Chapter 3

Materials and methods

3.1 COLLECTION OF PLANT SPECIMENS

Medicinal plants for the present study were selected after thorough literature survey. The plant specimens were collected within India, in their respective natural habitats. All the selected plants are common herbal crops available throughout the year; therefore needing no authorization from the Government of India for their collection in compliance with the United Nations Convention on Biodiversity. The details of collection sites for all plant specimens were presented in Table 3.1.

Table 3.1: Data showing the collection sites for the selected plant specimens.

<table>
<thead>
<tr>
<th>No.</th>
<th>Plant</th>
<th>Plant parts used</th>
<th>Site of collection</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td><em>Oroxylum indicum</em> Vent</td>
<td>Stem Bark</td>
<td>Mundoor forest range, Palakkad district, Kerala, India (10°47' N, 76°47' E).</td>
</tr>
</tbody>
</table>

3.2 PROCESSING AND EXTRACTION

Collected plant specimens were shade dried, powdered, sieved and preceded for extraction procedures.

3.2.1 METHODS OF EXTRACTION

Series of solvents according to their increasing polarity from petroleum ether, chloroform, ethyl acetate, methanol and water were used for sequential solvent extraction.
3.2.1.1 Soxhlet extraction

Each plant powder (50 g) was loaded in soxhlet apparatus with 300 ml of solvent and was serially extracted with the aforesaid solvents (with 1:6 /powder: solvent ratio) which has yielded 10 extracts; (5 extracts from each plant namely PHO, CHO, EHO, MHO and AHO from *Oroxylum indicum*, and PHR, CHR, EHR, MHR and AHR from *Rheum emodi*).

![A) Soxhlet extraction](image1)

![B) Soxhlet extraction](image2)

**Fig. 3.1:** Solvent extraction via soxhlet (A) and maceration (B).

3.2.1.2 Maceration

Amount of 50 g of each plant powder was added with 300 ml of solvent (with 1:6 /powder: solvent ratio) in a conical flask and shaken periodically. After incubation for a week at room temperature, the mixture was filtered with a Whatman no. 1 paper, and the raffinate was dried to sequentially extract with the aforesaid solvents. Maceration has yielded totally 10 extracts; (5 extracts from each plant namely PCO, CCO, ECO, MCO and ACO from *Oroxylum indicum*, and PCR, CCR, ECR, MCR and ACR from *Rheum emodi*).

3.2.2 CONCENTRATION OF EXTRACTS

All 20 extracts were then concentrated at 40 ºC under reduced pressure (petroleum ether: 180 mbar, chloroform: 474 mbar, ethyl acetate: 240 mbar, methanolic extract: 337 mbar, aqueous extract: 72 mbar) using a Rotavapor
R-215 (BÜCHI Labortechnik AG, Switzerland). Dried extracts were stored in a vacuum desiccator until further use (Pickett and Stephenson, 1980).

**Fig. 3.2:** Concentration of extract using Rotavapor R-215 (BÜCHI Labortechnik AG, Switzerland).

### 3.3 CELL CULTURE

#### 3.3.1 CELL LINES AND MAINTENANCE

The study has employed the use of MDA-MB-231 (human breast adenocarcinoma), MCF-7 (human breast ductal carcinoma) and WRL-68 (non-tumoral human liver embryonic) cell lines. MDA-MB-231 cells were maintained in L-15 (Leibovitz’s) culture medium whereas; MCF-7 and WRL-68 were maintained in Minimum essential medium (MEM) (Eagle) containing Non-essential amino acids. All the cultures were added with 10% fetal bovine serum (FBS), 100 μg/ml streptomycin, 250 μg/ml amphotericin B and 100 IU/ml penicillin. The cultures were maintained in a 25 cm² polystyrene-coated cell culture flasks (Freshney, 1992) with 1 x 10⁶ cells seeding density, and incubated in a humidified atmosphere at 37 °C in a Forma Series II water jacketed CO₂ incubator (Thermo Electron Corporation, MA, USA) with 5% CO₂ (except for MDA-MB-231).
3.3.2 SUBCULTURE

Maintenance of cell lines by regular passaging is indispensible in order to maintain a stable culture. Cells were harvested in the logarithmic phase of growth by adding 2 ml of trypsin-phosphate-versene-glucose (TPVG) solution (0.1% trypsin, 0.02% EDTA and 0.05% glucose in phosphate buffered saline) to the monolayer after thorough washing with PBS and with simultaneous incubation at 37 ºC for 2 min. Fresh growth medium containing serum was then added to the flask and gently aspirated to detach the loosely adhered cells and to dissociate the cell clusters. Suspension containing detached cells was centrifuged at 1500 rpm for 10 min, and the supernatant was discarded. To the pellet, 1 ml of fresh growth medium was added and the cells were counted using a haemocytometer by aspirating 0.2 ml of cell suspension with 0.4 ml of Trypan Blue (0.4% in PBS). From the counted cell suspension, 1 x 10^6 cells/25 cm² were used to seed a fresh flask.

