ice for 15 min with 600µl lysis buffer (0.2% Triton X-100; 10 mM Tris- HCl, pH 7.4, 10 mM EDTA). Centrifuge at 12,000g at 4°C for 20 min. Transfer the supernatant, which contains the DNA, into a new tube, add RNase A to a final concentration of 100 µg/mL, and mix completely by flicking the tube and incubate at 37°C for 1 h. Extract DNA by adding an equal volume (600 µL) of phenol: chloroform:isoamyl alcohol (25:24:1) to the sample and vortexing for a few seconds to properly mix the solutions and centrifuge at 12,000 RPM for 3 min at room temperature and aqueous phase was collected (repeat this step for 4 times). Add 600 µL of chloroform:isoamyl alcohol (24:1) to the sample and briefly mix by vortexing. Centrifuge at 12,000 RPM for 3 min at room temperature. Remove the top aqueous layer and place in a new tube. Precipitate the DNA by adding 25–30 µL of 5 M NaCl to a final concentration of 300 mM and add 2–2.5 volume of ice-cold 100% ethanol. Leave overnight at –20°C. Centrifuge the sample at 12,000 RPM for 30 min at room temperature. Carefully aspirate the ethanol off and wash the DNA pellet with 1 mL of 70% ethanol. Dislodge the pellet by inverting several times so that the ethanol can remove any excess salts. Centrifuge sample at 12,000 for 20 RPM min at room temperature. Dry the pellet for a couple of minutes to drain off ethanol. The DNA concentration was determined from the absorbance at 260 nm.

**2.2.6.3.2. Agarose Gel Electrophoresis and analysis of fragmented DNA**

Resuspend the dried DNA pellet by adding 18 µL of TE buffer (10 mM Tris-HCl, pH 8.0, 10 mM EDTA) and flicking the tube a few times. Add 2µL of 10 x loading buffer. Prepare 2% agarose to that added Ethidium at 0.5 µg/mL mix the ethidium bromide and pour the agarose into the gel-casting tray. After the gel has hardened (20–30 min), place gel into an electrophoresis tank containing sufficient 1 x TBE
(0.89 M Tris base, 0.89 M boric acid, 25 mM EDTA) electrophoresis buffer to cover the gel approx 1 mm. Aliquot the individual DNA samples into each well. Attach the leads so that the DNA migrates to the anode or positive lead, and Electrophoresis was carried out for 1 h at 100 V. Turn off the power supply when the bromphenol blue dye has migrated two thirds of the way down the gel. Photograph a stained gel directly on an UV transilluminator.

2.2.7. Determination of Total count and Differential Count.
Total WBC count was determined using haemocytometer as described in the textbook of practical Physiology, Chaudhari (2000 b).

**Principle:** The whole blood was diluted using a diluent which haemolyses red cells. Leaving all the nucleated cells intact. The number of white cells in a known volume and known dilution were counted using a counting chamber.

**Procedure:** Added 0.02 ml blood to 0.38 ml of diluting fluid charged the neubauer counting chamber with the well-mixed diluted blood. Counted the total number of white blood cells in the four large corner squares of chamber after 3-4 min. Total number of WBC = Number of cells counted x 50 count/mm3

2.2.8. Determination of Bone marrow cellularity
Bone marrow cellularity was determined by the method of Sreedni et al (1992). The animals were sacrificed; bone marrow was collected from the femur using a jet of PBS containing goat serum using a syringe. Bone marrow was made into single cell suspension and the cell number was determined using haemocytometer and expressed as total number of live cells/femur.

2.2.9. Determination of haemoglobin (Hb) in blood
Haemoglobin was determined according to the method of Drabkin and Austin (1932).

**Principle:** Haemoglobin was treated with a reagent containing potassium ferricyanide, potassium cyanide and potassium dihydrogenphosphate. The ferricyanide forms methaemoglobin, which is converted to cyanmethaemoglobin by the cyanide. The intensity of the color formed is measured at 546 nm against reagent blank. The optical density is directly proportional to the amount of haemoglobin present in the blood.
**Procedure:** The reagents used were from Agappe diagnostic kit. 0.02 ml of fresh whole blood was mixed with 5 ml of the cyanmeth reagent. The optical density was measured at 546 nm against reagent blank after 5 min incubation at room temperature. The O.D of standard solution corresponding to 60 mg/dl haemoglobin at 546 nm was read against reagent blank used for calculating the concentration of haemoglobin in the blood.

\[
\text{Haemoglobin (g/dl)} = \frac{\text{O.D of treated}}{\text{O.D of standard}} \times 60 \times 0.251
\]

**2.2.10. Determination of serum glutamate oxaloacetate transaminase (SGOT) activity**

Serum glutamate oxaloacetate transaminase [also called Aspartate Transaminase (AST)] activity was determined according to the method of Reitman and Frankle (1957).

