CHAPTER 3

METHODOLOGY

3.1 Herb Authenticication and Collection

The herb material was pulled together from the medicinal garden of Kerala Ayurveda. Plant material was authenticated by the botanist. A Voucher specimen is deposited in the college herbarium for future studies.

3.2 Pharmacognostical Studies

3.2.1 Preparation of Specimen

Specimens were prepared using solution mixture that contains acetic acid (5ml), formalin (5ml) and 70% of ethyl alcohol for one day. The material was dehydrated with a series of tertiary butyl alcohol (Sass 1940). For dehydration double stained and washed section was treated with increasing strength of alcohol for one minute in each strength with 30% alcohol, followed by 75%, 90% and 100%. This removes all moisture from the section. Infiltration was carried out by the addition of paraffin wax up to when super saturation was attained for tributyl alcohol. These wax blocks were used for sectioning (Khandelwal et al 2013).

3.2.2 Sectioning

Thin sections were obtained using the senior precision rotary microtome (MT-1090A, by Weswox, India). Normal procedures were used for de-waxing of the sections from the plant parts. (Johansen 1940). After de-waxing, sections are of about 10 to 12 µm the thickness were taken and toluidine blue was used for staining which is polychromatic stain. This stain will help for the clear vision of the sections under the microscope (O’Brien et al 1964). De-waxed sections were colored with safranin and fast green. Iodine solution was used for the characterization of starch in the sections. Provisional preparations of the above macerated / cleared materials were completed with glycerin. The powder microscopical studies were carried out by using powdered material that was cleaned with sodium hydroxide solution. These treated materials were mounted and viewed under the microscope.
3.2.3 Photomicrographs

Nikon lab photo 2 Microscopic units were used for taking the photographs of the stained sections with different magnifications. Lignified cells, starch and crystals were observed under the bright light (Easu 1964, Easu 1979).

3.3 Phytochemical Studies on *Urena lobata*

3.3.1 Preparation of the Extracts

3.3.1.1 Methanolic Extraction

For the extraction, the dried and coarsely powdered material was weighed, labeled and submitted to Green Chem Laboratory, Domlur, Bangalore, India. Briefly, 3.0kg of the powdered material was mixed in a round bottom flask; it should fixed to a reflux condenser. Methanol of about 15 L was added to the above still (apparatus) and operated for one hr at a temperature of 65°C, and then filtered. These processes were repeated and the extract was pooled. Buchi rotary evaporator was used for the drying the extract and the temperature were maintained at 95°C to obtain the required quantity of the extract in the form of powder. The extraction was repeated with 15 L of methanol for three times.

3.3.1.2 Aqueous Extraction

Aqueous powdered extract of *Urena lobata* was obtained as explained under the procedure previously, explained under methanolic extraction (3.3.1.1). However, in this case, the temperature was maintained at 100°C to reflux the mixture and the solvent used was distilled water (Remington 1985).

3.3.2 Qualitative Phytochemical Analysis

The extracts of *Urena lobata* were subjected to the following chemical tests for the detection of diverse phytoconstituents. The phytochemical examination of the methanolic and aqueous extract of *Urena lobata* Linn was carried out by using the standard procedures. Alkaloids, carbohydrates, flavonoids, glycosides, phytosterols/terpens, proteins, tannins, saponins and lipids were qualitatively analysed (Kokate et al 1999).

3.3.3 Phytoconstituents Estimation

3.3.3.1 Flavonoid Estimation

Methanolic and aqueous extracts of 3 g were refluxed with 50 mL of alcohol was added. Then it was kept on a water-bath for half an hour and then it was filtered. Twice,
the above process was repeated or continued the process until the bitterness was experiential in the residue. This resulted solution was then evaporated under vacuum. The process was repeatedly with the 15, 20, and 25 ml of warm water. 15, 20 and 25 ml of ethyl acetate was added to above aqueous extract was shaken repeatedly. Then it was collected and washed with the normal water. Finally, the extract was evaporated to dryness and weighed (Rajpal 2002).

3.3.3.2 Estimation of Total Tannins

Methanolic and aqueous extracts powder of 1 g was mixed with 50 ml of hot-water and the mixture was shaken continuously. When the powder will soluble completely in the hot water by shaking, then the solution was made up to 100 mL with distilled water. From the resulting solution about (10 mL) was pipetted out to 1000 mL conical flask, and then exactly 750 ml of distilled water was added to the conical flask. To the above solutions, added 25 mL of freshly prepared indigo sulphuric acid and then diluted to 1000 mL with distilled water.