3.3.3 STORAGE

Cells from confluent monolayer were harvested as mentioned in 3.3.2, from which 1.5 x 10^6 cells were dispensed in 1 ml of growth medium containing 10% DMSO. The suspension was then transferred to a 2 ml cryovial and labeled with the details of the culture inclusive of its passage number, name and date. The vial was reserved in a Nalgene freezing container for storage at low temperatures. Firstly, vial was maintained at −80 ºC overnight in a MDF-U32V V.I.P.™ Series –86 ºC Ultra-Low Temperature Freezer (Sanyo Biomedical, IL, USA), and then transferred to liquid nitrogen (−196 ºC) storage container (MVE XC47, Chart, Inc., GA, USA) for long term storage.

3.3.4 REVIVAL

The cells were recovered from the cell repository and immediately transferred to warm water maintained at 37 ºC. Once the cells were completely thawed, they were transferred into culture flasks (25 cm²) and gently added with 5 ml of growth medium (10% FBS) drop-by-drop. The flasks were then incubated at 37 ºC with/without 5% CO₂, and observed periodically for 3-4 h to check cell adherence. Post-adherence, the existing medium was decanted and replenished with fresh growth medium.
3.4 CYTOTOXICITY ASSAY – IN VITRO

The cytotoxicity of the crude extracts obtained were analyzed employing 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide (XTT) - formazan dye formation method (Weislow et al., 1989). MDA-MB-231 and WRL-68 cells (1 x 10^4 cells/well and 6 x 10^3 cells/well respectively) in 200 µl of their respective culture medium were seeded in a 96-well plate and incubated at 37 ºC for 24 h with/without 5% CO₂ supply. After 24 h, the control wells were replenished with fresh medium and the test wells were added with medium containing 25, 50, 100 and 200 µg/ml of the extracts. The plate was re-incubated for 24 h maintaining the same conditions. After incubation period, each well was replenished with 200 µl of fresh medium and 50 µl of XTT (0.6 mg/ml containing 25 µM PMS). The plate was incubated for further 4 h in the same conditions after which the absorbance was measured at 450 nm (with a 630 nm reference filter) in a Dynex Opsys MRTM Microplate Reader (Dynex Technologies, VA, USA). Percentage cytotoxicity was calculated by the following formula:

\[
\text{% Cytotoxicity} = \left( \frac{\text{Ac} - \text{At}}{\text{Ac}} \right) \times 100
\]

Ac is the mean absorbance of the control wells and At is the mean absorbance of test wells.

3.5. ASSAY FOR DETECTION OF APOPTOSIS

Apoptosis was determined by a photometric enzyme-linked immunosorbent assay (ELISA) kit (# 11-585-045-001; Roche Applied Science, Mannheim, Germany), in which, Bromodeoxyuridine (BrdU) labelled apoptotic DNA fragments in cytosolic extracts of culture supernatants was detected as per the supplier’s instructions. Cells (MDA-MB-231 and MCF-7) were labeled with 10 µM BrdU at 1 x 10^5 cells/ml density for 2 h. 1 x 10^4 BrdU-labeled cells in 100 µl medium were treated with varying concentrations (12.5, 25, 50, 100 and 200 µg/ml) of the extracts for a period of 4 h. The cells were then lysed and the supernatant containing apoptotic fragments were obtained after centrifugation at 1500 rpm for 10 min. The samples (100 µl) were then transferred to the anti-DNA coated wells in a 96-well flat-bottom microtiter plate and incubated for 90 min at 15-25 ºC. The wells were washed with washing buffer.
post-incubation and the DNA bound to coated wells were denatured by microwave irradiation (500 W for 5 min). The wells were then added with 100 µl of anti-BrdU-POD (Peroxidase) conjugate and incubated for 90 min. The wells were again washed thrice with washing buffer and added with 100 µl of 3,3',5,5'-Tetramethylbenzidine (TMB) substrate solution. The plate had been shaken until colour development was sufficient and the absorbance was read at 450 nm after addition of 25 µl stop solution. The absorbance recorded directly corresponds to the amount of apoptotic DNA fragments released.