**Principle:** Serum containing glutamate oxaloacetate transaminase catalyses the reaction between L-aspartate and α-ketoglutarate, to form oxaloacetate and glutamate. The unstable oxaloacetate is converted to pyruvate and reacts with 2,4-dinitrophenylhydrazine. The absorbance of the resultant brown colored phenylhydrazone is measured at 505 nm under alkaline conditions.

**Procedure:** Reagents used were from Span diagnostic kit. 0.1 ml of serum was added to 0.5 ml of the buffered substrate (2 mM of α-ketoglutarate and 100 mM L-aspartate in 100 ml phosphate buffer 0.1M, pH 7.4) at 37°C and incubated for 60 min. After the incubation, 0.5 ml of dinitrophenylhydrazine (19.8 mg/dl 1 N HCl) was added, mix well and kept at room temperature for 20 min. 0.4 ml of NaOH was added and read the absorbance after 10 min at 505 nm using the reagent blank. A control tube containing buffered substrate was treated with serum after the incubation at 37°C was also followed in the same manner. The enzyme activity was calculated from the standard (sodium pyruvate, 2 mM) calibration curve and expressed in IU/L.

**2.2.11. Determination of serum glutamate pyruvate transaminase (SGPT) activity**

Serum glutamate pyruvate transaminase [also called as Alanine Transaminase (ALT)] activity was determined according to the method of Reitman and Frankle (1957).

**Principle:** Serum containing glutamate pyruvate transaminase catalyses the reaction
between L-alanine and α-ketoglutarate, to form pyruvate and glutamate. The pyruvate thus formed was treated with 2, 4-dinitrophenylhydrazine. The absorbance of the resultant brown colored phenylhydrazone is measured at 505nm under alkaline condition.

**Procedure:** Reagents used were from Span diagnostic kit. 0.1 ml of serum was added to 0.5 ml of the buffered substrate (2 mM of α-ketoglutarate and 100 mM L-alanine in 100 ml phosphate buffer 0.1M, pH 7.4) at 37°C and incubated for 30 min. After the incubation, 0.5 ml of dinitrophenylhydrazine (19.8 mg/ml 1 N HCl) was added, mixed well and kept at room temperature for 20 min. 0.4 ml of NaOH was added and read the absorbance after 10 min at 505 nm using the reagent blank. A control tube containing buffered substrate was treated with serum after the incubation at 37°C was also followed in the same manner. The enzyme activity was calculated from the standard (Sodium pyruvate, 2 mM) calibration curve and expressed in IU/L of serum.

2.2.12. Determination of serum alkaline phosphatase (ALP) activity

Serum ALP activity was determined according to the method of King and Armstrong (1980).

**Principle:** ALP in the serum reacts with disodium phenyl phosphate under alkaline pH 10 release phenol. Phenol reacts with 4-aminoantipyrene in the presence of alkaline oxidizing agent to give a red colored complex, which is measured at 510 nm against reagent blank.

**Procedure:** Reagents used were from Span diagnostic kit. 0.05 ml of serum was incubated with 0.5 ml of the buffered substrate (1ml of 0.254 g of disodium phenyl phosphate dihydrate/dl water mixed with 1ml of the carbonate buffer pH 10) and 1.54 ml of distilled water at 37°C for 15 min. After the incubation, 2 ml chromogen (1ml of 0.6 g 4-aminoantipyrene/dl water and 1ml of potassium ferricyanide 2.4 g/dl water) reagent was added and measured at 510 nm. Phenol (10 mg%) was used as the standard for the calibration curve and serum alkaline phosphatase activity is expressed in KA units

\[
\text{Serum ALP (KA units)} = \frac{\text{O.D of sample} - \text{O.D of control} \times 10}{\text{O.D of standard} - \text{O.D of blank}}
\]

2.2.13. Determination of serum creatinine

Serum creatinine was determined according to method of Brod and Serota as described in Text book of Clinical Biochemistry, Varley (1980).
**Principle:** Creatinine forms a yellow-orange compound in alkaline medium with picric acid. The intensity of the color is measured at 500 nm. The concentration of the dye stuff formed over a certain reaction time is a measure of the creatinine concentration.