The solution which, was considered as (Vs) was titrated against 0.1 N potassium permanganate solutions (KMNO₄) with constant stirring to obtain an end point (golden yellow color). A blank titration was performed by titrating 25 mL of indigo sulphuric acid in about 750 mL distilled water which is considered as (Vb). The equivalent factor 0.1 N KMNO₄ solution were found to be 0.004157g and was equivalent to each ml of tannin compounds which was calculated as calculated as tannic acid (Rajpal 2002).

3.4 Pharmacological Evaluation

3.4.1 Antioxidant Activity

3.4.1.1 Preparation of DPPH Solution

DPPH stock solutions were prepared by standard procedure. From this stock solution, 0.375 mL was added to each test tube containing the extracts.

3.4.1.2 Preparation of the Test Solution

Table 21 represents the assay procedure of the aqueous and methanolic extracts of *Urena lobata*. Different concentrations of the methanolic and aqueous extracts (5-100 µg/mL) were prepared using distilled water. Different concentrations of the aliquot of the extracts were mixed with 1.5 mL of freshly prepared DPPH solution. Aliquot volume (2 ml)
was made using methanol (0.25 g/L), absorbance was measured under 515 nm, and standard used was ascorbic acid.

**Table 21. Assay protocol for aqueous extract and methanolic extract of *Urena lobta* Linn**

<table>
<thead>
<tr>
<th>Test tubes</th>
<th>Methanol (mL)</th>
<th>DPPH (mL)</th>
<th>Drug (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.625</td>
<td>0.375</td>
<td>-</td>
</tr>
<tr>
<td>Test tube 1</td>
<td>1.620</td>
<td>0.375</td>
<td>0.005</td>
</tr>
<tr>
<td>Test tube 2</td>
<td>1.615</td>
<td>0.375</td>
<td>0.010</td>
</tr>
<tr>
<td>Test tube 3</td>
<td>1.605</td>
<td>0.375</td>
<td>0.020</td>
</tr>
<tr>
<td>Test tube 4</td>
<td>1.575</td>
<td>0.375</td>
<td>0.250</td>
</tr>
<tr>
<td>Test tube 5</td>
<td>1.525</td>
<td>0.375</td>
<td>0.100</td>
</tr>
</tbody>
</table>

IC₅₀ was calculated and plotted between concentration and absorbance *(Amit et al 2010)*

### 3.4.2 Wound Healing Activity

#### 3.4.2.1 Experimental Animals

Animals were acquired from Biogen, Bangalore. Procured animals were sustained in the college animal house by giving food and water. All animal studies done under rules and regulation of IAEC and CPCSEA of India.

#### 3.4.2.2 Acute Toxicity Studies (LD₅₀)

##### 3.4.2.2.1 Animals

The nulliparous and non-pregnant female. The animals (Albino mice) of 20-30 g weight were used for the present study.

##### 3.4.2.2.2 Procedure

Saline/water was used for the solubilization of the methanolic extract of *Urena lobata*. According to OECD guidelines 423, the doses were prepared. The dose used was 3000 mg/kg and conducted in two phases. Phase one was called the observation day and phase, two for fourteen days after the first dose was administrated *(OECD 2008)*.
3.4.2.3 Drug Formulations

Two types drug formulations were prepared for testing the wound healing activity of the *Urena lobata*. First one was topical application and second was oral formulations. For the topical application, 10 and 20 g of the extract, which was incorporated with 90 g and 80 g of sodium alginate (2%) drug formulations was used for wound healing activity. Oral dosage was prepared by dissolving the methanolic extract in normal saline (Singh 2005).

3.4.2.4 Skin Irritation Studies

For skin irritation studies three areas were kept by shaving the dorsal side of about 500 sq.mm. One area was kept as control; second and third areas were applied with 10 and 20% of the MEUL gel.

The animal was kept in animal holder. The first area was kept as control, to which the vehicle (distilled water) was applied. 10% of the methanolic extract of *Urena lobata* (MEUL) was applied on the second area and the third area treated with 20% MEUL gel (Feller et al 1985). The follow-up proforma for wound healing activity is shown in Table 22.
### Table 22. Follow-up Proforma for Wound Healing