**Fig. 3.3:** Principle of the cellular DNA fragmentation ELISA (Roche instruction manual, 2005).

### 3.6 CASPASE-3 ACTIVATION ASSAY

Caspase-3 activation capability of the extracts was determined using a CPP32/Caspase-3 Colorimetric Assay Kit (BioVision Incorporated, CA, USA). The assay was performed according to the manufacturer’s instructions and the buffers used in this experiment were those supplied along with the kit.

#### 3.6.1 SEEDING OF CELLS

MDA-MB-231 and MCF-7 cells (8 x 10^5 per 12.5 cm^2 culture flasks) were seeded with their respective growth medium and incubated for 24 h at 37 °C with/without 5% CO₂.
3.6.2 TREATMENT OF CELLS

Confluent cells were added with the respective drug medium while maintaining an untreated group as a control. Cultures were incubated for 4 h maintaining the same conditions. 0.1 mM Hydrogen Peroxide (H$_2$O$_2$) and 10 µM vincristine were used as positive control for activation of caspase-3 (Dougherty et al., 2008; Abdelwahab et al., 2011).

3.6.3 PREPARATION OF WHOLE CELL PROTEIN EXTRACT

After treatment incubation, the detached cells in the supernatant were collected, and the adherent cells were trypsinized to add into the respective tubes. Subsequently, tubes were centrifuged at 1500 rpm for 10 min to acquire a cell pellet. Volume of 50 µl cell lysis buffer was added to the obtained cell pellet and incubated on ice for 10 min followed by centrifugation at 4000 rpm for 5 min. The supernatant containing cytosolic extracts were then transferred to fresh tubes and the protein concentrations of samples were determined by employing Bradford assay.

3.6.4 ACTIVE CASPASE-3 – DEVD-pNA ENZYME REACTION

Each protein sample (100 g) from the whole cell protein extracts, diluted to 50 µl with cell lysis buffer, was added in a 96 well plate for the reaction. Volume of 100 µl reaction buffer containing 10 mM dithiothreitol (DTT) was then added to all wells, which was followed by the addition of 5 µl of 4 mM Asp-Glu-Val-Asp (DEVD)-pNA substrate. The plate was then incubated at 37 °C for 90 min. Post-incubation, the plate was read at 405 nm in a Dynex Opsyx MR$^\text{TM}$ Microplate Reader (Dynex Technologies, VA, USA). Percentage caspase-3 activation was calculated using the following formula:

\[
\% \text{ Caspase-3 activation} = \left[ \frac{(A_t - A_c)}{A_c} \right] \times 100
\]

$A_t$ is the mean absorbance of test wells and $A_c$ is the mean absorbance of the control wells.
3.7.  PURIFICATION OF THE PHYTOCHEMICALS FROM MCO

Chromatography is a separation process that is achieved by distributing the components of a mixture between two phases, a stationary phase and a mobile phase.

3.7.1  THIN LAYER CHROMATOGRAPHY

Thin layer chromatography have been first developed and utilized by Schraiber in 1939. Unfortunately, Schraiber's work does not seem to have been heeded and the technique was rediscovered by Kirchner in 1951 (Scott, 2003). The technique was exerted using aluminium sheets coated with silica gel (TLC Silicagel 60 F_{254}) supplied by Merck, Germany. A small sample spot (1 to 2 mm diameters) was applied using a capillary tube, 1 cm above on one end of the plate, and was placed in the respective solvent chamber to obtain separation of mixtures. The adsorbent (silica gel) is known as the stationary phase and the solvent or solvent mixture is known as the mobile phase, which is drawn up the plate via capillary action against gravitational force due to which the separation of components in the sample mixture occurs. Various solvent mixtures (hexane, chloroform, ethyl acetate, and methanol) from non-polar to polar were employed to achieve maximum separation of the components. This developed chromatogram was used as a reference for fractions collected through column chromatography. The chromatogram was visualized through two different staining methods.

3.7.1.1  Iodine

Iodine vapor chamber was made by adding crystals of iodine with dry silica gel in a TLC jar. The TLC plates were introduced into the chamber for 1 min to develop brown spots respective to the number of compounds.

3.7.1.2  Ultraviolet Light

The TLC plates were irradiated in UV-transilluminator to visualize UV-active compounds.
Each component separated in the chromatogram is known by its $R_f$ value, which is calculated as mentioned below:

$$R_f \text{ value} = \frac{\text{distance traveled by sample}}{\text{distance traveled by solvent front}}$$

The $R_f$ values are strongly dependent upon the nature of the adsorbent and solvent. For this reason, the experimental $R_f$ values do not customarily agree with values specified in the literature.

### 3.7.2 COLUMN CHROMATOGRAPHY

Column chromatography typically subjects the samples with mixture of components through a physio-chemical separation process to obtain purified individual components.