**Procedure:** Reagents used were from Merk diagnostic kit. 0.2 ml of serum was mixed with 0.5 ml of buffer (313 mM NaOH and 12.5 mM phosphate, pH 8) and 0.5 ml of 8.73 mM picric acid. The absorbance is measured immediately after 1 min (O.Dt1) and exactly after 5 min (O.Dt2) at 500 nm. A standard creatinine solution (1 mg/dl) was treated in the same way.

\[
\text{Creatinine concentration (mg/100 ml) = } \frac{\text{O.D of sample} - \text{O.D of Blank} \times 3.0}{\text{O.D of standard} - \text{O.D of Blank}}
\]

2.2.14. Estimation of Urea

Serum Urea was determined by using commercial kit, span diagnostics Ltd.

**Principle:** The urea is hydrolyzed in the presence of water and urease to produce ammonia and carbon dioxide. Under alkaline conditions, ammonia so formed, reacts with hypocholite and phenolic chromogen to form coloured indophenol, which is measured at 578nm. Sodium Nitroprusside acts as a catalyst. The intensity of colour is proportional to the concentration of urea in the sample.

**Procedure:** 10µl of serum is added to 1.5 ml of reagent solution containing Phosphate buffer (20mM), urease (200000 U/L), sodium nitroprusside 3.2 (mM), sodium salicylate (60 mM/L). Mix well and incubate at 37oC for 3 minutes. Then add 1.5 ml of solution 2 containing 0.2% sodium hypochlorite and sodium chloride (m 400 m/L). Mix well and incubate at 37oC for 5 minutes. The intensity of chromogen developed was measured at 578 nm. Calculate the results as per the formula

\[
\text{Urea Concentration (mg/dL) = } \frac{\text{Absorbance of Test}}{\text{Absorbance of standard}} \times 50
\]

2.2.15. Determination of serum total protein

Serum protein was determined by the method of Reinhold, as described in Text book of Clinical Biochemistry, Varley (1980).

**Principle:** Protein reacted with cupric ions in alkaline medium to form a violet colored complex. The intensity of the complex was measured at 530 nm.

**Procedure:** The reagents used were from Span Diagnostic kit. 1 ml of the working Biuret reagent was mixed with 0.01 ml of serum and absorbance was measured at 530
nm against reagent blank. 0.01 ml of the standard solution was treated in the same way.

\[
\text{Serum total protein (g/dl) = } \frac{\text{Absorbance of sample} \times 6.5}{\text{Absorbance of standard}}
\]

2.2.16. Determination of superoxide dismutase (SOD) activity

Superoxide dismutase activity was determined according to the method of Mc Cord and Fridovich (1969).

**Principle:** Illumination of riboflavin solution in the presence of EDTA causes a reduction of the flavin. It then re-oxidizes and simultaneously reduces oxygen to \(O_2^-\), which is allowed to react with a detector molecule NBT, reduced the NBT to a formazan blue. The SOD in the sample will inhibit the formazan production.

**Procedure:** In the case of RBC, add 0.5 ml erythrocyte (RBC) haemolysate (1: 4 dilution) to 0.25 ml chloroform and 0.5 ml ethanol, mix well and centrifuge the mixture for 1 hour at 4 °C at 5000 rpm. Take 0.1 ml supernatant from RBC preparation or 0.1 ml of the tissue homogenate and mixed with 0.2 ml of 0.1 M EDTA (containing 0.0015% NaCN), 0.1 ml of 1.5 mM NBT and phosphate buffer (67 mM, pH 7.8) in a total volume of 2.6 ml. After adding 0.05 ml of riboflavin, the absorbance of the solution was measured against distilled water at 560 nm. Illuminated all the tubes uniformly for 15 min and absorbance of the blue color formed were measured again. Percent of inhibition was calculated after comparing absorbance of sample with the absorbance of control (the tube containing no enzyme activity). The volume of the sample required to scavenge 50 % of the generated superoxide anion was considered as 1 unit of enzyme activity and expressed in U/ mg protein for tissue and in U/ g Hb in the case of RBC.

2.2.17. Determination of catalase (CAT) activity

Tissue Catalase activity was determined according to the method of Beers and Sizer (1952).