<table>
<thead>
<tr>
<th>Sl. No</th>
<th>Wound Models</th>
<th>Observation</th>
<th>Made on Post Wounding day</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Excision</td>
<td>Wound tracing</td>
<td>0,2,4,6,8,10,12,14,16,18,20,22</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Wound photography</td>
<td>0,2,4,6,8,10,12,14,16,18,20,22</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Epithelialization</td>
<td>Observed on falling of scab without raw area.</td>
</tr>
<tr>
<td>2</td>
<td>Incision</td>
<td>Sutures removal</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tensile-strength measurement</td>
<td>10</td>
</tr>
<tr>
<td>3</td>
<td>Burn wound</td>
<td>Wound tracing</td>
<td>0,1,3,5,6,9,11,13,15,17,19</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Wound photography</td>
<td>0,1,3,5,6,9,11,13,15,17,19</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Epithelialization</td>
<td>Observed on falling of scab without raw area.</td>
</tr>
<tr>
<td>4</td>
<td>Dead space model</td>
<td>Granuloma weight</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hydroxyproline estimation</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Granuloma histopathology</td>
<td>10</td>
</tr>
</tbody>
</table>

### 3.4.2.5 Excision Wound Model

Each animals were anaesthetized using anaesthetic ether method used was open mask method. Before the experiment skin was shaved. On the dorsal thoracic region of the anaesthetized rat, an impression was made 1 centimeter away from vertebral-column. Then the wound was cleaned with normal saline. Six animals were placed into four groups. Group one considered as a control. Group two is represents as the standard and the animals were treated with povidone-iodine ointment 5% w/w, grouping three and four were treated with methanolic extract of the plant and applied with 10 and 20% of gel respectively. Drug was applied on the upper part of the skin until the epithelization was completed on the surface of...
the wound. The change on the wound was observed on alternative days (2\textsuperscript{nd} to 22\textsuperscript{nd}) of post wounding. The epithelization time is defined as the days (in numbers) which necessary for falling of outer layer with no any residual rare wound (Morton \textit{et al} 1972, Kuppast \textit{et al} 2003).

\textbf{3.4.2.6 Incision Wound Model}

Grouping of animals and drug administration were as same as the excision model (4.4.2.5). Incision were made 6 cm long on anesthetized animal on the para-vertebrally. When bleeding was stopped wound was closed by sutures of same distance which are 1 cm away. A Cotton swab dipped in 70\% of alcohol was used to clean the wounds. These stitches were detached on the nineth day of the post wounding. The tensile strength was deliberate using steady water supply scheme (Silambujanaki \textit{et al} 2011, Ehrich \textit{et al} 1969).

\textbf{3.4.2.6.1 Measurement of the Wound Breaking Strength of Incised Wound Model}

A model for measuring the breaking strength is shown Figure 9 and breaking strength was performed based on Lee \textit{et al} (2005) procedure with some modifications. Anaesthetized animal was placed on a table facing its abdomen towards the table. A thread was tied on the left side of the table of 0.5 cm with help of a clamp. The other side of the clamp was coupled to a water-proof poly-thene bag by means of a rubber tube and a container was placed on appropriate altitude. The location of the polythene bag was attuned (adjusted) so that bag was hanged freely. The addition of water to the polythene bag was hastily at steady speedy from the tank until the wound opened.
3.4.2.7 Burn Wound Model

Each all were grouped and anaesthetized (anesthetic ether) using open mask technique. Burn wound was formed by molten wax (4.0 g) which was heated at 80° C and placed on metal cylinder of 100 mm$^2$ on the backside of the animal. Wax got solidifies on 8 min and the cylinder was closed to skin and was detached, which left a circular wound of 100 mm$^2$. Until, the animals where recovered from anesthesia they were kept on separate cages (Yoganarsimhan 1996, Bairy et al 1997, Nayak et al 2008).

3.4.2.7.1 Epithelialization Period

The period of epithelialization was noted by the numeral of days that obligatory for the eschar to fall by parting a raw wound.

3.4.2.7.2 Wound Contraction

Wound contractions were evaluated on alternative days. The dimensions were traced on a tracing paper and then changed to a graph paper (1mm$^2$). The percentage of wound contraction was measured by taking the size of the first wound was considered as 100% using the following formula

$$\text{Percentage wound closure} = \left( \frac{\text{Initial area of wound} - \text{N}^{th} \text{ day area of wound}}{\text{Initial area of wound}} \right) \times 100$$
3.4.2.8 Dead Space Wound Model

In this model, the animals were grouped as the above model. On the tenth day of post wounding granulation tissue were formed. These tissues were entrenched on cotton pellets were cautiously aloof under anesthesia Fig 10. The drenched (wet) weight of the tissue was noted and these tissues were dried for 12 h at 60 °C. After drying, weight was noted and on the dried tissue. To the above tissue 6 N hydrochloric, acid was mixed and kept back at 110° C for one day. The hydroxyproline estimation was done using these hydrolysate tissues. For histological studies, the additional wet granulation tissue was preserved in formalin of 10 % strength. (Neuman et al 1950, Nayak et al 2009).