**Fig. 3.4:** Portrayal of column chromatography of sample MCO

#### 3.7.2.2 Preparation of column and sample loading

Currently, the study employed the use of a glass column with 450 mm length and 30 mm bore. The glass column was thoroughly washed with hexane and dried completely. The column was clamped vertically in a stand and was plugged with a piece of cotton at the bottom to prevent running out of silica gel. The column was then packed by wet method as follows. Hexane was filled till $3/4$th of the height of the column. Activated silica gel (60-120 mesh size; kept at 120 °C for 60 min) was mixed with hexane to prepare a fine slurry and was carefully added into the column by gentle tap on the sides with simultaneous draining of the column. Care was taken to prevent formation of any air bubbles while packing the column. The sample was prepared by mixing 1 g of extract (dissolved in methanol) with 10 g of silica gel (60-120 mesh) until the mixture turned powdery. The sample mixture was then carefully added on
top of the silica gel column to form a uniform sample layer. From this point, the column was never allowed to dry and the level of solvent was marked and maintained constantly.

3.7.2.3 Chromatography of the sample

The column run was performed using hexane and ethyl acetate with varying polarity range (from non-polar to polar). Elution was performed with a flow rate of 1 ml/min. Eluate collected was then analysed in TLC for the confirmation of single spot, with 60% hexane in ethyl acetate as reference solvent system. Fractions with same Rf values were pooled together to get a homogeneous solution of the same compound.

3.8 BIOACTIVITY OF ISOLATED COMPOUNDS

The bioactivity of the isolated compounds were analysed by employing XTT assay, cellular DNA fragmentation ELISA and caspase-3 activation assay as mentioned in section 3.4, 3.5 and 3.6 respectively.

3.9. CHARACTERIZATION OF ISOLATED COMPOUNDS

3.9.1 UV-VISIBLE SPECTRUM

The method yields an absorbance maximum (λmax) of the compound, which could be subsequently used in analytical identifications. The compound was dissolved in ethyl acetate and scanned for its λmax from 800 to 200 nm in a UV-Visible spectrophotometer.

3.9.2 HPLC

Sample was analyzed in HPLC using Waters 2487 HPLC system consisting of a dual detector, a Waters 1525 binary pump, and equipped with a Waters Symmetry C18 column (5 mm, 4.6150 mm) and Waters Sentry™ universal guard column (5 mm, 4.620 mm) (Waters Corporation, Milford, MA, USA).
Gradient HPLC program, as mentioned in table 3.2 was used with methanol and ethyl acetate as mobile phases (Rajkumar et al., 2012).

**Table 3.2:** Gradient HPLC program for isolated compound.

<table>
<thead>
<tr>
<th>Time (in min)</th>
<th>% Solvent A*</th>
<th>% Solvent B**</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>80</td>
<td>20</td>
</tr>
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<td>45</td>
<td>20</td>
<td>80</td>
</tr>
<tr>
<td>60</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>

* Methanol; ** Ethyl acetate.

3.9.3 **MASS SPECTRA**

EI-MS was measured with a Perkin Elmer GC - clarus 680 coupled with MS - clarus 600, fitted with an Elite - 5MS column (30.0 m, 0.25 mm ID, 250 μm df). Mass spectrum was obtained with a scan from 50 to 600 Da at 200 °C.

3.9.4 **NMR**

The \(^1\)H NMR and \(^{13}\)C NMR spectra were recorded on a Bruker DMX-500 NMR spectrometer at 400.1500354 and 100.6278593 MHz respectively using deuterated chloroform (CDCl\(_3\)) as solvent. The method employed the use of a 5 mm BBO probe and all spectra were recorded at 295.5 K. The carbon chemical shifts were referenced to the CDCl\(_3\) signal at 77 ppm.

3.9.5 **FTIR**

Pressed discs of the compound were prepared, employing KBr as transparent material. Infrared spectrum was recorded as KBr pellets on a Nicolet 170SXFT-IR spectrometer.
3.10 STATISTICAL ANALYSIS

All tests were carried out in triplicates. Data were represented as mean ± standard deviation (SD). Statistical analyses were performed by one-way ANOVA. Significant differences between groups were determined at P<0.05. To evaluate relationships between experimental parameters, results were analyzed for correlation and tested for significance (P<0.05) by Student’s t test. MATLAB ver. 7.0 (Natick, MA, USA), GraphPad Prism 5.0 (San Diego, CA, USA), SPSS ver. 9.05 (Chicago, IL, USA) and Microsoft Excel 2007 (Roselle, IL, USA) were used for the statistical and graphical evaluations.