**Principle:** Catalase catalyses the decomposition of \(H_2O_2\). In the ultraviolet range \(H_2O_2\) shows a continual increase in absorption with decreasing wavelength. The decomposition of \(H_2O_2\) can be followed directly by the decrease in extinction at 240 nm.
Procedure: 0.1 ml of the tissue homogenate (approximately 0.1 mg protein) was mixed with 1.9 ml of the phosphate buffer (0.5 M, pH 7). The decrease in extinction was measured at 240 nm, 1 min interval for 3 min immediately after adding 1 ml of 11 mM H₂O₂ solution in buffer. A sample control was placed in the reference cuvette containing 0.1 ml of tissue homogenate and 2.9 ml of the buffer. Activity of catalase was calculated using the molar extinction coefficient of 43.6 cm⁻¹ mmoles of H₂O₂ decomposed/min/mg protein or (U/mg protein) for tissue and expressed in κ/g Hb in the case of RBC.

\[
\text{Tissue catalase activity} = \left( \frac{\Delta A/\text{min} \times 1000 \times 3}{\text{mg protein}} \right) / 43.6
\]

\[
\text{RBC catalase activity} = \frac{2.303 \times \log \frac{\text{OD}_1 \times 1000}{15} \times \text{OD}_2}{\text{g Hb}}
\]

2.2.18. Determination of glutathione peroxidase (GPx) activity
Glutathione peroxidase activity was determined according to the method of Hafemann et al., (1974).

Principle: The activity of GPx was determined by measuring the decrease in GSH content after incubating the sample in the presence of H₂O₂ and NaN₃.

Procedure: 0.5 ml erythrocyte (RBC) haemolysate (1: 4 dilution) or tissue homogenate (approximately 0.5 mg protein) was incubated with 0.1 ml of 5mM GSH, 0.1 ml of 1.25 mM H₂O₂, 0.1ml of 25 mM NaN₃ and phosphate buffer (0.05 mM, pH 7) in a total volume of 2.5 ml at 37°C for 10 min. The reaction was stopped by adding 2 ml of 1.65 % HPO₃ and the reaction mixture was centrifuged at 1500 rpm for 10 min. 2 ml of the supernatant was mixed with 2 ml 0.4 M Na₂HPO₄ and 1ml of 1mM DTNB. The absorbance of the yellow colored complex was measured at 412 nm after incubation for 10 min at 37oC against distilled water. A sample without the tissue homogenate processed in the same way was kept as the nonenzymatic reaction. One unit of enzyme activity was defined as decrease in log GSH by 0.001/min after subtraction of the decrease in GSH per minute for the nonenzymatic reaction and is expressed as units/mg protein for tissue and units/g Hb for RBC.

2.2.19. Determination of reduced glutathione (GSH)
Reduced glutathione in the tissue was determined according to the method of Moron et al., (1979).
**Principle:** The acid soluble sulphhydryl groups (non-protein thiols of which more than 93% is reduced glutathione) form a yellow colored complex with dithionitrobenzene (DTNB). The absorbance of the colored complex was measured at 412 nm.

**Procedure:** 0.5 ml erythrocyte (RBC) haemolysate (1: 4 dilution) or 0.5 ml of the tissue homogenate was mixed with 0.1 ml of 25 % TCA and kept on ice for few minutes. These were then subjected to centrifugation at 3000 g for few minutes to settle the precipitate. 0.3 ml of the supernatant was mixed with 0.7 ml of 0.2 M sodium phosphate buffer (pH 8) and 2 ml of 0.6 mM DTNB (prepared in 0.2 M buffer, pH 8). The yellow color obtained was measured after 10 min at 412 nm against a blank which contained 0.1 ml of 5% TCA in place of the supernatant. A standard graph was prepared using different concentrations (10-50 nmoles) of GSH in 0.3 ml of 5 % TCA. The GSH content was calculated with the help of this standard graph and expressed as nmol/mg protein for tissue and units/g Hb for RBC.

**2.2.20. Determination of lipid peroxidation**

The level of lipid peroxidation was measured as malondialdehyde (MDA) according to the method of Ohkawa et al (1979).

**Principle:** The tissue malondialdehyde was allowed to react with TBA. The MDATBA adduct formed during the reaction in acidic medium was extracted to the organic layer and the absorbance was measured at 532 nm.

**Procedure:** 4 ml of reaction mixture containing 0.4 ml of the tissue homogenate, 1.5 ml of 0.8 % TBA, 1.5 ml of acetic acid (20 %, pH 3.5) and distilled water was kept for 1 h in a boiling water bath at 95°C. After 1 h, the reaction mixture was removed from the water bath, cooled and added 1 ml of distilled water. 5 ml of butanol:pyridine mixture (15:1) was added to the reaction tube, mix thoroughly and centrifuged at 3000 rpm for 10 min. Absorbance of the clear supernatant was measured at 532 nm against butanol:pyridine mixture. The MDA was calculated with the help of a standard graph made by using different concentrations (1-10 nmol) of 1'1'3'3'-tetramethoxypropane in 1 ml distilled water and is expressed as nmol of MDA/mg protein.