3.4.2.8.1 Chemicals used for Hydroxyproline Estimation in Dead space Wound Model

The different chemicals used are: 6 N HCl (200 mL): 107 mL of HCl and 93 mL water, 10 N NaOH (100 mL): was prepared using 40 gm sodium hydroxide in 100 ml of distilled water. NaOH 2.5 N (100ml) was prepared by adding 10 gm NaOH in 100 ml of distilled water, 2.5 N NaOH (100 mL): 10 g NaOH in 100 mL water. Standard hydroxy proline solution: 1 mg/mL (stock solution), 0.01 M CuSO₄ (100mL): 250 mg CuSO₄ in 100mL water, H₂O₂ (6%): Reagent bottle,Para-Dimethylaminobenzaldehyde (100 mL): 5 gm of in Para,
Dimethylaminobenzaldehyde (PDAB) 100 mL of n-Propanol (freshly prepared), n-Propanol and 3 N H$_2$SO$_4$ (100 mL): 9 mL H$_2$SO$_4$ in 100mL water.

3.4.2.8.2 Hydroxyproline Estimation

Estimation of hydroxyproline present in the dry tissue and control were measured using spectrophotometrically. Different concentrations of samples were taken and these were oxidized by mixing 1 ml of chloramines-T to every test tube which was then shaken well and kept it for 20 min at normal conditions. One ml of perchloric acid was added to each test tube to remove the chloramines-T. tube and then it was kept for 5 min. As a final point, 1 ml of PDAB was mixed to the above combination and shaken well. Development of the color in each test tube (Fig 11) was measured at 557 nm. Collagen content was determined by multiplying hydroxyproline content by the value of 7.46.

![Fig 11. Hydroxyproline Estimation](image)

3.4.2.9 Histopathological Study

On the 10$^{th}$ day, the granulation tissues were taken from the animals (both control and test) used for the histological studies. Van Geison stain was used for the better appearance of pink color on tissues.

3.4.2.10 Statistical Analysis

ANOVA (one-way analysis of variance) followed by Dunnet’s test was used for comparative studies on wound areas. Indicative significance of the P-values i.e: $> 0.05$. (Perez et al 2006).
3.5 Anticancer Activity

3.5.1 Experimental Animals

Swiss albino mice of 25 to 30 gm weight were procured from Biogen, Bangalore and maintained one week at Gautham College of pharmacy animal house at (27 ± 2°C tempaerature). Amala cancer research center was supplied the EAC cell lines. Cell lines were maintained and these animal studies were carried out with approval of IAEC and CPCSEA Govt of India. The cell lines were maintained in serial transplantation in mice, 1x10⁶ cells were injected i.p into a healthy mouse.

3.5.2 Determination of acute toxicity (LD₅₀)

3.5.2.1 Experimental animals

Female albino mice of 18-22 gm weight were used for the acute toxicity (LD₅₀) studies. These animals were non-pregnant. Experimental animals were maintained for one week in animal house before giving the drug.

3.5.2.2 Dose Preparation

Methanolic and the aqueous extract of *Urena lobata* were dissolved in the suitable solvents using Tween 80 to prepare a dose of 2000 mg/kg (OECD 2011).

3.5.3 In-vivo Anti-Tumor Activity

The dose dependent reduction and inhibition of tumour development property of methanolic and aqueous extracts of *Urena lobata* were determined in Swiss albino mice using ascitic tumour model.

3.5.3.1 Tumour Cell Line and its Maintenance

Amala Cancer Research Center, Thissur, Kerale, India, was supplied Ehrlich Ascites Carcinoma (EAC) cell lines. Theses cell lines were inoculated in to the mice. EAC cells were maintained by i.p transplantation in mice. Cell-lines were aspirated from the mouse peritoneum of a fully-grown tumour-using syringe with 18-gauge needle, washed thrice with 0.9 % saline and suspended in saline. About 1x10⁶ cells injected i.p., into new healthy animals.

3.5.3.2 Induction of Experimental Tumor

Aseptically the EAC cells were taken from the mice. Phosphate buffer saline was used to dilute the cells and 1x10⁶ EAC cells in 0.3 mL phosphate buffer were injected
intra-peritoneally using 18-gauge needle to obtain ascetic tumour in mice (Durairaj et al 2009).

3.5.3.3 Treatment Schedule

In an individual group of six where, each group containing six animals. 1\textsuperscript{st} group- Tumor Control, Receive NaCl solution (0.9%) (p.o), 2\textsuperscript{nd} group - 5-FU of 20 mg kg / day, i.p, 3\textsuperscript{rd} group- methanolic extract of 200 mg / kg b.w p.o, 4\textsuperscript{th} - methanolic extract of 400 mg / kg b.w; p.o, 5\textsuperscript{th} - aqueous extract of 200 mg / kg b.w; p.o and 6\textsuperscript{th} - aqueous extract of 400 mg/kg b.w ; p.o.

3.5.4 Evaluation of Antitumor Activity

3.5.4.1 Determination of the Mean survival time and the Percentage in increase in life span

MST and % ILS were calculated by the following: (Nipun et al 2011).
\[
\text{MST}^* = \frac{(FD) + (LD)}{2}^* \\
\text{FD-- First Death, LD--Last Death,*Time denoted by days} \\
\text{% ILS} = \left(\frac{\text{MST of treated Groups-MST of Control}}{\text{MST of Control}}\right) \times 100
\]

3.5.4.2 Tumor Volume

Tumour volume was measured by taking the ascitic fluid from the peritoial cavity of the tumour bearing mice using using graduated centrifuge marked graduated tube. (Durairaj et al 2009).

3.5.4.3 Viable/non viable cell count

Trypan blue assay was used for the viable and non-viable count. In tryphan blue assay the cells were stained with trypan blue (0.4% in normal saline) dye and the cells that did not take up the dye were viable and those cells that took the stain were non-viable. Cell count was calculated using the following formula (Nipun et al 2011).
\[
\text{Cell count} = \frac{\text{No of cells} \times \text{dilution factor}}{\text{Area} \times \text{Thickness of liquid film}}
\]

3.5.4.4 Tumor Mass

Tumour mass was calculated by taking the weight of the mice after and before the collection of ascitic fluid from the peritoneal cavity (Nipun et al 2011).
3.5.5 Haematological Parameters

Haematological parameters like WBC, RBC and Hb count by using an automatic analyzer (ERMA INC Tokyo, - PEC-21 OVET).

3.5.5.1 White Blood Cell Count

Blood was diluted with a suitable diluting fluid for 20 times, which destroys the red blood corpuscles and stains the nuclei of the white blood cells. The reagent used was Turk"s fluid (Glacial acetic acid 1.5 mL, Gentian violet 1 mL, and distilled water 100 mL). The leucocytes were then counted in a haemocytometer and their number in undiluted blood was calculated (Joshi et al 2005).

**Procedure:** The blood was taken up to 0.5 marks in the WBC pipette and diluted with Turk"s fluid up to 11 marks and mixed thoroughly. Initially, 1-2 drops were discarded and charged the diluted blood into counting chamber with cover slip. The cells were counted after 5 min under low power objective in the compound microscope. Total WBC in undiluted blood was calculated and expressed as cell x 10⁶/mL blood.

3.5.3.2 Red Blood Cell Count

The number of red cells in the blood is very high, so diluted for 200 times with an appropriate diluting fluid (Haeym"s fluid) before counting in a haemocytometer. The compositions of Haeym"s fluid are - NaCl 0.5 g (for is tonicity), sodium sulphate 2.5 g (as anticoagulant), mercuric chloride 0.25 g (as preservative) and distilled water 100 mL. Then number in undiluted blood was calculated.

**Procedure:** The blood was taken up to 0.5 marks in the RBC pipette and diluted with Haeym"s fluid up to 101 marks, mixed thoroughly. Initially, 1-2 drops were discarded and charged the diluted blood into counting chamber (hemocytometer) with cover slip. The cells were counted after 5 min under high power objective in the compound microscope. Total number of RBC in undiluted blood was calculated and expressed as cell x 10⁹/ mL blood.

3.5.5.3 Estimation of Haemoglobin Levels

**Principle:** The haemoglobin contained in a known quantity of blood is converted into acid haematin by reaction with HCl. This colour was then compared with a standard tube containing acid haematin.
**Procedure:** Five drops of 0.1 N HCl was placed in the graduated tube (Sahali”s tube) and placed it beside the colour comparator. Pipette out the blood with the help of micropipette up to the mark 20 mm, it was transferred immediately to the graduated tube, mixed thoroughly, added distilled water drop wise and stirred with the stirring rod until the colour of the solution matched with the standard on either side. Then it was compared under the natural light and observed the readings on the tube.

**3.5.5.6 Statistical Analysis**

Statistical analysis was done using ANOVA followed by Dunnett”s Test and software used was graph pad prism. Values were expressed in mean ± S EM and the P value was (>0.05) was considered significantly.