**2.2.21. Determination of tissue protein**

Protein content in the tissue was determined according to the method of Lowry et al. (1951).
**Principle:** The blue color developed by the reduction of the phosphomolybdichospho-
tungstic components in the Folin-Ciocalteau reagent by the amino acids tyrosine and
tryptophan in the protein plus the color developed by the biuret reaction of the protein
with the alkaline cupric tartrate were measured at 660 nm.

**Procedure:** 0.01 ml of the homogenate was mixed with 0.990 ml of distilled water, 5
ml of alkaline CuSO₄ (0.5 % CuSO₄ in 1 % sodium potassium tartrate and 2% 
Na₂CO₃ in 0.1 N NaOH mixed in the ratio 1:50) was kept for 10 min at room
temperature. 0.5 ml of 1 N Folin phenol reagent was added and absorbance was
measured after 20 min at 660 nm against the reagent blank. Protein content was
calculated from the standard graph prepared using different concentrations (0.1-0.5
mg/ ml) of bovine serum albumin (BSA).

**2.2.22. Histopathology**

After the termination of the experiments animals were sacrificed, liver was excised,
washed in phosphate buffered saline and a small portion was fixed in 40% formalin
solution and were given to Amala Hospital, Pathology Department for slide
preparation and diagnosis.

**2.2.23. Explant collection and preparation for tissue cultures**

Fresh tender leaves of *Ophiorrhiza incarnata* were collected at early morning and
were washed in tap water to remove dust and soil. Then were washed again using
commercial detergent Tween 20 and rinsed with distilled water to remove the
detergent. The leaves were then taken to laminar air flow, where it is again surface
sterilized by dipping in HgCl₂ for 3 minutes and washed in sterile distilled water for 5
times. The washed and surface sterilized leaves were cut in to pieces of 0.5 mm length
along with midrib. These leaf pieces were used as explants for culture.

**2.2.24. Culture media preparation**

Murashique and Skoogs (MS) basal medium supplemented with or without
phytohormones were used as culture media. 30% sucrose was used as carbon source.
7.5% of agar was used as solidifying agent for solid cultures. The pH of the medium
was adjusted to 5.7. Prepared medium after the addition of ingredients were
autoclaved at 121°C and 15 lbs pressure prior to culture. phytohormones such as
auxins (NAA, IAA and IBA) and cytokinins (BA and kinetin) at concentration ranging from 0.5 to 5 mg/litre in combinations or alone were used.

2.2.25. Estimation of camptothecin(CPT) and 10-Hydroxy camptothecin (HCPT) in Ophiorrhiza incarnata by HPLC

2.2.25.1. HPLC system and condition

The HPLC system is a Shimadzu SPD-10AVP HPLC system equipped with a multi solvent delivery system and UV-VIS detector. The column was a Purospher star column rp18, end capped, 5 µm, 250 x 4.60 mm (Merck, Germany). The mobile phase was composed of acetonitrile and water (60:40) with isocratic elution. The flow rate was 1ml / minute, with UV absorbance detection at 256 nm and sample injection volume was 20µl. The column temperature was kept at 25°C. For HPCT, the mobile phase was composed of acetonitrile and water (30:70) with isocratic elution. The flow rate was 1ml / minute, with UV absorbance detection at 266 nm and sample injection volume was 20µl.

2.2.25.2. Sample preparation for HPLC analysis

2.2.25.2.1. Plant sample preparation

The air dried wild plants, lyophilized in vitro cultured plantlets and calluses were powdered, and were subjected to extraction with methanol. The filtrate was concentrated to dryness in a rotary vacuum evaporator at 37°C. The concentrate was dissolved with acetonitrile: water (24:1 ratio) for CPT and in methanol for HCPT and centrifuged for 5 min at 2000 rpm. The supernatant was collected and was filtered with 0.45µm nylon syringe filter before HPLC analysis. 20µl from this is used for analysis

2.2.25.2.2. Standard preparation

A stock solution is prepared by dissolving 1 mg of standard camptothecin (Sigma) in 25 ml acetonitrile: water (24:1 ratio) and 1 mg 10-Hydroxy camptothecin in 25 ml methanol. 20µl from this stock is used for analysis.

2.2.26. Statistical analysis

The data were analyzed by one-way ANOVA followed by Dunnet multiple comparison test. p<0.05 taken as significant. The statistical calculation was done using InStat GraphPad 3.00 software for Windows, San Diego, California, USA.